

## Pharmacokinetics of 1,8-cineole, a dietary toxin, in the brushtail possum (*Trichosurus vulpecula*): Significance for feeding

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### Abstract

1,8-Cineole (cineole) is a *Eucalyptus* leaf toxin that defends against predation by herbivores such as the brushtail possum (*Trichosurus vulpecula*). The aim of the current study was to characterize the pharmacokinetics of cineole in the possum to improve understanding about how possums can avoid cineole toxicity when eating a *Eucalyptus* diet. Nine male possums were trapped in the wild and acclimated to captivity; a subcutaneous port was then implanted for venous blood sampling. Cineole was administered intravenously (10 and 15 mg kg<sup>-1</sup>) via a lateral tail vein and orally (30, 100 and 300 mg kg<sup>-1</sup>) by gavage, and blood concentrations of cineole and its metabolites were determined by gas chromatography. Cineole had a large terminal volume of distribution ( $V_z = 271 \text{ kg}^{-1}$ ) and a high clearance (43 ml min<sup>-1</sup> kg<sup>-1</sup>), equal to hepatic blood flow. The terminal half-life was approximately 7 h. Oral bioavailability was low ( $F = 0.05$ ) after low doses, but increased tenfold with dose, probably due to saturable first-pass metabolism. These findings indicate that when possums feed on a cineole diet, they eat until the cineole consumed is sufficient to saturate pre-systemic metabolism, leading to a rapid rise in bioavailability and cineole blood levels, and a cessation of the feeding bout. This is the first report on the pharmacokinetics of a dietary toxin in a wild herbivore, and provides insights into the interactions between the blood concentration of a plant secondary metabolite and the browsing behaviour of a herbivore.

**Keywords:** 1,8-Cineole, monoterpene pharmacokinetics, monoterpene metabolism, brushtail possum (*Trichosurus vulpecula*), herbivory

## Introduction

Plants produce a vast array of chemicals that have no evident role in plant metabolism and whose function is considered to be defensive (Fraenkel 1959; Freeland and Janzen 1974; Foley et al. 1999). These chemicals, termed plant secondary metabolites (PSMs), are thought to act by producing noxious effects in herbivores, although the mechanisms are mostly poorly understood. PSMs include terpenes, phenolics, glycosides, alkaloids and tannins, some of which have found uses as medicines (McLean and Duncan 2006). Since herbivores must eat plants, they have evolved countermeasures to overcome these chemical defences, principally by the metabolism of ingested PSMs and regulating their feeding to avoid consumption of intolerable amounts (Dearing et al. 2005). The entire field of xenobiochemistry, including the metabolism of therapeutic drugs, can be considered to relate to the development of offensive mechanisms by herbivores to overcome plant defensive chemicals (Gonzalez and Nebert 1990; Lewis et al. 1998; Karban and Agrawal 2002). A fuller understanding of plant–animal interactions requires a study of the pharmacokinetics, pharmacodynamics, and toxicology of plant secondary metabolites in mammalian herbivores.

*Eucalyptus* leaf is a major food for the brushtail possum (*Trichosurus vulpecula*) and other marsupial folivores despite it being well defended by PSMs. These defensive PSMs include monoterpenes such as 1,8-cineole (Lawler et al. 1999; Wiggins et al. 2003; Boyle and McLean 2004). Terpenes also constrain feeding in other plant–herbivore associations, such as *Juniperus monosperma* and generalist woodrats (*Neotoma algigula*; Dearing et al. 2000); Sitka spruce (*Picea sitchensis*) and red deer (*Cervus elaphus*; Duncan et al. 1994); and big sagebrush (*Artemisia tridentata*) and lambs (Burritt et al. 2000).

1,8-Cineole (cineole; eucalyptol; 1,3,3-trimethyl-2-oxabicyclo[2.2.2]octane) (Figure 1) is the major constituent of eucalyptus oil and is very lipophilic with a  $\log_{10}P$  octanol/water value of 2.74 (Griffin et al. 1999). Cineole has caused serious poisoning in humans after ingestion of even small doses ( $<1 \text{ g kg}^{-1}$ ; Tibballs 1995), and the oral  $\text{LD}_{50}$  in rats is  $2.5 \text{ g kg}^{-1}$  (Jenner et al. 1964).  $\text{LD}_{50}$  values have been reported in other species (Sweet 1987): Guinea pig,  $2.3 \text{ g kg}^{-1}$  intramuscularly (i.m.); dog,  $1.5 \text{ g kg}^{-1}$  subcutaneously (s.c.); and mouse,  $50 \text{ mg kg}^{-1}$  s.c.. Widely differing values have been reported elsewhere for the mouse:  $1070 \text{ mg kg}^{-1}$  s.c. and  $100 \text{ mg kg}^{-1}$  i.m. (Richardson 1993) and more than  $3.5 \text{ g kg}^{-1}$  orally (Santos et al. 2001), showing that these values need interpretation with caution. However, the brushtail possum can acclimate to an artificial diet with a daily cineole intake of about  $3.6 \text{ g kg}^{-1}$  (Lawler et al. 1999). This tolerance appears to be due to both metabolic and behavioural adaptations. Compared with humans and rats, cineole is more rapidly metabolized by possum liver microsomes, especially after induction through prior terpene exposure (Pass et al. 2001). Possums also regulate their rate of ingestion of dietary cineole, slowing their rate of feeding in response to higher levels of cineole in the diet (Wiggins et al. 2003) and observing a maximum daily intake of cineole (Boyle and McLean 2004). The present authors have also reported that possums will cease each feeding bout when the blood concentration of cineole reaches a critical level (Boyle et al. 2005). Although cineole intake appears to be regulated by plasma concentrations in possums, the specific mechanism (e.g. appetite suppression, toxicity) is not fully understood.

There have been previous reports on cineole pharmacokinetics in humans and laboratory and domestic animals. Inhalation exposure in mice (Kovar et al. 1987) and humans (Jager et al. 1996) showed a biphasic elimination but the data were limited. In particular, the doses were unknown, and blood was only collected for a short time (2 h in mice and 60 min in humans). The mice were exposed to rosemary oil, a mixture of seven terpenes including

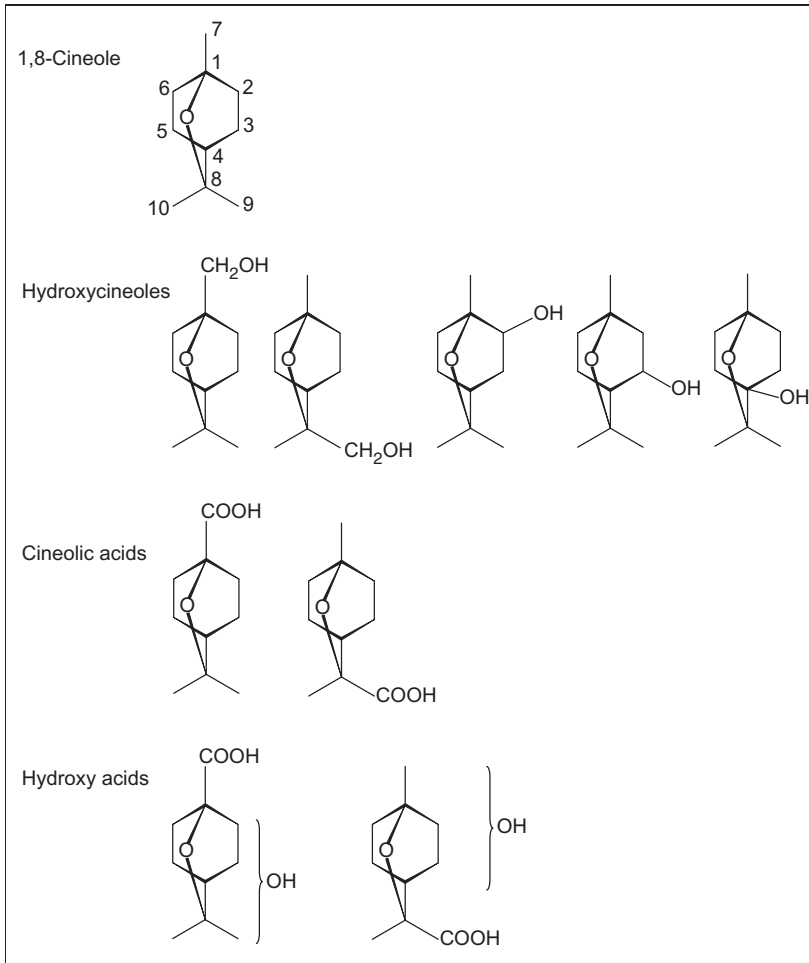


Figure 1. Cineole and its metabolites. Initial oxidation forms a hydroxycineole and oxidation of a second carbon forms a diol (structures not shown). 4-Hydroxycineole has only been found as a diol or hydroxy cineolic acid. The two primary alcohols can undergo further oxidation to form the two cineolic acids. When one of the primary alcohols has a second hydroxy group, the product of further oxidation is a hydroxy cineolic acid. There are many possible stereo-isomers of these metabolites (reviewed in Southwell et al. 1995).

39% cineole, allowing the possibility of metabolic interactions to affect pharmacokinetic values. Blood levels of cineole were also reported in humans after oral doses of myrtol (a mixture of cineole and  $\alpha$ -pinene), but this study was mainly assessing the influence of encapsulation on absorption (Zimmermann et al. 1995). A much fuller study provides pharmacokinetic data after intravenous and intra-ruminal dosing in lambs (Dziba et al. 2006), although this study did not include metabolite data. After intravenous infusion of cineole ( $40 \text{ mg kg}^{-1}$ ) over 40–50 min there was considerable variability in the terminal elimination half-life in different weeks of the study, ranging from 153 to 236 min. Oral doses ( $125 \text{ mg kg}^{-1}$ ) were given in oil as a bolus into the rumen, and the maximum plasma concentration was at 55 min, and terminal elimination half-life was 98 min.

The metabolism of cineole in the possum is known in general terms although the detailed structures have only been described for some metabolites (Carman et al. 1994;

Southwell et al. 1995; Carman and Garner 1996). The possum can oxidize cineole at all C-H sites (positions 7, 9 or 10, 2, 3, and 4; Figure 1), although 4-hydroxycineole is only found as the diol or hydroxyacid. Oxidation of the C7 or C9/10 methyl groups produces primary alcohols which can undergo subsequent oxidation to carboxylic acids. When oxidation also occurs at a second carbon, a number of possible diols and hydroxy acid metabolites can be formed. Quantitatively, the major urinary metabolites are hydroxycineolic acids (56% total metabolites), followed by cineolic acids (27%), hydroxycineoles (14%) and dihydroxycineoles (3%) (Boyle et al. 2000).

The metabolism becomes more complex because oxidation can convert the symmetrical cineole molecule into a chiral metabolite. Consequently, there are a large number of possible regio- and stereo-isomers of cineole metabolites. For example, 9-hydroxy-, 9-carboxy-, 2 $\alpha$ -hydroxy-, 3 $\alpha$ -hydroxy- and 3 $\beta$ -hydroxycineole are excreted in possum urine as partial racemates (Carman and Klika 1992; Carman et al. 1994). Although  $\alpha$  and  $\beta$  represent a trivial stereochemical nomenclature, the structures of metabolites are shown in the cited references. There has been a report that there is a sex difference in the enantiomeric ratio of the 9-hydroxy- and 9-carboxy cineoles, suggesting a possible pheromonal role for these metabolites (Carman and Klika 1992).

Microsomal studies have demonstrated the involvement of P450 enzymes in cineole metabolism, and shown species differences in regioselectivity. In human and induced rat liver microsomes the major metabolite was 2 $\alpha$ -hydroxycineole (Miyazawa et al. 2001), while 3 $\alpha$ -hydroxycineole has also been found in human microsomes (Duisken et al. 2005). Both these studies found that CYP3A enzymes catalysed cineole oxidation. In a comparative study, Pass et al. (2001) found that both possum and koala liver microsomes favoured oxidation at C9 over the ring carbons which were the major sites for rat and human liver microsomes. Inhibition by ketoconazole indicated that CYP3A enzymes were involved in 9-oxidation by possum liver microsomes (Pass and McLean 2002).

The present study details the pharmacokinetics of cineole and its metabolites in the brushtail possum. To the present authors' knowledge it is the first such study of a dietary PSM in a wild animal. Incorporation of PSM data into foraging models will enhance one's understanding of the ecology of herbivores and plants and could enable more effective management of change in habitat for both animals and plants.

## Materials and methods

### *Chemicals*

Cineole (99%) was supplied by Sigma Chemical Co. (Castle Hill, NSW, Australia) and (-)-linalool (3,7-dimethyl-3-hydroxy-1,6-octadiene; 97%) was from Robertet Natural Concentrates, supplied by Shiono Chemical Co. (Tokyo, Japan). Other chemicals and reagents were of analytical grade from commercial suppliers. Zoletil 50 (25 mg tiletamine plus 25 mg zolazepam per ml) was supplied by Virbac Australia Pty Ltd (Peakhurst, NSW, Australia).

### *Animals*

Possums were captured around the Hobart area and housed and maintained on an artificial terpene-free diet of chopped fruits and vegetable plus finely ground lucerne chaff as

previously described (Pass et al. 1999) The study was approved by the Parks and Wildlife Service (Tasmania) and the Animal Ethics and Experimentation Committee of the University of Tasmania. Only males were used to avoid possible gender differences as were reported in humans (Jager et al. 1996) and possums (Carman and Klika 1992); and because of the usual presence of pouch young in females. Animals remained within  $\pm 5\%$  of their capture weight ( $3.55 \pm 0.39$  kg; mean  $\pm$  standard deviation,  $n = 9$ ) throughout their captivity. All possums were allowed to acclimate to captivity and a terpene-free diet for a minimum of 4 weeks before experiments.

### Procedures

**Dosing.** As a dose-ranging study, two animals were given successive oral doses of cineole 30 and 300 mg kg<sup>-1</sup> followed by 15 mg kg<sup>-1</sup> intravenously (i.v.). Subsequently, four animals were given oral doses of 100 mg kg<sup>-1</sup> (twice) followed by 10 mg kg<sup>-1</sup> i.v. Three other animals were given a single oral dose of 100 mg kg<sup>-1</sup>. At least 1 week was allowed between doses, and this was sufficient to ensure that no cineole was detected in predose blood samples.

**Intravenous doses.** The i.v. dose was formulated by dispersing cineole (30 mg ml<sup>-1</sup>) in a 10% triglyceride emulsion (Intralipid 10% 500 ml, Baxter Healthcare Pty Ltd, Old Toongabbie, NSW, Australia). This method has been successfully used to administer other lipophilic substances intravenously (Knibbe et al. 1999; Musser et al. 1999). The cineole formulation was prepared aseptically by filtering the required volume of cineole (16.2 ml) directly into the sealed sterile triglyceride emulsion (500 ml) bottle using a 0.45 micron filter (Advantec MFS, Dublin, CA, USA), after first removing the equivalent volume of lipid emulsion. This was the smallest pore size through which cineole could be filtered. The bottle was then shaken vigorously before being placed on an orbital shaker overnight.

The stability and homogeneity of cineole in the emulsion was verified using the following assay. A 10 ml sample of the cineole lipid emulsion was removed aseptically from the sealed bottle and diluted 5000-fold (0.1 ml emulsion to 10 ml with methanol, then 1 ml methanol dilution to 50 ml with distilled water). A total of 50  $\mu$ l of the aqueous dilution were then transferred to a septum-sealed glass vial along with 25  $\mu$ l linalool standard (10 ng  $\mu$ l<sup>-1</sup>) and 25  $\mu$ l distilled water. Three replicates of each sample were prepared. Solid phase micro-extraction (SPME) was used to analyse the cineole concentration using the method for blood analyses (Boyle et al. 2002). Cineole concentrations were calculated from a calibration curve prepared with six cineole standards (10–400 ng 50  $\mu$ l<sup>-1</sup>). Two batches of cineole/lipid emulsion were assayed soon after formulation then again 2 months later. The combined mean  $\pm$  SD concentration for  $n = 6$  replicates was  $99.2\% \pm 1.4\%$  of the nominal concentration (30 mg ml<sup>-1</sup>).

Intravenous administration of cineole required the insertion of a catheter into a lateral tail vein. For this procedure possums were first anaesthetized with Zoletil (12 mg kg<sup>-1</sup>). The fur was clipped from the lateral aspects of the tail base and the insertion site cleaned. The catheter (3 Fr  $\times$  20 cm) was a single lumen (20 Ga) silicone midline catheter with a hydrophilic coated wire stylet and T Port extension (firstMidCath; Becton Dickinson Infusion Therapy Systems, Inc., Sandy, UT, USA). The catheter was inserted via a catheter introducer (Introsyte precision introducer, supplied with the firstMidCath), into the lateral tail vein and advanced proximally 15 cm to allow delivery of the cineole emulsion into the inferior vena cava. The mean dose volume was 1.2 ml injected manually over 2 min.

*Oral doses.* Oral doses of cineole ( $100 \text{ mg ml}^{-1}$ ) were formulated in 2% aqueous methyl cellulose 400 and administered by gavage using a paediatric nasogastric feeding tube (Boyle et al. 2002). Dose volumes were approximately 1, 5 and 10 ml for doses of 30, 100 and  $300 \text{ mg kg}^{-1}$ , respectively.

*Blood sampling.* Blood was taken from the jugular vein via a vascular access port (VAP model TI200; Access Technologies, Skokie, IL, USA), which was implanted subcutaneously at least 1 week before experiments began (Boyle et al. 2005). Blood samples (0.3 ml) were collected and immediately transferred to a heparinized glass vial which was chilled in ice/salt, and later stored at  $-18^\circ\text{C}$  until analysed (Boyle et al. 2005). Target collection times were 0, 5, 10, 20, 30, 40, 50 and 60 min and 1.5, 2, 3, 4, 6 and 8 h after oral dosing, with additional samples at about 3–4 min and 24 and 30 h after i.v. doses.

#### *Analysis of cineole*

Blood samples were thawed before analysis and  $50 \mu\text{l}$  analysed for cineole by headspace solid-phase microextraction and gas chromatography (Boyle et al. 2002). The limit of sensitivity was  $6.5 \text{ nM}$  cineole in blood.

#### *Extraction and chromatography of metabolites*

Conjugated and free metabolites of cineole were analysed in blood using methods modified from those previously developed for their urinary analysis (Boyle et al. 2000, 2001). Free metabolites were analysed by placing 0.1 ml thawed blood into a screw thread glass centrifuge tube and adding  $10 \mu\text{l}$  internal standard solution ( $0.1 \mu\text{g } \mu\text{l}^{-1}$  2,5-dimethyl benzyl alcohol in methanol), 0.3 ml saturated ammonium chloride solution and  $50 \mu\text{l}$  5 M HCl, vortexing briefly after the addition of each component. Metabolites were twice extracted with ethyl acetate ( $2 \times 1.5 \text{ ml}$ ) using a rotary mixer for 15 min followed by centrifugation at 2000 rpm for 5 min to separate the phases. The ethyl acetate extracts were combined and the volume reduced to 100–200  $\mu\text{l}$  with a rotary evaporator.

In some blood samples total metabolites were determined after hydrolysis of conjugates. Blood (0.1 ml) and internal standard solution ( $10 \mu\text{l}$ ) were mixed then buffered with 0.1 ml acetate buffer (1.1 M, pH 5.2) before adding  $20 \mu\text{l}$  extract of *Helix pomatia* (containing  $\beta$ -glucuronidase  $141\,000 \text{ units ml}^{-1}$  plus aryl sulfatase  $3950 \text{ units ml}^{-1}$ ; Boehringer Mannheim, Germany). The tubes were incubated at  $37^\circ\text{C}$  overnight, then saturated ammonium chloride (0.18 ml) and HCl were added and metabolites extracted as for the free metabolite assay.

Metabolites were derivatized with *N,O*-bis(trimethyl)trifluoroacetamide (BSTFA; Alltech Associates, Inc., Deerfield, IL, USA) to form trimethylsilyl derivatives of hydroxyl and carboxylic acid groups before analysis by gas chromatography mass spectrometry (GC-MS). About  $50 \mu\text{l}$  extract were transferred into a  $100 \mu\text{l}$  glass insert in an autosampler vial. BSTFA ( $25 \mu\text{l}$ ) was added and the vial was crimp sealed and heated at  $70^\circ\text{C}$  for 30 min. The Hewlett Packard instrument, HP-1 capillary column and operating conditions were as described before (Boyle et al. 2000), except that the injection volume was  $1 \mu\text{l}$ , He pressure was 15 psi, and the oven programme was  $60^\circ\text{C}$  to  $150^\circ\text{C}$  at  $5^\circ\text{C min}^{-1}$  then  $10^\circ\text{C min}^{-1}$  to  $290^\circ\text{C}$  and held for 9 min, with a solvent delay of 6 min. The instrument was programmed to analyse the derivatized metabolites in three groups by selected ion monitoring (SIM). The first group



(3- and 2-hydroxycineole and the internal standard) eluted before 14.5 min and were monitored by  $m/z$  108.1, 118.1, 119.1, 126.1 and 159.1. The second group (9- and 7-hydroxycineoles and -cineolic acids) eluted between 14.5 and 18 min and were monitored by  $m/z$  139.1, 183.1, 227.15 and 241.15. The third group (all hydroxycineolic acids) eluted after 18 min and was monitored by  $m/z$  93.05, 137.1, 156.1 and 227.15. A single ion was chosen for each metabolite for quantitation.

#### *Identification of metabolites*

The metabolites of cineole in the brushtail possum have been identified in urine (Boyle et al. 2000) enabling most of the blood metabolites to be recognized. Although 13 hydroxycineolic acids were at least partially identified in possum urine, only five were found at significant levels in blood, and their structures were not further reconciled because the original identifications were based on the methyl esters.

#### *Quantitation of metabolites*

Metabolite standards were isolated from brushtail possum and koala urine as described previously (Boyle et al. 2000, 2001) and their purities determined by GC-MS. The purities of 3-, 7- and 9-hydroxycineole were 92, 97 and 99%, respectively, and the ions monitored for quantitation were  $m/z$  159, 183 and 139, respectively. 2-Hydroxycineole could not be isolated and was quantitated using the 3-hydroxycineole calibration curve with correction for the different relative abundances of the ions measured ( $m/z$  108 (8.91%) and 159 (6.33%), respectively). The 7- and 9-cineolic acids could not be resolved and were used as the mixture (21 and 77%, respectively) and were quantitated by ions at  $m/z$  241 and 139, respectively. The five hydroxycineolic acids, whose structures were not further elucidated, were quantitated using the calibration curve for 7-hydroxy-9-cineolic acid (97% pure), a metabolite isolated from koala urine but not found in the brushtail possum. Again, the calibration curves were corrected for the differences in relative abundance of the ion monitored ( $m/z$  227): 227A (4.1%), 227B (12.1%), 227C (10.5%), 227D (8.3%), 227E (3.7%) and 7-hydroxy-9-cineolic acid (3.4%).

A mixed primary standard of the hydroxycineoles and cineolic acids and a primary standard of the single metabolite 7-hydroxy-9-cineolic acid were prepared in acetonitrile (about 5 mg of each compound in 25 ml). Primary standards were diluted with acetonitrile as appropriate before adding 5 or 10  $\mu\text{l}$  to 0.1 ml blood, as appropriate to produce the required concentrations (0.5–200  $\text{ng } \mu\text{l}^{-1}$  for the mixed standards and 10–1500  $\text{ng } \mu\text{l}^{-1}$  for the 7-hydroxy-9-cineolic acid). Calibration curves were prepared for each batch of analyses, and showed excellent sensitivity and linearity with  $r^2 > 0.99$ . All concentrations are of unconjugated metabolites, unless otherwise specified.

#### *Pharmacokinetic and statistical analyses*

Concentrations ( $C$ ) are expressed as nM or  $\mu\text{M}$  in blood. Concentration–time data were analysed by model-independent methods using Kinetica software (InnaPhase Corp., Philadelphia, PA, USA). After intravenous injection of cineole, the area under the curve to the last sampling time ( $\text{AUC}_{0-t}$ ) was calculated using the linear trapezoidal method, and the terminal elimination rate constant ( $\lambda_z$ ) by log-linear regression.  $\text{AUC}_{0-t}$  was extrapolated

to infinity ( $AUC_{0-\infty}$ ) by the addition of  $C_t/k$ , where  $C_t$  is the calculated concentration at the last sampling time. The terminal half-life ( $T_{1/2}$ ) was calculated as  $\ln 2/\lambda_z$ , systemic clearance (CL) as  $\text{dose}/AUC_{0-\infty}$ , and the post-distributional apparent volume of distribution ( $V_z$ ) as  $CL/\lambda_z$ . Mean residence time (MRT) was calculated as:

$$\frac{AUMC_{0-\infty}}{AUC_{0-\infty}},$$

where  $AUMC_{0-\infty}$  is the area under the first moment curve ( $C_t$  versus time). The apparent volume of distribution at steady-state ( $V_{ss}$ ) was the product of clearance and MRT. The extent of metabolite accumulation ( $R_m$ ) was given by the ratio of AUC metabolite/cineole. The maximum blood concentration ( $C_{max}$ ) and the corresponding time ( $T_{max}$ ) were the actual values directly derived from the concentration-time curves. Where the  $AUC_{0-\infty}$  was not adequately determined,  $AUC_{0-t}$  was used. Cineole and its metabolites derived after oral administration were treated as having been administered extravascularly. Systemic bioavailability ( $F$ ) was calculated as the ratio of  $AUC_{0-\infty}$  oral/intravenous corrected for the difference in doses (intravenous dose/oral dose). The fraction of elimination associated with the terminal elimination phase ( $f_2$ ) was calculated as:

$$\frac{\beta/\lambda_z}{AUC_{0-\infty}},$$

where  $\beta$  is the zero time intercept of the exponential terminal elimination line.

For the four animals given  $100 \text{ mg kg}^{-1}$  oral and  $10 \text{ mg kg}^{-1}$  i.v. doses, some blood samples (usually 6–12 after each dose) were analysed twice, before and after hydrolysis, to determine the percentage conjugation of each metabolite. The data were averaged for each animal, and mean  $\pm$  SD values for the four animals calculated.

Where values appeared to be normally distributed they are presented as mean  $\pm$  SD, and comparisons were made by analysis of variance (ANOVA) or Student's *t*-test for pairs of means. Otherwise, data are presented as median (range) and paired groups were analysed using the Wilcoxon test.

## Results

### *Identification of cineole metabolites*

This study only quantitated the major metabolites found in the blood: Namely four hydroxycineoles (2-, 3-, 7- and 9-); two cineolic acids (7- and 9-); and five hydroxycineolic acids. Because the derivatization procedure omitted the methylation step that we previously used before trimethylsilylation, it was not possible to assign individual structures to the hydroxyacid metabolites and they were identified by letters A–E in order of elution. Gas chromatography did not resolve all components, and quantitation by selected ion monitoring avoided these interferences and also ensured adequate sensitivity.

### *Blood concentrations after intravenous doses*

The blood concentrations of cineole and its metabolites after an intravenous dose of  $10 \text{ mg kg}^{-1}$  are shown for one possum in Figures 2 and 3, and mean pharmacokinetic values for six animals in Table I. Two animals were given a  $15 \text{ mg kg}^{-1}$  dose but, as their



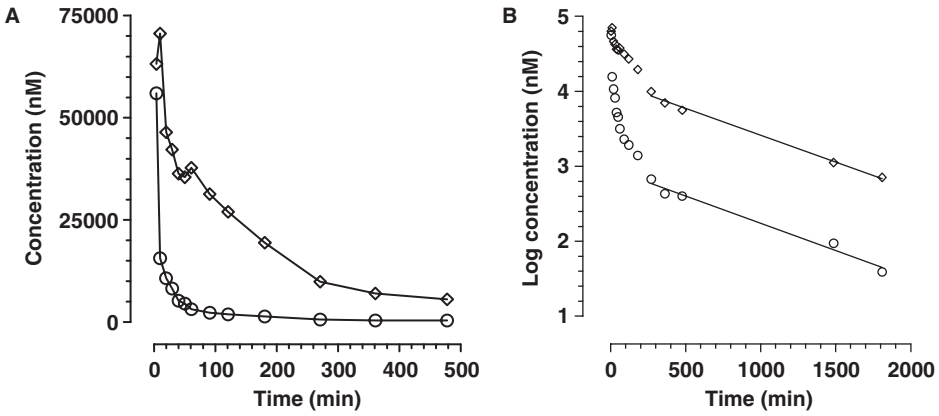


Figure 2. (A) Blood concentrations of cineole (o) and total metabolites (◇) after an intravenous dose of cineole  $10 \text{ mg kg}^{-1}$  in a single representative possum; (B) The same data, plotting log concentrations up to 2000 min.

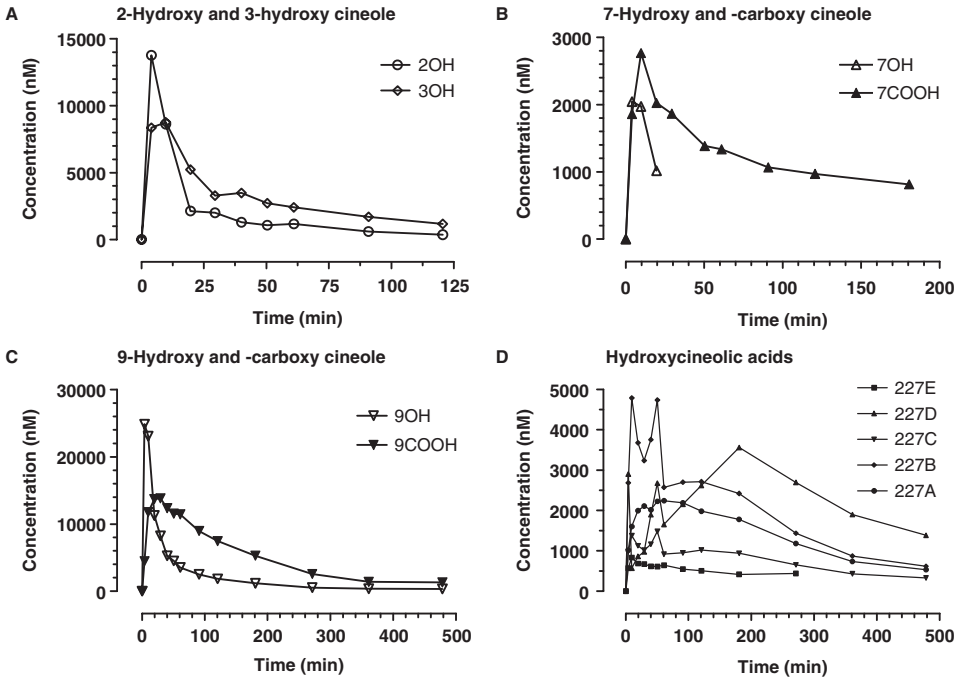


Figure 3. Blood concentrations of individual metabolites after the intravenous dose of cineole shown in Figure 2.

pharmacokinetic parameters were similar to those given  $10 \text{ mg kg}^{-1}$ , the concentration-dependent variables were changed in proportion to dose to enable their inclusion in the mean data.

After intravenous administration the cineole concentration fell rapidly with a correspondingly rapid formation of metabolites (Figure 2). The total metabolite concentration exceeded that of cineole from the first blood sample (taken at  $3.8 \pm 0.7 \text{ min}$ ), peaked early

Table I. Pharmacokinetic parameters for cineole and its metabolites after intravenous administration of cineole 10 mg kg<sup>-1</sup> to the brushtail possum.<sup>a</sup>

	Cineole	Hydroxy-cineoles	Cineolic acids	Hydroxy-cineolic acids
Dose (μmoles)	225 ± 27	–	–	–
C <sub>max</sub> (μM)	47.8 ± 14.7	36.9 ± 12.4	14.6 ± 5.2	17.8 ± 5.3
T <sub>max</sub> (min)	3.8 ± 0.7	8.2 ± 3.6	19.8 ± 11.4	76.4 ± 82.0
AUC <sub>0-∞</sub> (μM min)	1551 ± 323	1409 ± 390	1728 ± 681	10 760 ± 3099
T <sub>1/2</sub> (min)	426 ± 108	122.1 ± 80.4	144.1 ± 61.5	629.3 ± 267.1
CL (ml min <sup>-1</sup> kg <sup>-1</sup> )	43.0 ± 7.3	–	–	–
MRT (min)	294.2 ± 67.5	87.8 ± 34.6	160.1 ± 59.4	861.3 ± 435.8
V <sub>z</sub> (l kg <sup>-1</sup> )	26.5 ± 8.4	–	–	–
V <sub>ss</sub> (l kg <sup>-1</sup> )	12.8 ± 4.3	–	–	–
R <sub>m</sub>	–	0.98 ± 0.32	1.10 ± 0.46	4.40 ± 1.58

Data are the mean ± standard deviation ( $n = 6$ ). Animals weighed  $3.46 \pm 0.41$  kg.

<sup>a</sup>Two animals were given 15 mg kg<sup>-1</sup>, and their concentration-dependent values were adjusted by 10/15.

( $T_{\max} = 10.3 \pm 1.1$  min) and had considerably higher blood levels than cineole ( $R_m = 8.0 \pm 1.6$ ). The sequential formation of hydroxy- and carboxy-metabolites is clearly seen in Figure 3. The initial oxidation of ring (2- and 3-) and methyl (7- and 9-) carbons to form hydroxycineoles was very rapid (average  $T_{\max}$  for all alcohol metabolites was  $8.2 \pm 3.6$  min; Table I). The subsequent oxidation of the two primary alcohols (7- and 9-) to the corresponding cineolic acids was also fairly rapid ( $T_{\max} = 19.8 \pm 11.4$  min). Individual hydroxycineolic acids showed marked variability in  $T_{\max}$  ( $76.4 \pm 82.0$  min) and a mostly slower rate of accumulation (Figure 3), commensurate with the additional steps required for their formation. The apparent  $T_{1/2}$  of hydroxycineolic acids ( $629 \pm 267$  min) is probably an overestimate due to their continued formation well after the maximum level had passed. Evidence for this is the high proportion of the AUC<sub>0-∞</sub> of hydroxycineolic acids that was extrapolated after the last sampling time (AUC% extra):  $37.2 \pm 27.4\%$ , whereas for cineole it was  $4.4 \pm 2.6\%$ ; hydroxycineoles  $2.2 \pm 1.4\%$ ; and cineolic acids  $8.3 \pm 6.8\%$ .

Although cineole was rapidly metabolized its terminal elimination was slow ( $T_{1/2} = 426 \pm 108$  min), and not significantly different from the terminal elimination of total metabolites ( $292 \pm 91$  min, paired  $t$ -test,  $p > 0.05$ ,  $n = 6$ ). The mean clearance of cineole was  $43.0 \pm 7.3$  ml min<sup>-1</sup> kg<sup>-1</sup>. The slow terminal elimination is consistent with the large apparent volumes of distribution ( $V_z = 26.5 \pm 8.4$  l kg<sup>-1</sup>;  $V_{ss} = 12.8 \pm 4.3$  l kg<sup>-1</sup>). At steady-state, the amount of drug remaining in the body is the product of  $V_{ss}$  and  $C$ . After 6 h the mean concentration of cineole was 463 nM, indicating that only about 20.5 μmoles (9% dose) remained in the body.

The depressant effect of cineole on the central nervous system (CNS) was evident after each intravenous dose. Possums were anaesthetized with Zoletil to allow placement of the tail vein catheter but were usually showing signs of arousal by the time cineole was administered. The cineole caused an immediate decline in the level of consciousness lasting up to 40 min. The effects of cineole appeared to be additive to the anaesthetic effect of Zoletil.

#### Blood concentrations after oral doses

The blood concentrations of cineole and three groups of metabolites following an oral dose of cineole (100 mg kg<sup>-1</sup>) are shown for one possum in Figure 4, and pharmacokinetic data

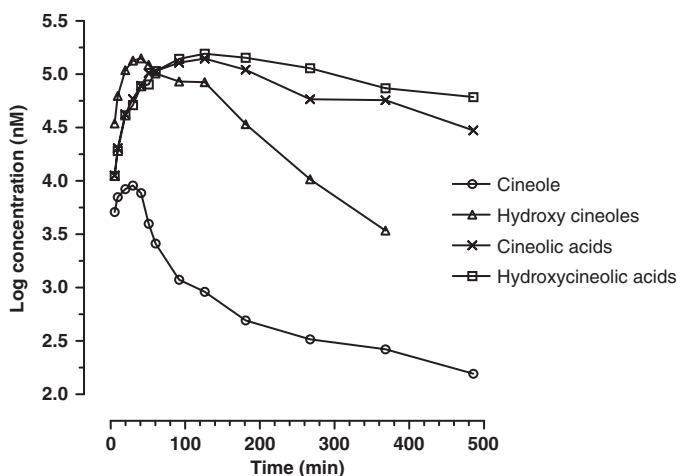


Figure 4. Blood concentrations (log scale) of cineole and its metabolites (in groups) after an oral dose of cineole  $100 \text{ mg kg}^{-1}$  in a single representative possum.

Table II. Pharmacokinetic parameters for cineole after oral administration of cineole to the brushtail possum.

Dose rate ( $\text{mg kg}^{-1}$ ) ( <i>n</i> )	30 <sup>a</sup> (2)	100 <sup>b</sup> (7)	300 <sup>a</sup> (2)
Dose ( $\mu\text{moles}$ )	698 (696–700)	2165 (1977–2710)	6982 (6963–7002)
$C_{\text{max}}$ ( $\mu\text{M}$ )	6.7 (4.5–9.9)	11.5 (2.0–107.6)	89.2 (68.6–109.8)
$T_{\text{max}}$ (min)	5.3 (5.0–5.6)	19.6 (4.8–30.0)	15.0 (10.0–20.0)
$\text{AUC}_{0-t}$ ( $\mu\text{M min}$ )	146 (128–164)	809 (97–6543)	12 845 (9882–15 808)
$T_{1/2}$ (min)	70 (60–81)	190 (137–312)	194 (125–263)
$F^c$	0.047 (0.034–0.057)	0.054 (0.008–0.685)	0.394 (0.346–0.443)

Data are the median (range). Metabolite data are shown in Table III.

<sup>a</sup>These animals were previously given the  $15 \text{ mg kg}^{-1}$  i.v. dose of cineole. Blood was collected for 360 min.

<sup>b</sup>Four of animals were previously given the  $10 \text{ mg kg}^{-1}$  i.v. dose of cineole. Blood was collected for 380 min.

<sup>c</sup> $F$  was calculated using  $\text{AUC}_{0-t}$  for the same time after oral and intravenous doses. Mean i.v. data were used to calculate  $F$  for the three animals which were not given an i.v. dose.

for nine possums and three doses in Tables II and III. There was considerable individual variation in some of the values, and the data are reported as median and range. Cineole was rapidly absorbed although  $T_{\text{max}}$  became longer as the dose increased (median  $T_{\text{max}} = 5\text{--}20$  min; Table II). Cineole metabolites were formed and accumulated in blood even more rapidly than the parent compound (Figure 4). Initial oxidation to the alcohol metabolites was followed in turn by their disappearance as the alcohols were further oxidized to cineolic acids and hydroxycineolic acids. This metabolic sequence was most clearly seen after the lowest dose of cineole, where the  $T_{\text{max}}$  values were in the order cineole < hydroxycineoles < cineolic acids < hydroxycineolic acids.

The median oral bioavailability of cineole was very low (0.05) after doses of 30 and  $100 \text{ mg kg}^{-1}$ , but increased to 0.40 after  $300 \text{ mg kg}^{-1}$ . Two of the seven animals given the  $100 \text{ mg kg}^{-1}$  dose showed a much higher bioavailability (0.21, 0.69) than the other five (range 0.01–0.07). The  $100 \text{ mg kg}^{-1}$  dose was repeated 1 week later in four animals, and there was no significant difference in the pharmacokinetic parameters  $F$ ,  $C_{\text{max}}$ ,  $T_{\text{max}}$  and  $\text{AUC}$  ( $p > 0.05$ , Wilcoxon matched pairs test), suggesting that the variability was due to real differences between individual animals rather than random error.

Table III. Pharmacokinetic parameters for cineole metabolites after oral administration of cineole to the brushtail possum.<sup>a</sup>

Dose rate (mg kg <sup>-1</sup> ) (n)	30 (2)	100 mg kg <sup>-1</sup> (7)	300 mg kg <sup>-1</sup> (2)
<i>Hydroxycineoles</i>			
$C_{max}$ (μM)	18.1 (17.9–18.3)	141.1 (70.4–240.3)	257.1 (159.2–355.0)
$T_{max}$ (min)	25.0 (10.0–40.0)	38.3 (19.0–48.9)	187.0 (134.0–240.0)
AUC <sub>0-t</sub> (μM min)	1611 (1427–1794)	12 926 (9420–37 969)	72 961 (43 531–102 391)
$T_{1/2}$ (min)	99 (92–105)	83 (53–110)	261 (221–302)
$R_m$	11.1 (11.0–11.2)	13.2 (5.8–113.2)	5.4 (4.4–6.5)
<i>Cineolic acids</i>			
$C_{max}$ (μM)	36.5 (33.5–39.5)	133.3 (95.7–170.5)	97.3 (84.8–109.8)
$T_{max}$ (min)	60 (60–60)	119 (91–126)	360 (360–360)
AUC <sub>0-t</sub> (μM min)	6362 (5418–7306)	31 217 (23 049–41 105)	25 324 (24 504–26 142)
$T_{1/2}$ (min)	68 (62–74)	125 (105–228)	— <sup>b</sup>
$R_m$	45.2 (33.1–57.2)	30.4 (4.5–69.5)	2.1 (1.6–2.7)
<i>Hydroxycineolic acids</i>			
$C_{max}$ (μM)	42.5 (26.8–58.1)	156.0 (74.5–228.3)	252.4 (123.3–381.5)
$T_{max}$ (min)	104 (60–148)	119 (119–181)	300 (240–360)
AUC <sub>0-t</sub> (μM min)	8232 (6276–10 188)	46 079 (16 479–61 593)	42 600 (16 078–69 122)
$T_{1/2}$ (min)	162 (125–199)	177 (111–246)	— <sup>b</sup>
$R_m$	59.1 (38.3–79.8)	50.5 (9.4–169.9)	3.0 (1.6–4.4)

Data are median (range).

<sup>a</sup>These are the same experiments as in Table II.

<sup>b</sup>Concentrations were not followed for long enough to establish  $T_{1/2}$ .

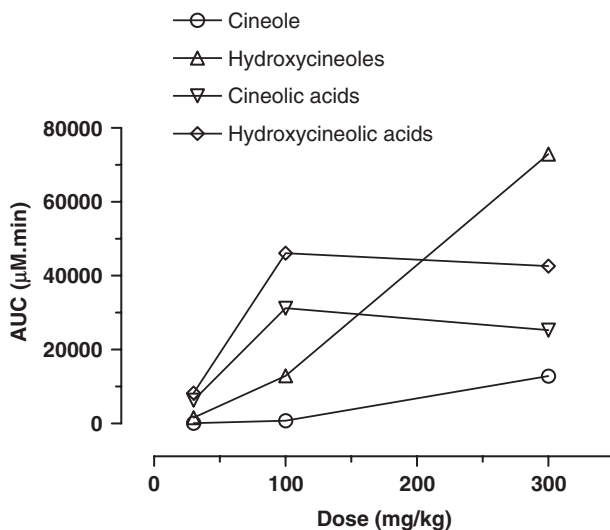


Figure 5. Effect of size of oral dose on median AUC<sub>0-t</sub> of cineole and its metabolites.

The median AUC<sub>0-t</sub> of cineole and its metabolites was plotted against oral dose and shown in Figure 5. From 30 to 100 mg kg<sup>-1</sup> AUC<sub>0-t</sub> increased with dose for cineole and its metabolites, but from 100 to 300 mg kg<sup>-1</sup> there was no increase in AUC for the acid metabolites, and hydroxycineoles became the major metabolites for the first 6–8 h after the dose. Thus although the initial oxidation of carbon increased with dose, the subsequent

oxidation of the alcohol metabolites to carboxylic acids appeared to become saturated by  $300 \text{ mg kg}^{-1}$ . Values for other pharmacokinetic parameters ( $T_{\text{max}}$ ,  $T_{1/2}$  and  $R_m$ ) were generally in agreement after the two lower doses, but not after the highest dose, which is also evidence of dose-dependent kinetics.

After oral doses blood was sampled for up to 480 min, which was generally insufficient time to establish terminal elimination constants, especially when the absorption of cineole was prolonged. Therefore, the  $T_{1/2}$  values in Tables II and III only describe the disappearance of cineole and its metabolites during this early period when elimination occurred together with formation and distribution, and the intravenous data better describe the terminal elimination phase.

#### *Conjugation of metabolites*

The extent of conjugation varied amongst the hydroxycineoles: 3-hydroxy,  $29.4\% \pm 1.4\%$ ; 2-hydroxy,  $36.0\% \pm 10.7\%$ ; 9-hydroxy,  $63.9\% \pm 7.8\%$ ; and 7-hydroxy,  $88.7\% \pm 4.1\%$ . The differences in conjugation amongst individual cineolic acids and hydroxycineolic acids were not significant and the data were combined, giving  $40.3\% \pm 17.2\%$  and  $27.6\% \pm 4.1\%$ , respectively.

## **Discussion**

#### *Cineole metabolites*

Oxidation was most facile at carbon 9, with higher blood concentrations of 9-hydroxy- and 9-carboxy-cineole than their positional isomers. The methyl hydroxyl groups (C7 and C9) were mostly further oxidized to the corresponding carboxylic acids, and hydroxycarboxylic acids were the major group of metabolites formed. Conjugation was also more extensive on the methyl alcohols (7 and 9) than the alicyclic moiety (2 and 3). These findings agree with previous reports on the metabolism of cineole in the brushtail possum: The excretion of urinary metabolites (Boyle et al. 2000) and the formation of metabolites by liver microsomes *in vitro* (Pass et al. 2001). In contrast, oxidation was primarily at C2 in human microsomes and C3 in rat microsomes (Miyazawa et al. 2001; Pass et al. 2001; Duisken et al. 2005). The extent of conjugation of metabolites in blood decreased with their polarity, as found for the urinary metabolites (Boyle et al. 2000).

#### *Intravenous dosing kinetics*

This is the first report on the metabolite pharmacokinetics of cineole, and one of few on any terpene, despite their importance as dietary constituents and in herbal and pharmaceutical preparations (Kohlert et al. 2000). After intravenous injection of cineole there was a prolonged distribution phase before a slower terminal elimination phase began, on average after the blood collection time of  $206 \pm 56 \text{ min}$  ( $n = 6$ ). The large volume of distribution shows that cineole was extensively taken up by tissues. The terminal distribution ( $V_z = 271 \text{ kg}^{-1}$ ) was twice the steady-state value ( $V_{\text{ss}} = 131 \text{ kg}^{-1}$ ), indicating that appreciable elimination occurs during the distribution phase. This can be confirmed by calculation of the fraction of elimination associated with the last exponential term ( $f_2$ ), which was

$58.5 \pm 13.7\%$  ( $n=6$ ). Therefore, about 40% of cineole was eliminated during the distribution phase.

This extensive distributional elimination is due to the relatively high concentrations of cineole presented to the organs of elimination together with their high capacity for elimination, shown by the CL value of  $43 \text{ ml min}^{-1} \text{ kg}^{-1}$ . This latter clearance value agrees extremely well with the published value for hepatic blood flow in the brushtail possum ( $42.5 \text{ ml min}^{-1} \text{ kg}^{-1}$ ; McDonald and Than 1976), suggesting that the rate of hepatic metabolism is only limited by the rate of perfusion and not by enzyme activity, except after high doses. The slow terminal elimination rate ( $T_{1/2} = 426 \text{ min}$ ) can be explained by the large terminal volume of distribution  $V_z$ .

Although some other studies have reported cineole to have a much shorter half-life, this is based on brief sampling times (1–2 h), which is insufficient to describe the terminal elimination rate. After intravenous infusion of cineole in lambs, the terminal elimination half-life was 202 min (Dziba et al. 2006). However, this was based on a 6-h sampling period, and later analyses might have found a longer half-life. The longer half-life of cineole reported here is commensurate with those found in humans for two other monoterpenes:  $\alpha$ -pinene (695 min) and limonene (750 min) (Falk et al. 1990; Falkfilipsson et al. 1993).

Elimination of cineole is almost entirely due to metabolism, as urinary and faecal excretion of unchanged cineole is negligible (Boyle et al. 2000; Boyle and McLean 2004) and respiratory elimination of intravenously injected monoterpenes generally is 1.5–5.0% (Kohlert et al. 2000). Metabolites were found in the first blood samples, and their total concentration was greater than that of cineole at all times (Figure 2). This is further evidence of the rapid metabolism of cineole, although the amounts formed of each metabolite cannot be estimated from their blood concentrations since their volumes of distribution are unknown.

A lipid emulsion was used as the vehicle for the cineole injection to avoid adverse effects, such as have been reported after i.v. administration of halothane (Musser et al. 1999). For the same reason the bolus dose was administered slowly, as recommended by the manufacturer of Intralipid, since the vehicle alone can cause fever, thrombosis and other adverse effects (Intralipid product information, Baxter Healthcare Pty Ltd, NSW, Australia, 2001). However, use of this lipid emulsion vehicle has been shown to affect the pharmacokinetics of propofol by decreasing the volume of distribution compared with an ethanol vehicle, although the elimination parameters were unchanged (Dutta and Ebling 1998). There were significant differences between this propofol study and the present work, indicating caution when applying their findings: Propofol is a phenol and, therefore, chemically dissimilar from cineole; its steady-state volume of distribution ( $3.21 \text{ kg}^{-1}$ ) was much less than that of cineole; and arterial blood was sampled instead of venous blood. However, even if the same vehicle-related effects occurred with cineole, it would not alter the essential findings of a large volume of distribution and a rapid metabolic elimination.

Adverse effects of terpenes on animals and humans have been reported in the literature and generally indicate CNS depression as a major mode of action for toxicity (Jenner et al. 1964; Patel and Wiggins 1980; Webb and Pitt 1993; Whitman and Ghazizadeh 1993). In the present study, there was evidence of CNS depression after the i.v. doses but not after the oral doses when blood levels of cineole were lower. Sleepiness and ataxia were also evident after an i.v. infusion of cineole ( $40 \text{ mg kg}^{-1}$  over 40–50 min) in lambs (Dziba et al. 2006). Although possums are unlikely to ingest enough cineole in the wild to reach equivalent blood levels, even a minor degree of CNS depression could be hazardous for arboreal animals.

The present authors encountered some practical difficulties during the sampling of blood from possums. Possums are wild, nocturnal animals and this made it very difficult to collect



blood samples during their active phase overnight (8–24 h after dosing). Furthermore, blood samples collected throughout the next day approached the analytical limit of the assay (Boyle et al. 2002).

#### *Oral dosing kinetics*

After oral administration cineole was rapidly absorbed and oxidized to its alcohol and carboxylic acid metabolites (Figure 4). There was considerable individual variability in pharmacokinetic parameters after the 100 mg kg<sup>-1</sup> dose, and this was confirmed when the dose was repeated in four animals. Individual variability in pharmacokinetics is a characteristic of drugs that have a low bioavailability due to extensive first-pass metabolism (Tam 1993; Hellriegel et al. 1996). Unlike inbred strains used as laboratory animals, brushtail possums are wild animals with variations in genetic and physiological status making them more like the human population with respect to individual variability in pharmacokinetics.

The bioavailability of cineole was very low for most animals after the 30 and 100 mg kg<sup>-1</sup> doses (median  $F=0.05$ ), and much higher after the 300 mg kg<sup>-1</sup> dose (median  $F=0.39$ ). No unchanged dietary cineole is excreted in the faeces (Boyle and McLean 2004), so the oral absorption is complete. Although efflux transporters such as P-glycoprotein are likely to regulate the absorption of some plant secondary metabolites in herbivores (Sorensen and Dearing 2006), it seems unlikely that they play a major role here. Even for drugs that are good substrates, efflux transport does not usually impede absorption when drugs are given at doses large enough (>50 mg) to exceed the low  $K_m$  values ( $\mu\text{M}$ ) of transporters (Lin and Yamazaki 2003). Efflux transport of well-absorbed drugs can increase residence time in the gut and prolong exposure to enterocyte enzymes, thus enhancing the intestinal metabolism. Although there is no direct evidence of enterocyte metabolism of cineole, it seems likely given the presence of CYP3A enzymes and their involvement in hepatic cineole metabolism. Therefore, the low oral bioavailability is most probably due to rapid metabolism in the liver and gastrointestinal tract during the absorption phase. Thus, after the lowest dose when  $F=0.05$ , only 5% of the dose reaches the post-hepatic circulation, and 95% is metabolized in the first pass through gut and liver. Figure 4 dramatically illustrates the rapid metabolism of oral doses, where the metabolite levels in blood greatly exceeded those of cineole at all sampling times. These results are consistent with extensive first-pass metabolism which, in most animals, became saturated at a dose between 100 and 300 mg kg<sup>-1</sup>, but for two animals was saturated at a lower dose.

The dose dependency of  $F$  is very likely due to saturable first-pass metabolism, as rapid absorption leads to high concentrations of cineole which exceed the capacity of enzymes in gastrointestinal and hepatic cells (Rowland and Tozer 1995). After an intermediate dose, differences between individuals in the Michaelis constant,  $K_m$ , of oxidizing enzymes will result in differences in bioavailability and other pharmacokinetic parameters. This occurs for a number of orally administered drugs that are highly extracted by the liver or intestinal tissues (Rowland and Tozer 1995). As expected, there was much less variability in pharmacokinetics after i.v. dosing even although  $C_{\text{max}}$  was much higher after i.v. doses (48  $\mu\text{M}$ ) than after the two lower oral doses (7 and 12  $\mu\text{M}$ ), because of avoidance of the first-pass effects of the gut and liver.

Cineole is oxidized to its alcohol metabolites by cytochrome P450 enzymes, notably CYP 3A4 and 3A5 in rat and human microsomes (Miyazawa et al. 2001; Duisken et al. 2005). The subsequent oxidation of the 7- and 9-hydroxycineoles to carboxylic acid metabolites is

presumably carried out by non-microsomal alcohol and aldehyde dehydrogenases. This is supported by the observation that there was little carboxylic acid formation when cineole was metabolized by possum liver microsomes *in vitro* (Pass et al. 2001). The  $K_m$  values for hydroxylation by microsomes from uninduced possums ranged from  $5\ \mu\text{M}$  for 7-hydroxylation to  $8\ \mu\text{M}$  for 9-hydroxylation, and for terpene-exposed possums from  $6\ \mu\text{M}$  for 3-hydroxylation to  $28\ \mu\text{M}$  for 2-hydroxylation (Pass et al. 2001). After the highest oral dose the concentrations in enterocytes and hepatocytes, which would initially be even greater than the maximum blood concentration ( $89\ \mu\text{M}$ ), would be expected to exceed these  $K_m$  values and saturate the enzymes. After the lower oral doses,  $C_{\text{max}}$  was similar to the  $K_m$  values and therefore liable to saturate enzymes for a briefer period.

The accumulation of metabolites ( $R_m$ ) was much greater after oral doses than *i.v.*, except after the highest oral dose when metabolism was saturated. This provides further evidence of extensive first-pass metabolism (Rowland and Tozer 1995). However, additional studies of metabolism by gut tissue will be required to confirm this. Figure 5 shows how the  $\text{AUC}_{0-t}$  of cineole and its metabolites varied with dose. The accumulation of cineole increased with dose, as did the formation of its hydroxy metabolites; however, the formation of carboxylic acid metabolites appeared to be saturated at the  $100\ \text{mg kg}^{-1}$  dose level. This suggests that the initial oxidation of cineole by a P450 enzyme is much less capacity-limited than is the subsequent oxidation of the alcohol to carboxylic acid by alcohol dehydrogenase. The accumulation of the hydroxycineole metabolites after the  $300\ \text{mg kg}^{-1}$  dose reflects the greater saturability of their further oxidation compared with their formation.

Ethyl alcohol is a well-known example of an alcohol whose metabolism is saturable in humans with a  $K_m$  of between  $100$  and  $760\ \mu\text{M}$  (Levitt and Levitt 1998; Norberg et al. 2003). After cineole doses of  $100$ – $300\ \text{mg kg}^{-1}$ , the  $C_{\text{max}}$  of hydroxycineoles measured in the jugular vein was  $141$ – $257\ \mu\text{M}$ , and therefore much higher in the portal and hepatic circulation during absorption, and plausibly high enough to exceed the  $K_m$  of alcohol dehydrogenase.

The elimination rates of cineole and its metabolites after oral dosage, reported as  $T_{1/2}$  (Tables II and III), are not directly comparable with the intravenous values for  $T_{1/2}$  (Table I). This is because the duration of sampling after the oral doses was not long enough to describe terminal elimination, and therefore the  $T_{1/2}$  value appears to be a hybrid with the distribution phase. It does, however, give a better measure of the fall in blood cineole concentration between frequent doses (Rowland and Tozer 1995), such as when a herbivore consumes cineole in a series of feeding bouts on eucalyptus leaf.

#### *Pharmacokinetic consequences for browsing behaviour*

Data from the present study on the pharmacokinetics of cineole after single doses can be combined with previous findings on blood levels during feeding on a cineole diet (Boyle et al. 2005) to explain how the brushtail possum copes with dietary cineole. When offered a diet containing between 1 and 4% cineole, possums ate until the (jugular vein) blood concentration of cineole reached  $52\ \mu\text{M}$ , having consumed on average  $335\ \text{mg}$  ( $2172\ \mu\text{moles}$ )  $\text{kg}^{-1}$  cineole during an initial feeding bout. The duration of feeding bouts varied with the percentage cineole in the diet (from 7 min on 4% to 16 min on 1%), and therefore the overall speed of absorption was correspondingly slower than would be expected following the immediate administration of bolus oral doses. However, cineole absorption from the artificial diet was rapid, and  $C_{\text{max}}$  corresponded to a cessation of feeding. Subsequently, cineole levels fell rapidly at first, then more slowly until the next feeding bout.

Metabolites accumulated but did not seem to affect feeding bouts, which were closely associated with the rise and fall of blood cineole concentrations.

Based on the findings of the present study, it seems likely that possums can eat the cineole diet while high first-pass metabolism and rapid distribution keep the systemic blood concentration of cineole low. When a critical dose is exceeded, the concentration of cineole in the portal circulation is sufficient to saturate the enzymes which metabolize cineole, and the systemic concentration of cineole starts to rise steeply. The dose that will saturate first-pass metabolism, found in this study to be between 100 and 300 mg kg<sup>-1</sup>, is similar to the amount of cineole consumed in the first feeding bout (335 mg kg<sup>-1</sup>), especially allowing for the slower absorption of dietary cineole due to the slower intake in food compared with a bolus dose. The  $C_{\max}$  of cineole at which feeding ceased was 52 μM, which lies between the  $C_{\max}$  values after doses of 100 and 300 mg kg<sup>-1</sup> (12 and 89 μM, respectively), and exceeds the  $K_m$  values for terpene-induced enzymes (6–28 μM; Pass et al. 2001).

The rapidly rising cineole concentration results in increasing CNS depression, which is detected by the animal as a signal to cease feeding. The concentration of cineole then falls rapidly through a combination of distribution and metabolism, and after an interval it is low enough for the continuing (although now diminished) hunger to cause the animal to resume feeding.

Over a longer time period daily consumption of cineole will be limited by the terminal elimination from the body rather than by the more rapid distribution process. The elimination of cineole from the possum is prolonged beyond the feeding bout. For practical purposes, the time required by possums to eliminate the cineole ingested, regardless of the amount, is about 35 h (five half-lives equivalent to 97% of dose eliminated; Rowland and Tozer 1995). When consumed daily, therefore, cineole will always be present in the circulation, albeit falling to low levels between feeding sessions.

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