PROPOFOL HYDROXYLATION BY DOG LIVER MICROSOMES: ASSAY DEVELOPMENT AND DOG BREED DIFFERENCES

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(Received October 7, 1998; accepted June 15, 1999)

This paper is available online at http://www.dmd.org

ABSTRACT:
Pharmacokinetic studies indicate that clearance of propofol, an anesthetic agent, is slower in greyhounds compared with other dog breeds. Biotransformation of propofol to 2,6-diisopropyl-1,4-quinol (4-hydroxypropofol) by cytochrome P-450 in the liver is proposed as a critical initial step in the elimination of this drug in dogs. Breed differences in the activity of this enzyme could therefore explain pharmacokinetic differences. An in vitro propofol hydroxylase assay was developed and then used to compare enzyme activities in liver microsomes from male greyhound, beagle, and mixed-breed dogs (five each). HPLC of incubate identified only one NADPH-dependent metabolite, which had a chromatographic retention time and UV absorbance, fluorescence, and mass spectra that were identical with authentic 4-hydroxypropofol standard. HPLC with fluorescence detection provided a highly sensitive quantitation method for 4-hydroxypropofol with a quantitation limit of 8 ng/ml using optimized excitation/emission wavelengths (288 nm/330 nm, respectively). Estimates of apparent \( K_m \) and \( V_{max} \) for propofol hydroxylation by microsomes from a male beagle dog were 7.3 \( \mu \)M and 3.8 nmol/mg/min, respectively. At a substrate concentration of 20 \( \mu \)M, propofol hydroxylase activity was significantly lower \( (p=0.032) \) in greyhound microsomes (1.7 \( \pm \) 0.4 nmol/mg/min) compared with beagle microsomes (6.1 \( \pm \) 1.3 nmol/mg/min) but was not statistically different \( (p=0.42) \) compared with mixed-breed microsomes (3.1 \( \pm \) 1.2 nmol/mg/min). These results indicate that there are breed differences in propofol hydroxylase activity and that deficient hydroxylation of propofol by one or more hepatic cytochrome P-450 isoforms may contribute to slow pharmacokinetic clearance of propofol by greyhounds.

Propofol (2,6-diisopropylphenol) is rapidly gaining widespread utility for i.v. anesthesia and sedation in dogs and humans (Robertson et al., 1992; Zoran et al., 1993; Mandsager et al., 1995). One of the major advantages of this drug over other injectable anesthetic agents is the rapid and complete recovery that occurs even after relatively prolonged i.v. infusions (Mandsager et al., 1995). This property is attributable to rapid and extensive biotransformation of the parent compound, primarily by the liver, to multiple inactive metabolites, which are excreted in the urine. The relative contribution of individual metabolic pathways has been found to vary among species.

In humans, more than 50% of an administered dose of propofol is metabolized by glucuronidation and sulfation to yield propofol glucuronide and sulfate, respectively (Simons et al., 1988; Sneyd et al., 1994). However, in dogs the major metabolites of propofol are conjugates of the intermediate and sulfate, respectively (Simons et al., 1988; Sneyd et al., 1994). Consequently, biotransformation of propofol to 4-hydroxypropofol by oxidative enzymes in the liver is likely to be a major rate-determining step in the elimination of propofol in the dog. Evidence from in vitro studies of propofol hydroxylation in human and rat liver microsomes indicates that this reaction is mediated by cytochrome P-450 (CYP; Guitton et al., 1997, 1998).

Materials and Methods

Dr. Court is the recipient of a Special Emphasis Research Career Award from the National Institutes of Health (K01-RR-00104). This work was also supported in part by National Institutes of Health Grants MH-34223 and MH-19924 and a Hill’s Resident Research Grant.

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1 Abbreviations used are: 4-hydroxypropofol, 2,6-diisopropyl-1,4-quinol; quinone, 2,6-diisopropyl-1,4-quinone; GC-MS, gas chromatography-mass spectrometry; SPE, solid phase extraction; \( \lambda_{max} \), maximum UV absorption wavelength; CYP, cytochrome P-450; DMSO, dimethyl sulfoxide.

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Drugs and Chemicals. Pure propofol was provided by Zeneca Pharmaceuticals (Wilmington, DE) in a sealed 2-ml glass ampule with argon overlay.
transferred to a Teflon-sealed glass vial with nitrogen overlay, and stored at –80°C. A small quantity (4 mg) of 4-hydroxypropofol was a gift from Dr. J. Guitton (Université Claude Bernard, Lyon, France). This had originally been provided by Zeneca Pharma (Cergy, France) as a powder and stored at room temperature. Thymol (5-methyl 2-isopropylphenol), NADP⁺, isocitrate dehydrogenase, NADH, dimethyl sulfoxide (DMSO), and 50 mM potassium phosphate buffer (pH 7.5) were purchased from Sigma Chemical Company (St. Louis, MO). Acetonitrile and methanol (chromatographic grade) were obtained from Fisher Scientific (Fairlawn, NJ). Stock solutions of propofol (8.9 mg/ml) and thymol (10 ug/ml of methanol) were stored in screw-capped polypropylene tubes with neoprene O-rings at –80°C. In this discussion, 2,6-diiosopropyl-1,4-quinol is referred to as 4-hydroxypropofol and 2,6-diiisopropyl-1,4-quinol is called the quinone.

Liver Microsomes. The liver tissue used in this study was obtained from mature male greyhound, beagle, and mixed-breed dogs (five each) within minutes of pentobarbital overdose and immediately frozen at –80°C. Liver microsomes were prepared from this tissue by differential centrifugation as described previously (Court et al., 1997). The final microsomal pellet was resuspended in phosphate buffer containing 20% glycerol for storage at –80°C. An aliquot of this suspension was used to determine protein concentration by the bicinchoninic acid assay technique (Pierce, Rockford, IL).

Propofol Solubility. Pure propofol is an oily liquid at room temperature with limited solubility in aqueous solutions and moderate volatility. To obtain appropriate concentrations of propofol in an aqueous incubation system, some form of solubility enhancement was needed. The minimal concentrations of three solubility enhancers, including DMSO, methanol, and acetonitrile, which completely solubilize propofol at a 500 μM concentration in phosphate buffer at room temperature, were determined. Complete solubilization was substantiated by visual inspection of solutions in glass culture tubes after 1 min of rapid vortexing. Undissolved propofol appeared as oily droplets floating in the solution.

HPLC. HPLC apparatus consisted of a dual-head pump with autoinjector (Models 515 and 717; Waters, Milford, MA) serially connected to a UV absorbance detector (model 486; Waters) and a fluorescence detector (model 474; Waters). Unless otherwise indicated, the UV detector was set with an absorbance wavelength of 270 nm and the fluorescence detector was set with an excitation wavelength of 276 nm, emission wavelength of 310 nm, and 15-nm slit widths. The HPLC mobile phase consisted of 50% acetonitrile, 40% water, and 10% methanol with a flow rate of 1.8 ml/min. The column was a 3.9 mm x 30 cm C₁₈ Bondapack (Waters).

Incubations. Incubation containers consisted of either 1.5-ml glass gas chromatograph vials closed with Teflon seals or 2-ml polypropylene screw-capped tubes with neoprene O-rings. Sealed tubes were used to reduce evaporative loss of propofol, which became significant (>10% decrease) after only 10 min in open tubes at 37°C. Typical incubations were performed as follows. All working solutions were made up daily and were kept on ice. Liver microsomes were diluted with phosphate buffer and 50 μl was added to each tube. Next, 100 μl of an NADPH-regenerating system (final concentrations of 0.5 mM NADPH, 6 mM magnesium chloride, 3.75 mM NADH, and 1 U/ml isocitrate dehydrogenase) in phosphate buffer was added. Tubes were then vortexed briefly and placed in a shaking water bath at 37°C for 2 min. Reactions were started by adding 100 μl of propofol working solution, prepared by diluting propofol/DMSO stock solution in phosphate buffer. A 500-fold dilution yielded 100 μM propofol and 0.2% DMSO in a total incubation volume of 250 μl. Incubations were stopped by the addition of 250 μl of acetonitrile containing thymol as the internal standard (generally 100–250 ng/ml final concentration) to each tube. The tubes were immediately vortexed and placed on ice. Tubes were spun at 14,000 rpm in a microcentrifuge for 5 min in a cold room. The supernatant was then transferred to HPLC vials (1-ml glass vial with 250-μl insert and polypropylene cap) for immediate assay or for storage at 4°C until assay by HPLC, within 2 weeks.

Metabolite Detection. Microsomal incubates were generated, as described previously, using 100 μM propofol, 0.5 mg/ml protein, and 10 min incubation, and subjected to HPLC analysis. Chromatograms generated with and without NADP⁺ in the cofactor mix were compared to identify potential NADPH-dependent metabolite peaks using both the fluorescence detector at 276-nm excitation and 310-nm emission wavelengths and the UV absorbance detector at wavelengths between 200 and 300 nm. Metabolite Purification. To facilitate identification, a small amount of this metabolite was purified by solid phase extraction (SPE). The procedure used was as follows. The incubation was scaled up to a 10-ml volume with 1 mM propofol, 2% DMSO, and 1.36 mg/ml microsomal protein; it was then incubated for 30 min at 37°C. After centrifuging for 5 min at 14,000 rpm, the supernatant was applied to a 0.5 g C₁₈ SPE cartridge (Supelclean LC-18; Supelco, Bellefonte, PA) that had been conditioned with 4 ml of methanol followed by 4 ml of phosphate buffer. The cartridge was then washed with 8 ml of water and eluted with 1-ml aliquots containing increasing concentrations of acetonitrile in water (from 10 to 100% in 10% increments). The initial incubate flowthrough, wash solution, and aliquoted eluate solutions were assayed for metabolite by HPLC. Metabolite-containing solutions (60 and 70% acetonitrile eluates) were pooled, dried down to 50% of initial volume in a centrifugal vacuum concentrator (Centrivap Concentrator; Labconco, Kansas City, MO) to remove acetonitrile, and reconstituted with water. This was then reapplied to a second SPE cartridge, which had been conditioned with 4 ml of methanol and 4 ml of water. The cartridge was washed with 8 ml of water and 8 ml of 30% acetonitrile in water and then eluted with 1-ml aliquots of acetonitrile in water (concentrations increasing from 40 to 50% in 1% increments). The 40, 41, and 42% acetonitrile eluates, which were found to contain all of the metabolite, were pooled, divided into two equal volumes, completely dried down in the centrifugal vacuum concentrator, and stored at room temperature in polypropylene tubes for subsequent analysis by UV absorption spectrophotometry and mass spectrometry.

UV Absorption Spectrophotometry. 4-hydroxypropofol standard was diluted in HPLC mobile phase to 200 μM in a volume of 100 μl and placed in a 50-μl quartz cuvette. After first establishing a baseline using HPLC mobile phase, the UV spectral profile (between 200- and 400-nm absorbance wavelength) of this metabolite was determined using a scanning absorption spectrophotometer (Uvikon 860; Kontron Instruments, Zurich, Switzerland). This profile was then compared with the profile generated in an identical fashion using the SPE-purified metabolite. A small quantity (0.5 μl) of 2 N NaOH was added to each of these solutions and the spectral profiles were redetermined and compared. Small aliquots of each of these metabolite-containing solutions were also analyzed by HPLC for changes in apparent HPLC retention and sensitivity to detection by fluorescence and UV absorption techniques.

Mass Spectrometry. Negative ion electrospary mass spectral analysis was performed on a tandem mass spectrometer (VG Quattro I; Fisons Instruments, Manchester, United Kingdom). Samples dissolved in 0.01 M ammonium acetate in water/acetonitrile (1:1, v/v) were introduced into the electrospray source by flow injection at 10 μl/min using the same solvent as the delivery solvent. Spectra were collected in the first analyzer using a cone potential of 25 V. Collision-induced dissociation spectra of the m/z 193 ion were generated using a collision energy of 25 eV with argon as the reagent gas. The spectrum of 0.01 M ammonium acetate in water/acetonitrile (1:1, v/v) was subtracted from the sample spectra to correct for solvent background ions. Scans of both microsomal metabolite and 4-hydroxypropofol standard were collected for comparison.

Fluorescence Spectrophotometry. 4-hydroxypropofol standard was diluted to 10 μM in a volume of 2 ml with HPLC mobile phase in a quartz cuvette and placed in a scanning fluorescence spectrophotometer (Model LS50B; Perkin-Elmer, Beaconsfield, Buckinghamshire, United Kingdom). Excitation and emission slit widths were set at 5 nm. Initial estimates of optimal excitation and emission wavelengths were obtained using the rapid scan function of the spectrophotometer. Using these estimates as starting points, alternating excitation and emission scans of wavelengths between 200 and 350 nm (excitation scans) and between 250 and 400 nm (emission scans) were performed. This process was repeated using the wavelength showing the maximal fluorescent output from the previous scan for the following scan, until both excitation and emission and wavelength maxima changed by less than 1 nm. Similar scans were also performed using SPE-purified metabolite and 10 μM solutions of propofol and the quinone (derived by alkaline treatment of 4-hydroxypropofol solution similar to that described for UV spectrophotometry).

Sensitivity of HPLC Detection Methods and Metabolite Recovery. The sensitivity of fluorescence and UV absorbance HPLC detection methods was determined by the analysis of a series of sequentially diluted 4-hydroxypropofol standard solutions (10 μM–5 nM). Each of these standard solutions were
similar in composition to a microsomal incubate, containing 0.5 mg/ml microsomal protein, NADPH cofactor mix and thymol, but lacked propofol and were not incubated. Samples were then prepared as described above for HPLC injection (10 μl volume). Analysis was performed twice, once with UV absorbance wavelength of 270 nm and fluorescence wavelengths of 276 nm (excitation) and 310 nm (emission) and once with UV absorbance wavelength of 288 nm and fluorescence wavelengths of 288 nm (excitation) and 330 nm (emission). Slit widths were maintained at 15 nm. The minimal detectable concentration was defined as the concentration of 4-hydroxypropofol that resulted in a HPLC peak height greater than 3 times the level of baseline noise.

Recovery of the metabolite from protein containing solutions was also determined during this experiment by preparing a similar set of standard solutions for HPLC analysis with substitution of phosphate buffer for microsomes and NADPH cofactor mix.

**Stability of Metabolite.** Small quantities (0.5 mg) of pure 4-hydroxypropofol standard powder were dissolved in 1 ml of methanol, phosphate buffer, and DMSO, aliquoted into polypropylene screw-capped tubes with neoprene O-rings, and stored at 4, −20, and −80°C. The change in 4-hydroxypropofol concentration and appearance of the quinone was monitored by HPLC with UV absorbance detection at 270 nm for up to 1 month. The stability of measured compounds (including metabolite, substrate, and internal standard) in a typical incubate prepared for HPLC analysis were also evaluated over a 2-week period by measuring the change in concentration of each of these compounds in HPLC vials stored at 4°C versus room temperature.

**Metabolite Mass Balance.** The relationship between the appearance of metabolite to the disappearance of propofol was examined over a range of substrate concentrations (5–100 μM). Assays were conducted in duplicate at each substrate concentration both with and without added NADPH. For each tube, DMSO concentration was 0.2%, microsomal protein concentration was 0.3 mg/ml, and incubation time was 10 min. Thymol (125 ng in 250 μl of acetonitrile) was added to each tube as an internal standard. In addition, a standard curve was generated by serially diluting pure 4-hydroxypropofol (final concentrations of 20 μM to 625 nM) to 250 μl with phosphate buffer. After adding 250 μl of internal standard in acetonitrile, these samples were analyzed in a similar manner by HPLC with fluorescence detection.

Propofol concentrations were plotted against propofol peak height ratios (relative to internal standard) for samples incubated without NADPH. The slope of this line was then derived by linear regression forced through the origin and used to calculate the concentration of propofol that remained in tubes incubated with NADPH. These values were then subtracted from the initial propofol concentrations and divided by the protein concentration and incubation time to give the rate of propofol consumption at each substrate concentration. These values were then plotted against the respective rates of metabolite formation, derived from metabolite peak height ratios and the pure 4-hydroxypropofol standard curve, and analyzed by linear regression.

**Enzyme Kinetics.** The effects of incubation time (up to 60 min), protein concentration (up to 2 mg/ml), and substrate concentration (1–100 μM propofol with 0.2% DMSO) on the production of the metabolite were determined by HPLC with fluorescence detection. Metabolite production was quantified in a similar manner to that described above by use of a standard curve generated with purified metabolite diluted in phosphate buffer. Intra- and interassay c.v. values for this assay procedure were also determined by analysis of activity data replicates generated during a single assay at substrate concentrations between 1 and 100 μM and activity data generated over three separate assays at 100 μM substrate concentration. Enzyme kinetic parameters, including $K_m$ and $V_{max}$, were derived by nonlinear least-squares curve fit of substrate concentration and velocity data to the Michaelis-Menten equation (Court et al., 1997).

**Dog Breed Comparisons.** Propofol hydroxylase activity was determined using liver microsomes from greyhounds, beagles, and mixed-breed dogs (all males, five each). Substrate concentration, incubation time, and microsomal protein concentration were 20 μM, 10 min, and 50 μg/ml, respectively. Activity determinations were performed for each microsomal preparation in duplicate and averaged. Statistical comparisons of these data were performed using the SigmaStat software package (Jandel Scientific, San Rafael, CA). The independent variable was dog breed and the dependent variable was propofol hydroxylase activity. Data were initially evaluated for normality of distribution and equality of variance. Because of unequal variance, pairwise comparisons of these data were subsequently performed using the Mann–Whitney rank sum test. Differences between groups were considered significant when the p value was less than .05. For each set of data, the c.v. was calculated by expressing the S.D. as a percentage of the mean, and fold variation in the range was calculated by dividing the highest value by the lowest value.

**Results**

Propofol (500 μM in phosphate buffer) was completely solubilized with 1% DMSO, whereas concentrations of methanol or acetonitrile up to 10% were ineffective. Consequently, DMSO (up to 0.2% final concentration) was used in all incubations as a solubility enhancer for propofol. A preliminary study showed no difference in propofol hydroxylase activity in incubates containing 0.4% compared with 0.2% DMSO.

Only one NADPH-dependent propofol metabolite could be identified by HPLC (Fig. 1). Compared with thymol (4.6 min) and propofol...
these solutions with NaOH resulted in a decrease in the minor fragment ions at 177 and 4-hydroxypropofol standard (B) for collision-induced dissociation analyses of the isolated metabolite (A) were approximately 200 peaks. Concentrations of microsome-derived metabolite and pure 4-hydroxypropofol were approximately 200 μM.

(7.2 min) this compound had a short retention time (2.8 min), which was identical with authentic 4-hydroxypropofol standard (data not shown). A small quantity of this metabolite (approximately 80 μg) was successfully purified to apparent 100% homogeneity (as determined by HPLC; Fig. 1) from 10 ml of microsomal incubate by SPE procedures.

Purified metabolite and 4-hydroxypropofol standard diluted in mobile phase had similar UV spectral profiles with identical maximum UV absorption wavelengths (λmax) of 288 nm (Fig. 2). Treatment of each of these solutions with NaOH resulted in a decrease in the λmax from 288 to 258 nm and a 5-fold increase in absorbance at the λmax (Fig. 2). HPLC analysis of each of these solutions with the UV absorbance detector set at 270 nm showed an increase in peak retention time from 2.8 to 7.3 min and a 2.5-fold increase in peak height with alkaline treatment (Fig. 1). On the other hand, when monitored with the fluorescence detector set at 276-nm excitation/310-nm emission wavelengths, no peaks were visible after alkaline treatment (Fig. 1).

The negative ion electrospray mass spectra of both the microsomal isolate and 4-hydroxypropofol reference standard showed a major ion at m/z 193. This was thought to be consistent with the ionized molecular ion (M-H)− of 4-hydroxypropofol, assuming a molecular weight of 194. A second ion was also detected at m/z 209, consistent with an ammoniated adduct of the (M-H)− ion. Figure 3 shows the collision-induced dissociation analyses of the isolated metabolite (A) and 4-hydroxypropofol standard (B) m/z 193 ions. Both spectra show minor fragment ions at m/z 177 and m/z 150. The m/z 177 was probably due to the loss of one oxygen atom, whereas the m/z 150 ion was consistent with the loss of one of the isopropyl groups.

Both propofol and 4-hydroxypropofol were similarly fluorescent with optimal excitation/emission wavelengths of 274 nm/300 nm and 288 nm/330 nm, respectively (Fig. 4). Transformation of 4-hydroxypropofol to the quinone by alkaline treatment resulted in a greater than 10-fold decrease in fluorescence.

Using the fluorescence detector, minimal detectable 4-hydroxypropofol concentrations by HPLC were 8 ng/ml (40 nM) at 288 nm/330 nm (excitation/emission wavelengths) and 30 ng/ml (156 nM) at 276 nm/310 nm. Detection by UV absorbance was over 30-fold less sensitive with minimal detectable 4-hydroxypropofol concentrations of 240 ng/ml (1.25 μM) at 288 nm (absorbance wavelength) and 1.9 μg/ml (10 μM) at 270 nm. Plots of 4-hydroxypropofol concentration versus peak height ratio were found to be linear from the lowest quantifiable concentration up to 10 μM. Recovery of 4-hydroxypropofol from the incubation mixture was 99 ± 3% over the concentration range examined.

Studies of metabolite stability showed that 4-hydroxypropofol standard dissolved in methanol converted into the quinone at an approximate rate of 1% per day, regardless of storage temperature. On the other hand, 4-hydroxypropofol dissolved in phosphate buffer or pure DMSO showed no decomposition over the course of these studies (1 month) at 4°C or less. Because solubility of 4-hydroxypropofol in phosphate buffer was relatively limited (<1 mM) compared with DMSO, stock solutions of 4-hydroxypropofol standard (5 mM) for these studies were maintained at −20°C in pure DMSO.

Stability studies of HPLC injectate solutions prepared from microsomal incubate were also conducted. At room temperature, concentrations of both metabolite and thymol changed by less than 5% over 24 h, whereas propofol concentration decreased by 19% over the same time period (average 0.8% decrease per hour). Consequently, when
samples were batch processed, total run times were kept to less than 12 h. For longer-term storage, it was found that samples could be maintained at 4°C for at least 2 weeks with less than 10% change in the concentration of measured compounds.

The mass balance study showed a linear relationship between propofol consumption and metabolite appearance at substrate concentrations from 5 to 100 μM with apparent conservation of mass ($y = 5 + 1.0563\times x; r^2 = 0.9497$).

At a substrate concentration of 200 μM, metabolite formation was linear with respect to time for up to a 15-min incubation (0.5 mg/ml microsomal protein concentration), and was linear with respect to protein concentration up to 0.6 mg/ml (15-min incubation). A similar study at 5 μM propofol concentration established linear metabolite formation up to a 10-min incubation (0.1 mg/ml microsomal protein concentration), and up to 0.1 mg/ml microsomal protein concentration (10-min incubation). At substrate concentrations between 5 and 100 μM, the intra-assay c.v. averaged 3.5%, whereas at a substrate concentration of 100 μM, the interassay c.v. was 6.5%.

Propofol concentrations versus reaction velocity data were adequately described by the Michaelis-Menten equation (Fig. 5). Derived values for apparent $K_{m}$ and $V_{max}$ were 7.3 μM and 3.8 nmol/mg/min, respectively.

FIG. 5. Effect of substrate concentration on propofol hydroxylase activity in liver microsomes from a male beagle dog.

A, activities are expressed as a reaction velocity: nanomoles of 4-hydroxypropofol formed per minute per milligram of microsomal protein. The solid line connects best-fit estimates of these data determined by nonlinear least-squares curve fit to the Michaelis-Menten equation. Estimates for apparent $K_{m}$ and $V_{max}$ were 7.3 μM and 3.8 nmol/mg/min, respectively. B, Eadie-Hofstee transformation of these data shown by plotting reaction velocity ($V; \text{nmol/mg/min}$) versus reaction velocity divided by propofol concentration at that velocity ($V/S; \text{nmol/mg/min/μM}$).

Interindividual variability in the rates of microsomal hydroxylation was greater in mixed-breed microsomes (c.v. = 84%; 11-fold range) than in beagle (c.v. = 57%; 4-fold range) and greyhound microsomes (c.v. = 57%; 4-fold range).

Discussion

Incubation of propofol with dog liver microsomes and an NADPH-regenerating system resulted in production of a single chromatographically discernable peak. This was subsequently identified as 4-hydroxypropofol based on the following criteria. Firstly, HPLC retention
times of microsomal metabolite and 4-hydroxypropofol standard were identical. Secondly, UV absorbance and fluorescence spectra of purified metabolite and 4-hydroxypropofol standard were superimposable. Thirdly, alkalization of both of these solutions, which transforms 4-hydroxypropofol to the quinone, resulted in a similar shift in the UV spectral profile and loss of fluorescence. Finally, negative-ion mass spectrometry of purified metabolite showed a major molecular ion (M-H) at *m/z* 193, which is consistent with a monohydroxylated derivative of propofol. In addition, the collision-induced fragmentation pattern of this ion indicated aromatic hydroxylation and was essentially identical with that produced by 4-hydroxypropofol standard. Although these findings do not exclude the possibility of this metabolite being 3-hydroxypropofol, such a regioisomer or its conjugates have not been identified by *H*-NMR of metabolites in dogs or any other species (Simons et al., 1991).

The physicochemical properties of both substrate and metabolite presented a number of challenges to the development of this assay. Propofol is relatively insoluble in aqueous solutions, requiring the addition of a small amount of DMSO as a solubility enhancer to ensure appropriate substrate concentrations. Although DMSO has been shown to adversely affect certain CYP-associated activities in human microsomes, concentrations less than or equal to 0.2% appear to have minimal effect (Chauret et al., 1998). In addition, we were unable to demonstrate any difference in propofol hydroxylase activity in incubates containing 0.4% compared with 0.2% DMSO. An alternative technique of substrate addition that we commonly use in this laboratory (and was attempted initially) involves the addition of propofol dissolved in methanol to an empty incubation tube, which is then dried down to remove methanol (Janicki et al., 1992). The substrate is then solubilized directly by addition of the microsomes. Results using this method were quite variable. Subsequent investigations indicated significant loss of propofol by evaporation. A similar problem was encountered during microsomal incubations and sample preparation before HPLC, which was resolved by using capped tubes for incubations and storing samples in sealed tubes at 4°C. Similar reports of significant loss of propofol from stored blood samples in pharmacokinetic studies support this finding (Weaver et al., 1995). Review of the techniques used in previous studies of the in vitro metabolism of propofol (Janicki et al., 1992; Audibert et al., 1993; Le Guelllec et al., 1995; Raoof et al., 1996; Guitton et al., 1997, 1998) indicates that in all but one study (Raoof et al., 1996) incubations were performed in open containers. It is therefore possible that evaporative losses of propofol during microsomal incubations adversely affected the accuracy and precision of data from these studies. Although pure 4-hydroxypropofol is stable when dry, previous work indicates instability of this compound when dissolved in aqueous solutions (Vree et al., 1987; Guitton et al., 1997). In contrast to these reports, we found that 4-hydroxypropofol was stable in aqueous solution, at least if buffered to pH 7.5. Instability, represented by slow decomposition to the quinone at the rate of approximately 1% per day, resulted from dissolving the compound in methanol, a common solvent used for stock solutions in in vitro metabolic studies. Review of the aforementioned papers revealed that methanol was in fact used to dissolve 4-hydroxypropofol in each case and is the most likely basis for the reported instability. 4-hydroxypropofol, however, has limited solubility in aqueous solutions. We therefore examined the potential of using pure DMSO for concentrated stock solutions of metabolite and found no evidence of decomposition after 1 month at 4°C or less. Apart from not promoting degradation, DMSO also has antioxidant properties and may also help to limit oxidation during long-term storage.

One other method for quantitation of 4-hydroxypropofol in microsomes has been reported (Guitton et al., 1997). This technique involves alkaline transformation of 4-hydroxypropofol to the quinone, organic extraction, and quantitation by gas chromatography-mass spectrometry (GC-MS) with selected ion monitoring. Although this method is quite sensitive (limit of quantitation: 25 ng/ml) and avoids any problems associated with decomposition of the metabolite to the quinone, it is somewhat more labor intensive and uses a less widely available analytical technique (GC-MS) compared with HPLC. Using fluorescence detection with detector settings optimized for 4-hydroxypropofol (288-nm emission/330-nm excitation wavelengths) we were able to achieve 2 to 3 times the sensitivity of GC-MS. Although more limited in sensitivity, it was also possible to use UV absorbance detection with the detector set at 288 nm for quantitation of 4-hydroxypropofol in microsomal incubates, provided injection volumes were increased to as much as 10 µl/injection. An alternative HPLC technique that we considered was to alkaline transform 4-hydroxypropofol to the quinone and then use UV detection at an absorbance wavelength of 258 nm. Based on our UV spectral data, we predicted as much as a 5-fold increase in sensitivity of quantitation for the quinone compared with 4-hydroxypropofol. Unfortunately, using the current HPLC system, the quinone and propofol had similar retention times and could not be satisfactorily resolved.

A previous in vivo study of propofol metabolism in dogs, rabbits, and rats indicates that although hydroxylation of one of the isopropyl groups occurs to a limited extent in rabbits (<7% total metabolism), 4-hydroxypropofol is the principal, if not sole, oxidative metabolite in these species (Simons et al., 1991). We were able to substantiate this finding using an in vitro mass balance technique. A similar study using human liver microsomes has also confirmed this to be true in that species (Guitton et al., 1998).

Hydroxylation of propofol to 4-hydroxypropofol appears to be a critical initial step in the metabolism and elimination of propofol in the dog (Simons et al., 1991). Consequently, we had hypothesized that breed differences in the pharmacokinetic clearance of propofol should...
be reflected by similar differences in the rate of propofol hydroxylation by hepatic microsomes. As a first step, we have shown that propofol hydroxylase activity is dependent on the presence of NADPH and is therefore most likely attributable to one or more CYP isoforms. We have also obtained preliminary enzyme kinetic parameters (K_m = 7.3 μM and V_max = 3.8 nmol/mg/min) for propofol hydroxylase activity in dog liver microsomes, which appear to be consistent with average values reported for human liver microsomes (K_m = 18 μM and V_max = 2.6 nmol/mg/min) (Guitton et al., 1998). Although this does not rule out the possible contribution of multiple CYP isoforms having similar K_m values, or making quantitatively small contributions. Finally, we have shown that there are significant breed-related differences in propofol biotransformation by dog liver microsomes. Greyhounds were compared with beagles, one of the most commonly used dog breeds in biomedical research, as well as to mixed-breed dogs. Confirming our original hypothesis, greyhounds showed on average 3-fold lower microsomal hydroxylase activities relative to beagles. This is similar to the reported difference in propofol clearance values between greyhounds (2-fold lower) and nongreyhound dogs (Zoran et al., 1993). Mixed-breed dogs were studied to give an indication of the possible range of activity values in the dog, which could be considered independent of the effect of breed. As expected, values from mixed-breed dogs were more variable (84% c.v. and greater than 10-fold range) and essentially spanned the range of greyhound and beagle data.

Relatively slow clearance of thiopental, thiamylal, and methohexitol has also been observed in greyhounds (Sams et al., 1985; Court, 1999). Like propofol, these are all short-acting general anesthetics and are likely to be primarily metabolized by hepatic CYP (Sams et al., 1985). On the other hand, there are no breed differences in the disposition of pentobarbital, a longer-acting anesthetic (Sams et al., 1985). Greyhounds are likely to metabolize other drugs relatively slowly. However, this has yet to be reported probably because the pharmacodynamic consequences of reduced metabolism of these unidentified drugs are not as remarkable as the consequences of slowed anesthetic drug metabolism.

Based on the foregoing discussion we speculate that the metabolism of a number of anesthetic drugs, including propofol, thiopental, thiamylal, and methohexitol is polymorphic in the dog. A review of the current literature indicates that this may be the first reported example of a drug metabolism polymorphism in the dog. Furthermore, we propose that greyhounds represent (or at least consist of a high proportion of) the “slow metabolizer” phenotype, which results from dysfunction of one or more CYP isoforms with propofol hydroxylase activity. In future studies we hope to identify CYP isoforms responsible for the metabolism of propofol, thiopental, thiamylal, and methohexitol in the dog and ascertain the biochemical and molecular genetic basis for the polymorphism. This is of importance not only because it will enable a more rational approach to pharmacotherapy of dogs in both clinical and research settings, but also because such a defect may serve as an animal model of similar drug metabolism polymorphism in humans.

Acknowledgments. We thank Dr. P. Beaune (Center Universitaire des Saints-Peres, Institut National de la Sante et de la Recherche Medicale U 490, Paris) and Dr. J. Guitton (Université Claude Bernard, Lyon) for obtaining the 4-hydroxypropofol standard.

References