TOXICOLOGICAL EVALUATION AND METABOLIC DISPOSITION OF WESTERN RED CEDAR EXTRACTIVES

by

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Toxicological Evaluation and Metabolic Disposition of Western Red Cedar Extractives

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ABSTRACT

The purposes of this work were to study the potential toxicities of the aqueous extractives of Western red cedar, *Thuja plicata* Donn, and the toxicokinetics of β-thujaplicin (BT), an antifungal component of the extractives, in laboratory animals. The extractives have been used as a preservative for wood stored in the lumberyard since the extractives contain chemicals which have antifungal activities.

Toxicity tests of the extractives were carried out according to the Organization for Economic Cooperation and Development guidelines. These tests included oral and dermal LD_{50}s, acute eye and dermal irritation/corrosion tests and subchronic (90 days) oral toxicity. The oral and dermal LD_{50}s could not be determined since there was no mortality at the highest doses tested. The extractives were non-irritating to the eyes and skin of the tested animals. In the subchronic study, the treated and control animals showed no difference in fluid consumption, behaviour or weight gain. A dose-related change in hematology, clinical chemistry and pathology between the treated and control animals also was not detected.

The toxicokinetics of BT were examined in the rat after intravenous (i.v.) and oral administration. Following i.v. administration of a single dose of BT to the rat, BT concentrations in the blood declined exponentially with time. The toxicokinetics of BT in the blood after i.v. administration

were best described by a one- or two-compartment open model. BT was rapidly absorbed into the blood after oral administration to the rat. The toxicokinetics of BT in the blood after oral administration were best described by a one-compartment open model. BT underwent enterohepatic recycling and was excreted rapidly in the urine of the rat.

The results of these studies suggest that Western red cedar extractives are non-toxic to mammals.

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ALT	alanine aminotransferase
AP	alkaline phosphatase
AST	aspartate aminotransferase
ATPase	adenosine triphosphatase
AUC	area under blood-concentration time curve
BHA	butylated hydroxyanisole
BHT	butylated hydroxytoluene
BUN	blood urea nitrogen
BT	β-thujaplicin
C_B	blood concentration at time t
C_b	bile concentration at time t
C_o	initial concentration at time zero
Canfor	Canadian Forest Products Limited
Cl	total body clearance
COMT	catechol-o-methyltransferase
cor	correlation
CK	creatine kinase
F	fraction of dose absorbed
GGT	γ-glutamyl transferase
GT	γ-thujaplicin
Hb	hemoglobin
HPLC	high pressure liquid chromatography
Ht	hematocrit
IDH	isocitrate dehydrogenase
ip	intraperitoneal
i.v.	intravenous
k	elimination rate constant
k_a	absorption rate constant
LC_{50}	median concentration lethal dose
LD_{50}	median lethal dose
MeOH	HPLC grade methanol
MW	molecular weight
NAD	nicotinamide adenine dinucleotide
NADP	nicotinamide adenine dinucleotide phosphate
OECD	Organization for Economic Cooperation and Development
RBC	red blood cell
TH	thujaplicins
$t_{(1/2)k}$	half-life of elimination from blood
USP	United States Pharmacopeia
UV	ultraviolet
V_c	volume of central compartment
V_d	volume of distribution
WBC	white blood cell

PART A

INTRODUCTION

Western red cedar (*Thuja plicata* Donn) is an abundant and economically important wood species in coastal British Columbia. Western red cedar is a massive tree which grows up to 150-200 feet high and attains a diameter of 8 feet or more. It has small dark-green, shiny scale-like leaves. Cedar grows well on moist soils and sphagnum peat bogs. Therefore, Western red cedar flourishes in the coastal areas of British Columbia where the rainfall is high and the soil is rich (Hosie, 1975).

Western red cedar has many potential uses in agricultural industries where decay resistance is desirable (Barton and MacDonald, 1971). For example, it is especially valuable for shingles, poles, posts, boat-building, green house construction and exterior siding. The antifungal property of Western red cedar has been responsible for its use as a natural fungicide.

The chemistry of Western red cedar has been reviewed by Barton and MacDonald (1971). Western red cedar wood extractives contain a multitude of chemicals which may be classified into volatile and non-volatile components.

The major substances in the volatile fraction of the extractives (and their % in red cedar heartwood) are α-, β-, and γ-thujaplicin (0.51%), β-dolabrin (0.0003%), β-thujaplicinol (0.07%), thujic acid (0.08%) and methyl thujate (0.17%) (Barton and MacDonald, 1971). The chemical structures of these substances are identified as seven-membered cyclic rings with various isopropyl, ketone and hydroxyl groups at different ring

positions (Fig. 1). The proportion of each chemical constituent depends on the methods of extraction and the part of the tree extracted. In total, the volatile components account for 1.0-1.5% of the heartwood.

β-Dolabrin and β-thujaplicinol are structurally similar to the thujaplicins (Gardner and Barton, 1958). Therefore, they display similar fungicidal activities (Rudman, 1962; Anderson *et al.*, 1963; Röff and Whittaker, 1959).

Thujic acid and its methyl ester, methyl thujate, have a characteristic cedar fragrance. Hach and coworkers (1973) tested the derivatives of thujic acid for potential commercial uses. They found that an ethyl amide derivative of thujic acid was a highly effective repellent of the yellow fever mosquito *Aedes aegypti* and of other insect species. This finding resulted in a patent on insect repellent animal collars impregnated with thujic acid and its derivatives (Ford, 1977).

The α-, β-, and γ-thujaplicins (2-hydroxy-3-, 4-, and 5-isopropyl-2,4,6-cycloheptatriene-1-one) also were found in other plant species. The isolation and identification of these substances was first reported by Erdtman and Gripenberg (1948). They identified thujaplicins (TH) in Western red cedar (*Thuja plicata* Donn), hence the name "thujaplicin". MacLean and Gardner (1958) found up to 0.56% of TH in the dry wood of cedar. The proportions of β-thujaplicin (BT) and γ-thujaplicin (GT) were 40% and 60%, respectively. No α-thujaplicin was detected. TH

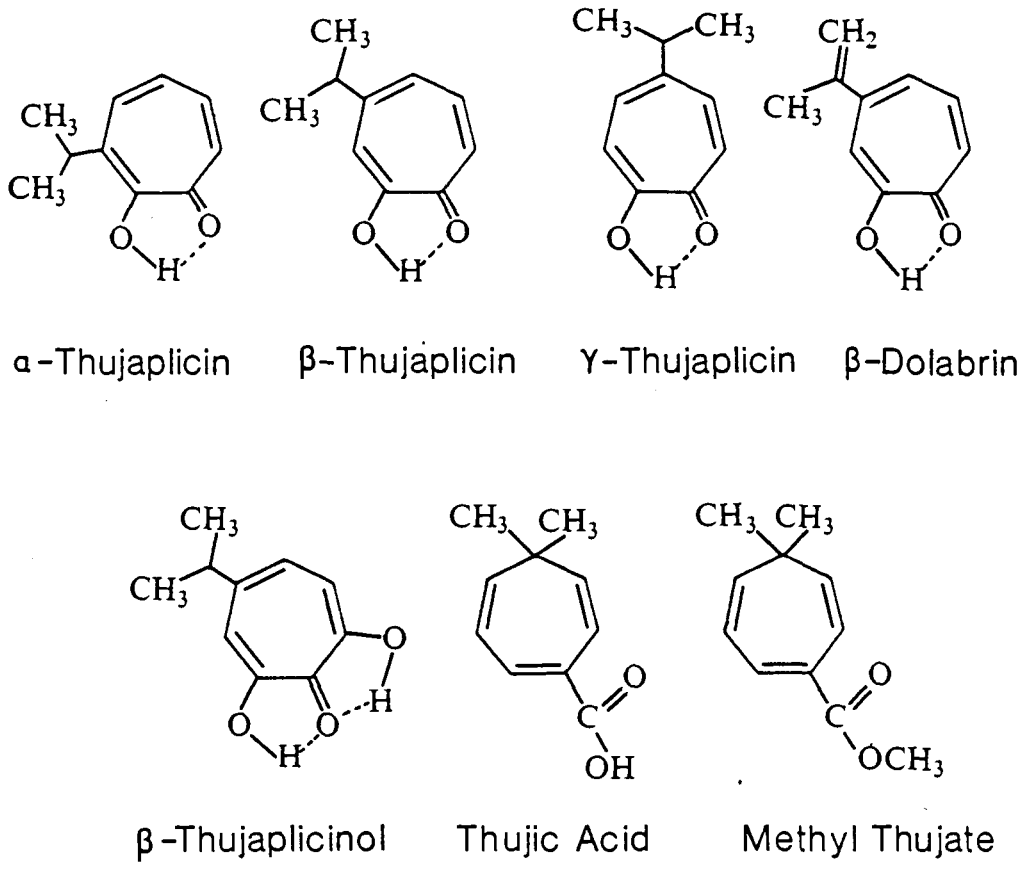


Fig. 1: Compounds found in the volatile fraction of Western red cedar.

were also identified in the *Cupressales* order (Senter *et al.*, 1975; Enzell *et al.*, 1962 and Yuan-Lang, 1962). Kwang-Ting *et al.* (1964) found TH in the *Libocedrus* order. Han *et al.* (1980) quantified TH in *Thymus magnus* of the *Lamiales* order. Recently, Li *et al.* (1985) identified TH in *Illicium verum* of the *Magnoliales* order. Therefore, TH were found in widely different plant species from widely different orders.

The following is a summary of the chemical characteristics of BT:

* it has an empirical formula of $C_{10}H_{12}O_2$ and a molecular weight of 164 daltons
* it has a pk_a of 7.3 and a partition coefficient of 7.0 in n-octanol and chloroform (Skidmore, 1964)
* it is relatively lipophilic (Skidmore, 1964)
* the absorbance maximum of this compound in the UV range is 242 nm (Coombs, 1973)

Release of TH from Western red cedar chips causes corrosion of steel digesters by forming an iron chelate (Barton and MacDonald, 1971). Moreover, many *in vitro* effects of TH are caused by their metal chelating abilities. TH chelates Mg^{2+} and other divalent cations (Lyr *et al.*, 1980). Bohme *et al.* (1980), using the isolated rat liver mitochondria, found that BT bound to cations in the mitochondrial matrix and caused a dramatic inhibition of oxidative phosphorylation. At a concentration as low as 0.37 nmole/L, BT was able to decrease mitochondrial Mg^{2+}

by 2/3 (*in vitro*) after a 10 min exposure period. ATPases from isolated mitochondrion also could be inhibited by BT. This effect was prevented if the ATPase was pretreated with Mg^{2+}. Raa *et al.* (1965) showed that the inhibition of glycolysis by BT could be counteracted by the addition of Fe^{2+}. It was hypothesized that Fe^{2+} chelated with BT and prevented it from binding with Mg^{2+}. Lyr (1966) observed that succinate dehydrogenase in isolated rat liver mitochondrion was inhibited by BT. This effect was probably caused by chelation of the enzyme's iron-sulfur center by BT (Lehninger, 1982).

The chelation effects of TH were not limited to respiration enzymes. For example, two enzymes involved in catecholamine synthesis, tyrosine hydroxylase (Goldstein, 1967) and dopamine β-hydroxylase (Goldstein *et al.*, 1965), were also inhibited *in vitro* by BT. However, the addition of Fe^{2+} partially restored the activities of these enzymes. Catechol-o-methyl transferase (COMT), an enzyme involved in the inactivation of catecholamines, was also inhibited by BT (*in vitro*) (Belleau *et al.*, 1963). The mechanism of COMT inhibition was more complex than that of the respiration enzymes; BT deprived COMT of Mg^{2+} as well as bound with the active site of the enzyme which would normally be occupied by catechols (Belleau and Burba, 1963). Furthermore, since BT and catechols were shown to be biochemically isosteric, BT displayed *in vitro* blockade of β-receptors. Therefore, the effects of BT on COMT were not reversed by adding excess Mg^{2+}. The mechanism of inhibition was

hypothesized to involve a reaction with COMT, S-adenosyl methionine and BT (Borchardt, 1973).

TH have various effects on whole animals, many of which may be explained by their *in vitro* effects. Wyse and Halliday (1969) found that intraperitoneal administration of 2 mg/day of GT to rats for 13 weeks resulted in decreased catecholamine content of the heart. Apparently, the inhibitory effects of GT on tyrosine hydroxylase and dopamine β-hydroxylase resulted in decreased catecholamine synthesis. Subcutaneous injection of BT to rats caused a decrease in urinary catecholamine metabolites probably due to COMT inhibition (Mavrides, 1963). However, a single intravenous injection of BT to the dog caused increases in catecholamine release, cardiac output and vascular pressure (Maxwell, 1969). These effects were explained by COMT inhibition which resulted in decreased catecholamine degradation. Intravenous injections of 50 mg/kg of BT also caused convulsions in rats (Halliday, 1959). This effect was assumed to be caused by a combination of BT chelating with Ca^{2+} necessary for muscle activity and BT binding with other divalent cations involved in neurotransmitters (Gilman *et al.*, 1975).

The thujaplicins have gained much attention in the commercial market. For example, they were used in many fungicidal preparations (Tanaka *et al.*, 1975; Takasago Perfumery Company, 1973; and Kafuku, 1976). Lyr (1962) proposed that the fungicidal effects of TH were caused by inhibition of fungal respiration. BT inhibited mitochondrial respiration of wood

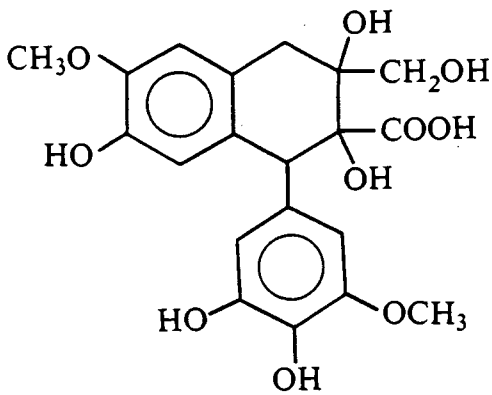
destroying fungi. The antifungal effects were temporary because wood enzymes such as laccase, tyrosinase and peroxidases detoxified TH and rendered them innocuous to wood destroying fungi. However, Luthardt (1967) found that BT or GT inhibited tyrosinase. Anderson *et al.* (1963) quantified antifungal ingredients and their nonfungicidal dimers in *Libocedrus;* they hypothesized that nonfungicidal dimers were endproducts of enzymatic action on antifungal monomers. It was hypothesized that the degree of fungicidal activity of TH depended on a combination of fungus susceptibility and TH detoxification by fungal oxidases (Anderson *et al.*, 1963).

Thujaplicins were also used in hair and skin lotions since the bactericidal and antiinflammatory activity of BT helped to reduce irritation of the scalp. Suga (1963) and Kubo (1985) used BT in hair conditioning shampoos which contained several other substances such as resorcinol, sorbitol, and glycerol. The hair lotions of Yamato (1984) and Taisho Pharmaceutical Company (1984) contained 0.05% and 0.1% of BT, respectively. Fukuda (1980) found that a mixture of 0.01% BT-palmitate in cold cream was an effective melanin inhibitor and could be used as a skin "vanishing cream". The mixture was found to inhibit tyrosinase, an enzyme involved in melanin synthesis. The same author patented a lotion containing 0.05% BT, amino acetic acid, pyridoxine, and propylene glycol as a prevention against sunburn. The mode of action may be through UV absorption since BT has an absorbance maximum in the UV range. The efficacy of

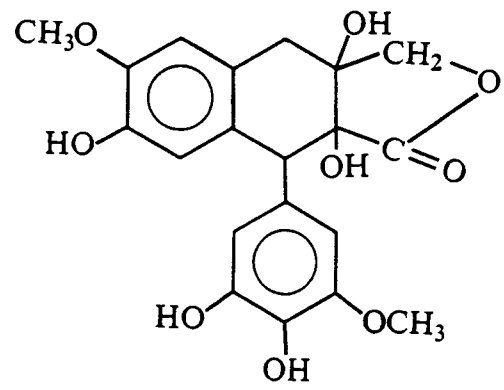
the lotions and creams were demonstrated in humans. However, the effect of each component of the product was not identified.

The non-volatile fraction of Western red cedar extractives accounts for approximately 5-15% of the heartwood and consists primarily of phenolic compounds. Four of the isolated and identified phenols account for up to 90% of the phenolic mixture; they are plicatic acid, plicatin, thujaplicatin and thujaplicatin methyl ether (Fig. 2). The predominant substance is plicatic acid ($C_{20}H_{22}O_{10}$, MW=422), a heat and light sensitive strong organic acid (Gardner *et al.*, 1959, 1960, 1966). The other three substances are structurally similar to plicatic acid and may have been formed from it (Gardner *et al.*, 1971).

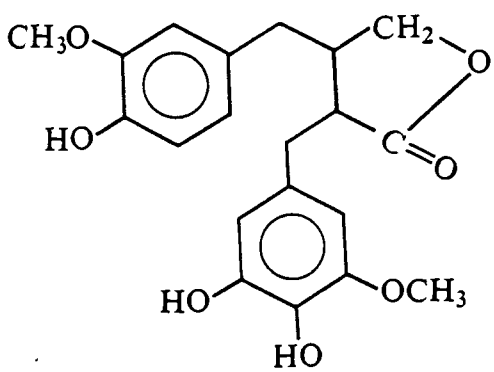
Plicatic acid and its derivatives are useful antioxidants since the unetherified neighbouring phenolic hydroxyl functional groups in the plicatic acid chemical structure possess strong antioxidant properties (Gardner *et al.*, 1959). Several workers have found methods of preparing plicatic acid and its amide, acid hydrazide and ester derivatives (Buchholz and Reintjes, 1974, 1972; Howard and McIntosh, 1969). ITT Rayonier Incorporated (1970) found that a mixture of plicatic acid and its derivatives (50-150 ppm) was an effective antioxidant for fresh lard as compared to other antioxidants in a 450 h peroxide test. Karchmar and McDonald (1969) found that a mixture of plicatic acid and its derivatives (100-150 ppm) added to pork fat or to safflower oil were more effective than butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) in a 450



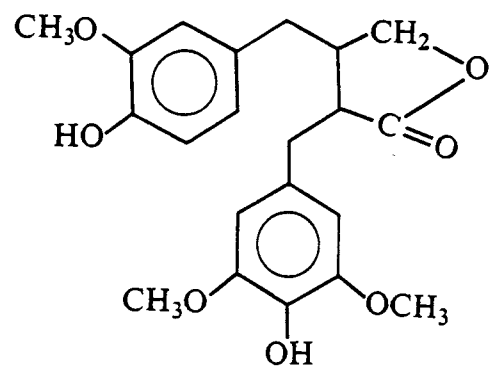
Plicatic acid



Plicatin



Thujaplicatin



Thujaplicatin methyl ether

Fig. 2: Compounds found in the non-volatile fraction of Western red cedar.

h/57 °C peroxide test. Bishov and Henick (1975) showed that plicatic acid and its derivatives were as effective as BHT as an antioxidant of corn oil.

Plicatic acid and its derivatives also have many other commercial uses. Kronstein (1970) found that plicatic acid applied to an iron surface before painting improved the durability of the surface. Other industrial uses of plicatic acid included electrolytic refining of lead (Kerby and Jackson, 1978) and cable manufacturing (Langille, 1975). Russell (1970) found that 10% plicatic acid-iron complexes were effective in treating iron-deficiency chlorosis of plants when applied to soil or foliage. Karchmar and Nejelski (1975) used 0.2% n-propyl plicatate as a preservative for photographic developers. It extended developer life by 65% as compared to a control solution without the plicatic acid derivative. In addition, plicatic acid was shown to be equivalent to zinc chloride in fungicidal activity (Roff and Atkinson, 1954).

Western red cedar effluents have been shown to be toxic to various aquatic species. Wong (1980) found that among the effluents from pulping of trembling aspen, black spruce and Western red cedar, Western red cedar was the most toxic. Peters and coworkers (1976) showed that water soluble extractives of Western red cedar were toxic to aquatic organisms. The volatile fraction was more toxic than the non-volatile fraction. The volatile fraction at 2.7 mg/L caused 50% mortality (96 h exposure period) in coho salmon *Oncorhynchus kisutch*. In

addition, this fraction was found to be less toxic to invertebrates than to free-swimming stages of the tested fish. Fry were the most sensitive stage of coho salmon to the volatile fraction. They also found that exposure of coho fry to sublethal levels of chelatable cations moderated toxic effects of the volatile fraction. It was concluded that direct releasing of cedar leachate into streams should be avoided.

β-Thujaplicin was more toxic to fish than to mammals. For example, *Lebistes reticulatus* died in a 0.02% and 0.002% solution of BT within 10 min and 3 h, respectively, after exposure (Erdtman and Gripenberg, 1948). In contrast, the oral LD_{50}s of BT in the rat, rabbit, cat and dog were 162, 40, 70, and 40 mg/kg, respectively (Halliday, 1959).

Western red cedar extractives and its constituents can elicit allergic responses in humans. Chan-Yeung and coworkers (1980, 1973, and 1971) found that human exposure to plicatic acid could lead to asthmatic attacks and wheal formation on the skin. Prick testing with 1-5% plicatic acid caused more wheal formation than 1-5% Western red cedar extractives. The test subjects were mill workers previously exposed occupationally to cedar dust. It was noted that plicatic acid could activate the complement system *in vivo* thus causing an inflammation response. The complement system consists of plasma proteins which normally circulate in the blood in an inactive state. Activation consists of fragmentation or structural rearrangement of the protein molecules. The active proteins exert their effects on the

inflammation process (Vander *et al.*, 1980). Giclas (1982) performed further studies on the *in vitro* activation of human complement by plicatic acid. He found that activation of the complement system by plicatic acid could be explained by the classical pathway. Therefore, it is likely that plicatic acid is the ingredient in Western red cedar extractives which is responsible for the allergic responses in humans. This hypothesis was supported by the finding that human or pig lung tissue incubated with Western red cedar dust (5-100 mg/mL) caused release of significant amounts of histamine from these tissues (Evans and Nicholls, 1974).

Very few studies have been performed on the pharmacokinetics of BT in rabbits. Kazuo (1961) studied the absorption and excretion of BT administered to rabbits by different routes and found that BT in blood decreased to an unmeasurable level within 1 h after i.v. administration. In contrast, BT was detectable in the blood 3 h after subcutaneous injection. BT concentration in the urine rose to a maximum at 1 h after i.v. administration. Hino (1961) reported that the excretion of BT in the rabbit urine after oral, intramuscular and intraperitoneal injections were 16.2, 5.3, and 7.0% of the dose, respectively. The urinary excretion rate reached a maximum at 2 h and lasted for 13-19 h after chemical administration. Both conjugated and free BT were found in the urine. The ratios of conjugated/free form were 4.2/1, 1.5/1 and 2.7/1 in the oral, intramuscular and intraperitoneal routes of administration, respectively. Only a

very small amount of BT was excreted in the rabbit bile.

Canadian Forest Products Limited (Canfor) has been using Western red cedar extractives as a wood preservative on wood stored in the lumberyard. Lumber is sprayed with the solution in a rectangular enclosure equipped with a conveyor belt. Wood coming in from one end of the enclosure, is sprayed and then passes out of the other end. The sprayed wood is picked up by a worker and stacked for storage in the lumberyard. The worker does not come in contact with the actual spraying process.

The extractives formulation used by Canfor consisted of: water (97.8%), borax (2.0%), insoluble carbohydrate (0.1%), plicatic acid (0.08%), L-arabinose (0.004%) and β- and γ-thujaplicin (0.0048%) (Anon, 1982). The antifungal properties of the formulation presumably were due to the presence of plicatic acid, thujaplicins and borax. Rennerfelt (1948) and Roff and Atkinson (1954) noted that the thujaplicins and plicatic acid were equivalent to pentachlorophenol and zinc chloride, respectively, in fungicidal activity.

In addition to its presence in the Canfor formulation, borax ($Na_2B_4O_7 \cdot 10H_2O$) and the related compound boric acid (H_3BO_3) are present in numerous industrial and commercial products. For example, they were used in porcelain and glass manufacturing, printing and dyeing, and have antibacterial and antifungal properties (Merck Index, 1976). Borax was used as a food additive in Thailand (Monsereenusorn, 1982). Belenkov and Serov

(1975) showed that 0.4% borax was effective in protecting pine sapwood against the fungus *Ophiostoma coeruleum*. The Lion Corporation (1982) patented a bactericidal eye lotion which contained 0.1% di-K glycrrhizinate, 0.1% benzalkonium chloride, 1.85% H_3PO_3, 0.032% borax and water. However, Gilman and coworkers (1975) noted that borax alone was a poor bacteriocide when compared to other commercial products available.

The toxicological effects of borax and boric acid were studied extensively. The LC_{50}s of borax were 100, 155, and 46 ppm for trout, catfish and goldfish (Birge and Black, 1977). Boric acid fed to rats at 1 g/kg per day for 3 weeks resulted in retarded weight gain and inhibition of DNA synthesis in the liver (Dani and coworkers, 1971). However, such effects could be produced only at a dosage higher than 1 g/kg per day. In contrast, borax and boric acid were relatively non-toxic to the mammalian species. Weir and Fisher (1972) found that the oral LD_{50}s of borax and boric acid in male and female Sprague-Dawley rats were between 4.5-5 g/kg. In a 90-day feeding study, rats tolerated up to 4630 ppm borax (Weir and Fisher, 1972) whereas dogs showed no toxic signs at up to 175 ppm borax or 525 ppm boric acid. The rat had to be exposed to 15430 ppm borax before toxic signs such as growth suppression, decreased food utilization, degeneration of the gonads and skin desquamation were detected. These authors also showed that 3087 ppm borax could be tolerated by rats and dogs with no ill-effects in a 2-year dietary feeding study. Shaipanich and Anukrahanon (1978)

showed that rats consuming 1.5% borax for 3 weeks experienced no mortality. However, rats exposed beyond this level resulted in weight loss, elevated serum enzyme aspartate aminotransferase and alanine aminotransferase and atrophy of the spermatozoa. Thus, the studies of Shaipanich and Anukrahanon (1978) and Weir and Fisher (1972) demonstrated a similar finding; exposure to boron in the form of boric acid or borax might result in degeneration of the reproductive system. Verbitskaya (1975) found that the oral LD_{50}s of borax and boric acid ranged between 300-700 mg/kg in the rabbit, guinea pig, dog and rat. Aspartate aminotransferase, alanine aminotransferase, aldolase, and lactate dehydrogenase activities were inhibited in rabbits fed 31 mg/kg of boron 5 times a week for 2 months.

Western red cedar extractives may replace chlorinated phenols as the predominant industrial wood preservative for wood stored in the lumberyard. However, its human health effects remain relatively unknown. This work deals with some aspects of the toxicological effects of Western red cedar extractives on mammals.

PART B

MATERIALS AND METHODS

Laboratory Animals

Male and female Wistar rats (200-350g) and male Duncan Hartley guinea pigs (350-400g) were obtained from the Animal Care Facility, University of British Columbia, Vancouver, British Columbia. Male New Zealand White rabbits (2.0-3.0kg) were obtained from R & R Rabbitry (Stanwood, Washington). They were individually housed in stainless steel cages and maintained in a temperature (20°C) and light (12 h dark/ 12 h light) controlled room at the Animal Care Facility, Simon Fraser University, Burnaby, British Columbia. All animals were acclimatized for one week prior to use. Purina Laboratory Chow (Ralston Purina Company, St. Louis, Missouri) and water were available *ad libitum*. Health status of the animals was determined by visual observation and physical examination. Rats were tail marked, guinea pigs were individually caged and rabbits were eartagged for identification. Identification was confirmed at each cage change and at sacrifice.

Chemicals

Sodium heparin was obtained from Sigma Chemical Company, St. Louis, Missouri. Hexane and methanol were of high pressure liquid chromatography (HPLC) grade and obtained from Caledon Laboratories Ltd., Georgetown, Ontario. β-Thujaplicin was

obtained from Columbia Organics, Cassatt, South Carolina and purified by repeated recrystallization from hexane. The purity of BT determined by HPLC (HP 1090, Hewlett Packard Inc., Toronto, Ontario) and elemental analysis (Elemental Analyzer Model 1106, Strumentazione Carlo Erba, Milan, Italy) exceeded 99%. Western red cedar extractives were obtained from Canfor. The solution was stored in dark glass bottles at 4°C. The same batch was used throughout the study.

I. ACUTE TOXICITY OF WESTERN RED CEDAR EXTRACTIVES

Acute oral LD_{50}:rats

This study was carried out according to OECD guideline #401. The extractives were diluted to different concentrations with tapwater. Four concentrations of extractives were used in this study: control (tapwater), low (33%), medium (66%) and high (undiluted). Ten female and ten male rats (200-225g) were used for each dose level. The extractives were administered as a single dose (45 mL/kg) by gastric gavage using a 10 mL glass syringe fitted with a curved roundball needle. Food was withheld the night before chemical administration. The animals were observed for signs of toxicity for 14-days after dosing. After the observation period, all animals were necropsied and the internal organs examined *in situ* for gross pathological changes. In addition, the liver was removed, macroscopically examined, weighed and preserved in 10% formalin.

Acute dermal LD_{50}:rats

This study was carried out according to OECD guideline #402. The extractives were diluted to different concentrations with tapwater. Four concentrations of extractives were used in this study: control (tapwater), low (33%), medium (66%) and high (undiluted). Ten female and ten male rats (200-225g) were used

for each dose level. The dorsal area (about 6 cm^2) of each rat was clipped free of fur with animal clippers 24 h before chemical administration. Care was taken to avoid abrading the skin. The extractives (3 mL/kg) were administered to the clipped area with a 1 mL glass syringe fitted to a roundball needle. The solution was massaged gently into the skin. A 2 inch square gauze patch was applied over the test area and held in place for 24 h with Transpore® surgical tape (3M Medical Products Division, St. Paul, MN). Animals were observed for signs of toxicity for 14-days after dosing. After the observation period, all animals were necropsied and the internal organs examined *in situ* for gross pathological changes. In addition, the liver was removed, macroscopically examined, weighed and preserved in 10% formalin.

Acute dermal irritation/corrosion

These studies were carried out according to OECD guideline #408. Six rats (200-225 g), three rabbits (2.0-3.0 kg), and three guinea pigs (350-400 g) were clipped free of fur (about 6 cm^2) on the dorsal surface. The extractives were applied undiluted (0.5 mL per animal) to the clipped area of each animal. A 2 inch square gauze patch was applied over the test area. The patch was removed after 24 h and the skin evaluated for signs of irritation according to Draize *et al.* (1944). Adjacent skin was used as the control.

Acute eye irritation/corrosion:rabbit

This study was carried out according to OECD guideline #405. The extractives (0.1 mL) were applied to the conjunctival sac of the left eye of 3 different rabbits with a roundball needle fitted to a 0.5 mL glass syringe. The right eye of each rabbit served as control. The eyes were observed for signs of irritation at 0 h, 2 h, 4 h, and 24 h after chemical instillation according to the scoring system of Draize *et al.* (1944). A hand lense and laboratory illumination was used for eye examination.

II. SUBCHRONIC ORAL TOXICITY OF WESTERN RED CEDAR EXTRACTIVES IN RATS

Test material administration

The cedar extractives were diluted to 2, 5 and 7% with tapwater and dispensed in 500 mL opaque polypropylene mouse bottles. Ten female and ten male Wistar rats (200-225 g) were used at each dose level. The test animals drank the diluted mixture *ad libitum* for 90-days. Ten females and ten males which drank only tapwater were used as controls. Fluid consumption was measured and drinking water was changed every second day for all the animals. All rats were given Purina laboratory rodent chow *ad libitum* throughout the period of test material administration.

Clinical Observations

The rats were observed twice daily for overt signs of toxicity. Physical examination of the rats was performed weekly. Individual body weights were recorded prior to chemical exposure, weekly thereafter and at sacrifice.

Blood Sampling

At the conclusion of 90-days chemical treatment, the animals were weighed and anesthetized with diethyl ether. Food was withheld the night before blood sampling. Blood obtained by orbital sinus with a glass capillary tube was dispensed into 50 mL glass centrifuge tubes with ground glass stoppers and 1 mL EDTA-Vacutainers® (Becton-Dickinson, Rutherford, N.J.) on ice. The blood in the centrifuge tubes were allowed to clot for 30 min before centrifugation at 3000 g in a IEC Model K Centrifuge (International Equipment Company, Needham Hts., Mass) to collect serum which was dispensed into 0.5 mL Microfuge® tubes (Bio Plas Inc., San Francisco, CA) and stored at -70 °C until analyzed. The blood in the EDTA-Vacutainers® was analyzed immediately as described below (see Hematology and Clinical Chemistry).

Hematology and Clinical Chemistry

Hematology was assayed with whole blood. A Model TA II Coulter cell counter (Coulter Electronics, Hialeah, FL) was used to determine the erythrocyte (RBC), leucocyte (WBC) and platelet counts. The operating conditions of the Coulter cell counter were as follows: 100 μm aperture tube; aperture resistance 13,000 Ω; aperture current 3.2 mA; manometer volume 0.5 mL;

aperture matching switch 10; scope display Pulse Amp; and counting time 12 sec. The diluent for RBC and WBC was Isoton II® (Coulter Electronics Limited). The diluent for platelets was Zap-o-globin® (Coulter Electronics Limited). Hematocrit (Ht) was determined with 75x1.1mm hematocrit tubes (Dade Diagnostic Inc., Aguada, Puerto Rico) and a Microhematocrit centrifuge (Clay Adams, Parsippany, NJ). Differential leucocyte counts were performed manually using Wright-Giemsa stain (Camco-Quik Stain®, Cambridge Chemical Products Inc., FT. Lauderdale, FL) and oil-immersion light microscopy. One hundred cells were counted from each slide. Hemoglobin was determined spectrophotometrically by the cyanomethemoglobin method using a clinical chemistry kit (Diagnostic Chemical Limited, Charlottetown, PEI).

Biochemical parameters, serum enzyme activities and serum electrolytes (calcium and chloride) were determined with standard clinical chemistry kits (Diagnostic Chemical Limited, Charlottetown, PEI) on a Perkin-Elmer Lambda 3B/UV VIS Spectrophotometer equipped with a thermostatted sample cell holder and a R100 Chart Recorder (Perkin-Elmer Corporation, Oak Brook, Illinois). The operating conditions of the spectrophotometer were as follows: mode absorbance; lamp visible; and cell holder at 37 °C for enzymes and 22 °C for the remaining biochemical parameters. The chart recorder operating conditions were as follows: sensitivity 500 mV and chart speed 6 cm/min.

a. The biochemical parameters (and accompanying methods) determined were as follows: albumin (Bromcresol green), BUN (urease), creatinine (creatinine picrate), glucose (hexokinase), phosphorous (ammonium molybdate), protein (Biuret), globulin (glyoxylic reaction).

b. The serum enzymes (and their methods of determination) were as follows: ALT (NADH-NAD), AST (NADH-NAD), AP (p-nitrophenyl phosphate), CK (NADP-NADPH), GGT (p-nitroaniline), IDH (NADP-NADPH).

c. Calcium and chloride were determined by cresolpthalein complexone and ferric thiocyanate, respectively.

d. Sodium and potassium were determined flame photometrically (Corning Flame Photometer Model 1430, Corning Inc., Toronto, Ontario). The flame photometer operating conditions were as follows: mode Sodium/Potassium; automatic dilutor on; internal standard 3000 mEq/L of Lithium.

Reliability of test results were monitored by routine use (every 30 samples) of pooled sera.

Necropsy

The external body surface and organs of the animals were examined at the time of sacrifice. The following organs were removed, macroscopically examined and fixed in 10% formalin: brain, heart, lungs, liver, spleen, kidneys, gonads, thyroid,

thymus, trachea, pancreas, urinary bladder and stomach. The first seven aforementioned organs were weighed before fixation.

Statistical Analysis

Group means and standard deviations were calculated for each parameter. Statistical analyses which compared the treatments with the control groups was performed. Body and organ weights, fluid consumption, hematology and clinical chemistry data were compared using computer programs by Tallarida and Murray (1981) for analysis of variance and Dunnett's Multiple comparison test.

III. METABOLIC DISPOSITION OF β-THUJAPLICIN IN THE RAT

Time course of β-thujaplicin in the blood

Cannulation of the rat jugular vein

One day before the experiment, the jugular vein of the male rat (300-350g) was cannulated by a modified procedure of Migdalof (1976). Under diethyl ether anesthesia, a 1 cm incision was made at the dorsal midline of the rat approximately 3 cm behind the ears. A 2 cm skin incision was made at the ventral midline along the animal's neck. The connective tissue around the jugular vein was removed. After a small incision was made on the vein with fine pointed scissors, a Silastic Medical Grade Tubing (Dow Corning Company, Midland, MI; i.d. 0.02in., o.d. 0.037in.) was inserted into the vein. Subsequently, using a pair of hemostatic forceps, the free end of the cannula was inserted past connective tissue and underneath the skin to the dorsal incision. The incisions were closed with Autoclips® (Clay Adams, Parsippany, NJ). The cannula was filled with heparinized saline (100 USP units/mL), plugged and attached to the rat's back. The entire procedure lasted approximately 20 min. After the animals recovered from anesthesia, they were housed individually in metabolic cages.

Chemical administration and blood sampling

Twenty-four h after surgery, each rat was administered a single dose of BT (30 or 20 mg/kg dissolved in 0.1 N Na_2CO_3) either through the jugular vein cannula or by gastric gavage. Glass syringes were used for both procedures. Food was withheld 24 h before chemical administration. Immediately after i.v. administration, 0.5 mL of heparinized saline (50 USP units/mL) was injected into the cannula to ensure the entire dose entered the circulatory system. After chemical administration, a 0.35 mL blood sample was withdrawn through the cannula with a glass tuberculin syringe at 1, 3, 5, 7, 10, 15, 20, 25, 30, 45, 60, 75, 90, 120, 150, 180 min. After each sample, an equal volume of heparinized saline (50 USP units/mL) was injected into the cannula to prevent blood clotting and replace removed blood.

Analysis of β-thujaplicin in the blood

A 0.2 mL aliquot of each blood sample was pipetted into a 12 mL centrifuge tube with a Teflon® cap. Sulfuric acid (1 N, 0.4 mL) was added to the tube to deproteinize and acidify the blood. The mixture was extracted twice with 1.5 mL hexane in an Eberbach reciprocating shaker (Eberbach Corporation, Ann Arbor, Michigan) for 10 min. The organic layer of the mixture was separated by centrifugation at 1000 g for 15 min in a IEC Model K Centrifuge (International Equipment Company, Needham Hts., Mass). The hexane layer was removed, combined and evaporated to

dryness in 1 mL borosilicate dark glass HPLC vials (Supelco Canada Limited, Oakville, Ontario) under a gentle stream of nitrogen in a Meyer Analytical Evaporator (Organomation Inc., South Berlin, MA). The residue was redissolved in 0.25 mL of HPLC grade methanol (MeOH) containing 2,4-dichlorodiphenyl ether (2,4-DCDE) (10 μg/mL) which served as the internal standard. The mixture was analyzed with a Hewlett Packard HPLC Model 1090 equipped with a reverse phase 100X2.1mm, Hypersil ODS 5μm column and a Diode-Array UV Detector. Calibration curves were prepared with 1, 4, 10, 15, 30, 40, 50 μg BT each dissolved in 0.25 mL of MeOH which contained 10 μg/mL of 2,4-DCDE. Spiked biologic samples and calibration standards were analyzed with each batch of biologic samples. The HPLC operating conditions were as follows: wavelength 242 nm; oven temperature 40 °C; eluting solvent system 72% MeOH: 28% Ammonium carbonate; and flow rate 2 mL/min.

Urinary excretion of β-thujaplicin

Urine collection

Urine was collected from the rat after i.v. administration of BT (30 mg/kg). Timed urine samples (every 2 h, for 10 h) were obtained by grasping the animal and depressing the abdominal region to induce urination into preweighed glass liquid scintillation vials. The samples were stored at -20 °C until analysis.

Analysis of β-thujaplicin in the urine

Urine was analyzed for unchanged BT by the same method as previously mentioned for blood samples. In addition, the remaining aqueous layer was retained for analysis of conjugated BT. The aqueous layer was acid hydrolyzed for 2 h (heated in a 80 °C water bath with agitation) and then extracted and analyzed for unchanged BT.

Biliary excretion of β-thujaplicin

Bile collection and analysis

Male Wistar rats (300-350 g) were anesthetized with urethane (0.8 g/kg; Sigma Chemical Company, St. Louis, Missouri) ip. An incision was made on the abdominal wall to expose the common bile duct and the inferior vena cava. The bile duct was cannulated with a PE-10 tubing (Clay Adams, Parsippany, NJ). After the body temperature (37 °C) and bile flow rate (2 mL/min) stabilized, a single intravenous dose of BT (30 mg/kg, dissolved in 0.1 N Na_2CO_3) was administered through the inferior vena cava. Body temperature of the rat was monitored with a rectal thermometer and maintained at 37 °C with a heating lamp. Bile samples were collected in preweighed glass liquid scintillation vials at 15 min intervals for 3 h. Bile volumes were calculated from their weights. Samples were stored at -20 °C until analyzed. The concentration of BT in the bile was determined by

the same method used in urine analysis.

Enterohepatic recycling of β-thujaplicin

Bile was collected continuously for 3 h from a bile duct cannulated rat treated intravenously with BT (30 mg/kg). The bile sample was subsequently administered by gastric gavage to a recipient rat whose bile duct was previously cannulated. Timed bile samples (every 15 min, for 6 h) were collected from the recipient rat after donor bile administration. Unchanged and conjugated BT in the bile samples were determined according to the method described for urine analysis.

Mathematical Analysis of Blood and Bile Data

The blood concentration-time curves and biliary excretion rate were analyzed by NONLIN® (Metzler *et al.*, 1974), a nonlinear least-squares regression program. The data were fitted to exponential equations. The overall goodness of fit of the equation was determined by the value of the correlation (cor), r^2 and by the scatter of the observed data values around the calculated values.

For the blood-concentration time curves, the parameters estimated from nonlinear regression analysis were used to calculate the secondary pharmacokinetic parameters which depending on the model included k_{12} the rate constant for transfer from the central compartment into the peripheral

compartment, k_{21} the rate constant for transfer from the peripheral compartment into the central compartment, k_a the absorption rate constant, k the elimination rate constant from the blood, $t_{(1/2)k}$ the half-life of elimination from the blood, F the fraction of the dose absorbed, V_c the volume of the central compartment, V_d the volume of distribution, Cl the total body clearance and AUC the area under the blood-concentration time curve (Gibaldi and Perrier, 1975).

PART C

RESULTS

I. ACUTE TOXICITY OF WESTERN RED CEDAR EXTRACTIVES

Acute oral and dermal LD_{50}:rats

There was no mortality in the oral or dermal LD_{50} tests. The animals displayed no sign of toxicity during the observation period. No gross lesion was found upon macroscopic examination of the internal organs during necropsy. Liver to body weight ratios for the acute oral and dermal studies are presented in tables 1A and 1B, respectively. Statistical analysis (analysis of variance & Dunnett's Multiple comparison test) also did not indicate any difference in liver to body weight ratios between the control and treated groups. Therefore, the oral and dermal LD_{50}s of the extractives in rats are greater than 45 mL/kg and 3 mL/kg, respectively.

Table 1A: Liver to body weight ratios of Wistar rats after a single oral dose of cedar extractives[1].

	DOSE[2]			
	0%	33%	66%	100%
Sex				
male	3.96±0.23	4.01±0.13	4.03±0.13	3.96±0.30
female	3.49±0.19	3.36±0.05	3.36±0.11	3.48±0.19

[1]values are expressed as (mean±SD) times 100;n=10
[2]45 mL/kg; % extractives diluted with tapwater

Table 1B: Liver to body weight ratios of Wistar rats after a single dermal dose of cedar extractives[1].

	DOSE[2]			
	0%	33%	66%	100%
Sex				
male	3.87±0.19	4.00±0.24	3.97±0.23	3.93±0.19
female	3.52±0.20	3.49±0.18	3.45±0.15	3.49±0.17

[1]values are expressed as (mean±SD) times 100;n=10
[2]3 mL/kg; % extractives diluted with tapwater

Acute dermal and eye irritation/corrosion

No sign of irritation was observed on the skin of the test animals immediately or 24 h after chemical application. There was no sign of erythema or oedema on the test skin as compared to the control skin. The extractives also did not cause irritation on the rabbit eye. No redness, swelling or ulcerations developed in the test eye as compared with the control eye. There was no difference in appearance of the cornea, iris and conjunctivae of the test eye as compared to the control. The rabbits did not exhibit discomfort upon application of the test substance or during the observation period.

II. SUBCHRONIC ORAL TOXICITY OF WESTERN RED CEDAR EXTRACTIVES IN RATS

Clinical Observations

The behaviour and appearance of the control animals were not different from those of the treated animals. None of the animals became ill in the experiment. There was no mortality during the entire exposure period. The only unusual condition noted during this period was a dried red substance around the eyes, nose and mouth of the rats. This condition occurred randomly at all dose levels and in the controls. Each rat recovered to its original appearance within 3-5 days. There were no significant differences in body weight and average weekly fluid consumption between the control and treated groups. The rats gained weight at the same rate regardless of treatment.

Hematology and Clinical Chemistry

The hematology and clinical chemistry of the treated and control animals were not different. The hematological profile, biochemical parameters, and serum enzyme and electrolyte data are presented in tables 2-5.

Table 2A: Hematological profile of male Wistar rats treated with cedar extractives for 90-days[1].

	DOSE[2] 0%	2%	5%	7%
WBC $X10^3(/\mu L)$	12.7±3.8	11.3±3.4	13.7±2.5	11.9±4.2
RBC $X10^6(/\mu L)$	8.1±0.5	7.8±0.4	8.0±0.6	7.8±0.3
Platelets $X10^6(/\mu L)$	1.30±0.21	1.29±0.15	1.30±0.16	1.13±0.24
Hematocrit (%)	41.6±1.5	43.5±2.4	41.6±1.1	42.3±1.9
Hemoglobin (g/dL)	18.4±1.6	19.0±1.9	18.9±1.7	18.4±1.6
Lymphocytes (%)	74.8±10.0	76.0±5.1	82.4±7.6	77.2±5.3
Neutrophils (%)	25.2±18.0	24.0±4.0	17.6±8.3	22.8±5.0

[1]values are expressed as mean±SD;n=10
[2]%extractives in drinking water

Table 2B: Hematological profile of female Wistar rats treated with cedar extractives for 90-days[1].

	DOSE[2] 0%	2%	5%	7%
WBC $X10^3(/\mu L)$	11.7±2.4	11.5±2.8	11.8±1.4	11.8±1.4
RBC $X10^6(/\mu L)$	7.6±0.5	7.3±0.6	7.5±0.3	7.8±0.5
Platelets $X10^6(/\mu L)$	1.22±0.41	1.30±0.19	1.32±0.12	1.26±0.26
Hematocrit (%)	41.9±1.8	41.9±1.6	42.8±3.7	41.1±2.9
Hemoglobin (g/dL)	17.5±1.5	17.5±1.1	17.2±2.3	18.3±1.0
Lymphocytes (%)	83.4±8.8	88.0±5.5	81.3±8.8	91.2±9.7
Neutrophils (%)	16.6±7.5	12.0±9.4	18.7±2.3	8.4±6.4

[1]values are expressed as mean±SD;n=10
[2]%extractives in drinking water

Table 3A: Biochemical parameters determined in male Wistar rats treated with cedar extractives for 90-days[1].

	DOSE[2]			
	0%	2%	5%	7%
Albumin (g/dL)	2.8±0.2	3.0±0.2	2.7±0.2	3.0±0.2
BUN (mg/dL)	12.9±2.9	13.0±2.4	13.5±2.4	12.9±2.2
Creatinine (mg/dL)	0.76±0.15	0.74±0.08	0.70±0.07	0.66±0.04
Glucose (mg/dL)	125.0±13.0	123.0±9.0	130.0±11.0	125.0±14.0
Phosphorous (mg/dL)	6.0±0.5	5.8±0.5	6.3±0.3	6.5±0.5
Protein (g/dL)	5.9±0.6	6.0±0.3	5.8±0.5	5.5±0.4
Globulin (g/dL)	2.0±0.2	1.9±0.1	1.9±0.1	1.9±0.1

[1]values are expressed as mean±SD;n=10
[2]%extractives in drinking water

Table 3B: Biochemical parameters determined in female Wistar rats treated with cedar extractives for 90-days[1].

	DOSE[2]			
	0%	2%	5%	7%
Albumin (g/dL)	3.1±0.2	3.0±0.5	3.3±0.2	3.2±0.1
BUN (mg/dL)	15.0±3.0	13.4±2.5	16.3±3.4	15.0±3.0
Creatinine (mg/dL)	0.78±0.10	0.92±0.09	0.84±0.08	0.90±0.21
Glucose (mg/dL)	109.0±17.0	96.4±14.0	100.0±9.0	90.0±12.8
Phosphorous (mg/dL)	5.5±0.6	5.1±0.9	5.4±0.5	5.6±0.7
Protein (g/dL)	6.1±0.5	6.2±0.6	6.2±0.4	5.9±0.1
Globulin (g/dL)	2.0±0.2	2.0±0.1	2.1±0.1	2.0±0.1

[1]values are expressed as mean±SD;n=10
[2]%extractives in drinking water

Table 4A: Serum enzyme activity[1] determined in male Wistar rats treated with cedar extractives for 90-days[2].

	DOSE[3]			
	0%	2%	5%	7%
ALT	33.9±4.2	34.±5.4	32.9±6.7	37.3±4.2
AST	85.8±12.0	87.1±13.6	87.1±14.7	87.6±3.9
AP	70.1±10.4	68.1±14.0	65.4±12.2	71.3±14.1
CK	87.8±32.0	118.0±39.1	197.4±41.3	122.3±30.4
GGT	10.9±3.6	4.6±1.7	10.4±3.1	7.5±2.1
IDH	3.0±0.6	2.3±0.5	3.2±1.0	2.9±0.7

[1]enzyme activities are expressed in U/L
[2]values are expressed as mean±SD;n=10
[3]%extractives in drinking water

Table 4B: Serum enzyme activity[1] determined in female Wistar rats treated with cedar extractives for 90-days[2].

	DOSE[3]			
	0%	2%	5%	7%
ALT	35.3±7.0	32.7±6.5	40.3±4.0	35.3±4.5
AST	80.8±10.2	81.9±13.6	80.2±10.9	81.5±12.0
AP	55.7±10.5	63.4±11.7	46.2±4.5	45.7±4.1
CK	49.7±25.0	78.2±34.0	92.0±19.4	65.8±27.2
GGT	7.83±4.10	3.24±0.97	7.67±1.70	7.05±2.4
IDH	3.16±1.30	2.62±0.64	3.51±0.84	3.51±1.25

[1]enzyme activities are expressed in U/L
[2]values are expressed as mean±SD;n=10
[3]%extractives in drinking water

Table 5A: Serum electrolytes determined in male Wistar rats treated with cedar extractives for 90-days[1].

	DOSE[2]			
	0%	2%	5%	7%
Ca(mg/dL)	9.82±0.4	9.56±0.39	9.51±0.44	9.61±0.52
Cl(meq/L)	97.0±40.1	95.5±29.2	100.3±22.7	99.0±29.1
K(meq/L)	5.0±0.3	4.7±0.3	5.0±0.3	5.2±0.4
Na(meq/L)	135.2±2.0	136.5±1.0	136.1±1.0	136.3±1.0

[1]values are expressed as mean±SD;n=10
[2]%extractives in drinking water

Table 5B: Serum electrolytes determined in female Wistar rats treated with cedar extractives for 90-days[1].

	DOSE[2]			
	0%	2%	5%	7%
Ca(mg/dL)	9.93±0.60	9.73±0.80	10.6±0.61	9.80±0.43
Cl(meq/L)	99.5±36.1	97.8±39.4	97.5±39.6	101.0±48.9
K(meq/L)	5.0±0.6	4.7±0.8	5.1±0.4	5.2±0.4
Na(meq/L)	135.2±2.0	136.0±2.0	137.2±2.0	136.1±2.0

[1]values are expressed as mean±SD;n=10
[2]%extractives in drinking water

Although some parameters of the treated animals were significantly different from those of the control, a dose-related response was not detected. For example, CK activities at the 5% dose level were significantly ($p \leq 0.05$) higher than that of the control group; however, these values were within the range of biological variation. Furthermore, the values for this enzyme at the 7% dose level did not significantly differ from the control values. Similarly, GGT activities at the 2% dose level differed significantly ($p \leq 0.05$) from control values. However, the values at the 5% and 7% dose levels did not differ significantly from the control values. No toxicological significance could be attributed to these findings since no dose-related response was found.

Necropsy

The organ to body weight ratios are presented in tables 6A and 6B. No statistical difference between the control and treated groups was found in the weighed organs. Furthermore, macroscopic examination of the organs during necropsy revealed no gross lesions.

Table 6A: Organ to body weight ratios of male Wistar rats treated with cedar extractives for 90-days[1].

	DOSE[2]			
	0%	2%	5%	7%
Brain	4.62±0.15	4.61±0.24	4.71±0.25	4.65±0.16
Heart	2.95±0.40	2.92±0.18	2.84±0.47	2.89±0.42
Lung	4.02±0.60	3.70±0.52	3.40±0.27	4.07±0.59
Liver	30.5±4.1	29.6±2.3	29.5±4.7	29.1±3.2
Spleen	1.89±0.24	1.70±0.16	1.90±0.40	1.59±0.30
Kidney	7.14±0.71	6.90±0.49	6.87±0.92	7.32±0.78
Testes	7.38±0.39	7.04±0.71	7.39±0.82	7.03±0.54

[1]values are expressed as (mean±SD) times 1000;n=10
[2]%extractives in drinking water

Table 6B: Organ to body weight ratios of female Wistar rats treated with cedar extractives for 90-days[1].

	DOSE[2]			
	0%	2%	5%	7%
Brain	7.82±0.44	7.54±0.36	7.89±0.27	7.81±0.20
Heart	3.30±0.25	3.22±0.19	3.52±0.18	3.27±0.20
Lung	4.87±0.83	4.81±0.70	5.41±1.0	5.41±0.83
Liver	29.6±3.1	31.3±5.3	30.7±2.6	29.6±2.4
Spleen	2.49±0.34	2.34±0.37	2.66±0.41	2.29±0.34
Kidney	7.14±0.71	6.92±0.49	7.48±0.92	7.14±0.78

[1]values are expressed as (mean±SD) times 1000;n=10
[2]%extractives in drinking water

III. METABOLIC DISPOSITION OF β-THUJAPLICIN IN THE RAT

Analysis of β-thujaplicin

A typical HPLC chromatogram of the blood extractive is shown in Fig. 3. β-Thujaplicin and 2,4-DCDE had retention times of 1.80±0.10 min and 2.50±0.10 min, respectively. Recoveries of β-thujaplicin from the blood, urine and bile ranged between 72-84%, 75-85% and 70-85%, respectively.

Time course of unchanged β-thujaplicin in rat blood after i.v. administration

Fig. 4 shows the time course of unmetabolized BT in the rat blood after i.v. administration (30 mg/kg). The figure represents a semilogarithmic plot of the averaged data of 4 animals (mean±SD). Unmetabolized BT concentration declined biphasically with time and reached the HPLC detection limit of 7-12 ng/μL in about 1.5-2 h after administration. These data could be fitted to a biexponential equation: $C_B=Ae^{-\alpha t}+Be^{-\beta t}$, where C_B is the blood concentration (μg/mL) of BT at time t, A and B are the initial concentrations of each component (μg/mL), α and β are the rate elimination constants (min^{-1}), and t is time (min). This equation represents a two-compartment open pharmacokinetic model with first order absorption and elimination. The computer generated estimates for A, B, α, and β and the calculated values for the secondary pharmacokinetic

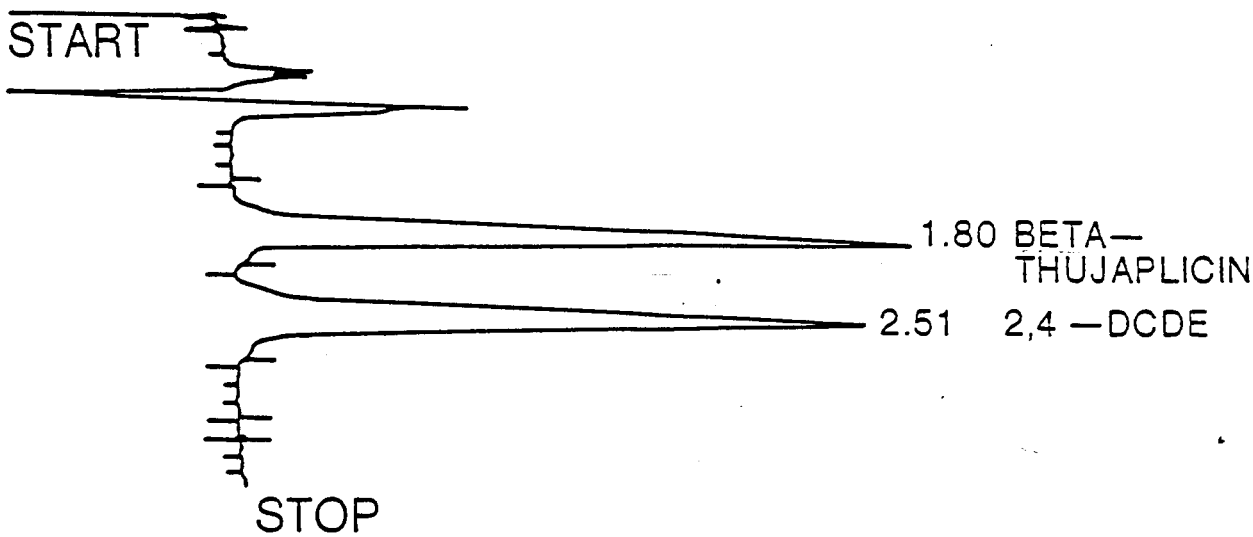
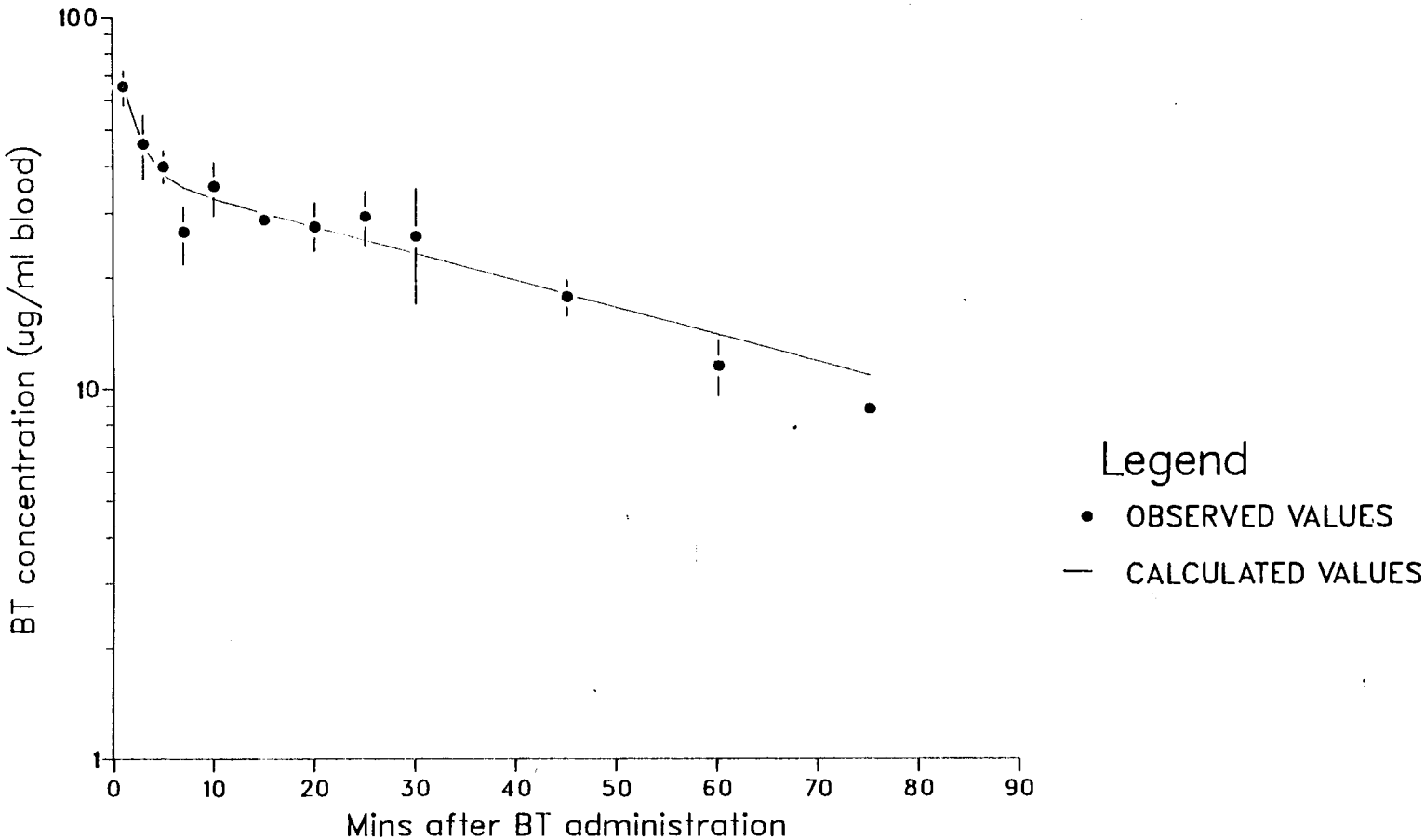


Fig. 3: A typical HPLC chromatogram of the blood extractive

FIG. 4: MEAN TIME COURSE OF UNCHANGED
BT IN THE RAT BLOOD AFTER IV ADMINISTRATION OF 30 mg/kg



parameters: k_{12}, k_{21}, k, $t_{(1/2)k}$, V_C, Cl and AUC for the averaged data are presented in table 7. The time course of unchanged BT in blood of individual rats is shown in Appendix I. These data could not be adequately curve-fitted since they were widely dispersed.

Fig. 5 shows the time course of unmetabolized BT in the rat blood after i.v. administration (20 mg/kg). The curve represents a semilogarithmic plot of the averaged data of 4 animals (mean±SD). Unmetabolized BT concentration declined monophasically with time and reached the HPLC detection limit at 1.5-2 h after administration. These data could be fitted to a monoexponential equation: $C_B=C_0e^{-kt}$, where C_B is the blood concentration (μg/mL) of unchanged BT at time t, C_0 is the initial concentration (μg/mL), k the elimination rate constant (min^{-1}), and t is time (min). This equation represents a one-compartment open pharmacokinetic model with first order absorption and elimination. The computer generated estimates for C_0 and k and values for the secondary pharmacokinetic parameters: $t_{(1/2)k}$, V_d, Cl and AUC for the averaged data are presented in table 7. The time course of unchanged BT in blood of individual rats is shown in Appendix II. These data could not be adequately curve-fitted since they were widely dispersed.

FIG. 5: MEAN TIME COURSE OF UNCHANGED
BT IN THE RAT BLOOD AFTER IV ADMINISTRATION OF 20 mg/kg

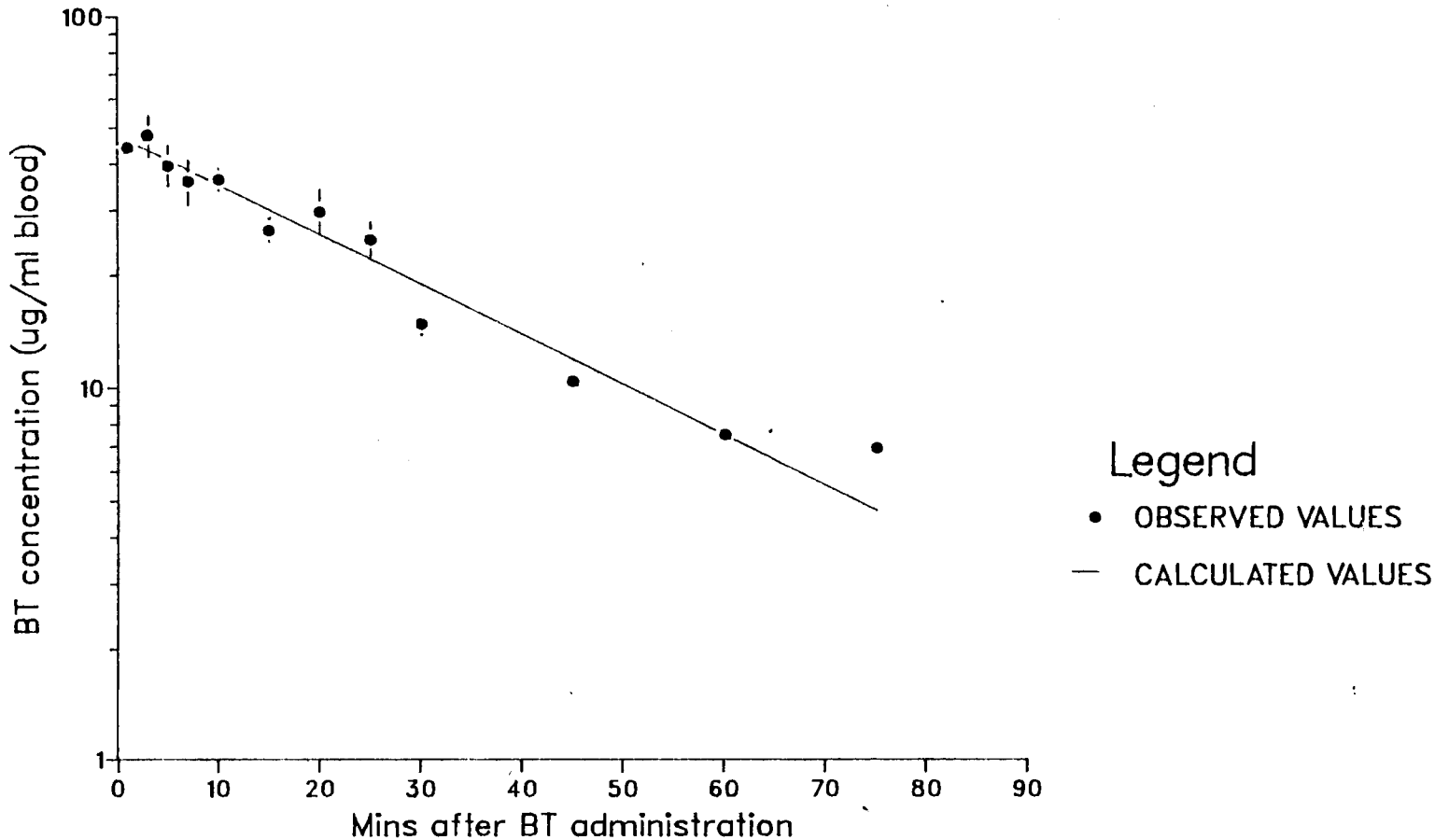


Table 7: Pharmacokinetic parameters of β-thujaplicin from rat blood after i.v. administration

	DOSE	
PARAMETER	30 mg/kg	20 mg/kg
C_0 (μg/mL)	n/a[1]	47.6
A (μg/mL)	50.7	n/a
B (μg/mL)	38.4	n/a
α (min^{-1})	0.593	n/a
β (min^{-1})	0.0169	n/a
k_{12} (min^{-1})	0.307	n/a
k_{21} (min^{-1})	0.266	n/a
k (min^{-1})	0.0377	0.0309
$t_{(1/2)k}$ (min)	18.4	22.4
V_c (mL/kg)	336	n/a
V_d (mL/kg)	n/a	420
Cl ($mLkg^{-1}min^{-1}$)	18.5	15.2
AUC (μg/mL)(min)	1621	1313

[1] n/a not applicable

Time course of unchanged β-thujaplicin in rat blood after oral administration

Fig. 6 and 7 show the time course of unmetabolized BT in the blood after oral administration of 30 mg/kg or 20 mg/kg to rats, respectively. Each figure represents a semilogarithmic plot of the averaged data of 4 rats (mean±SD) since the dispersion of blood concentrations in individual animals (see Appendix III and IV) made it impossible to appropriately curve-fit these data. Unmetabolized BT concentration in blood were detected within 1 min and peaked between 10-15 min after oral administration. Furthermore, unmetabolized BT could not be detected in the blood approximately 1.5-2 h after oral administration. The averaged data could be fitted to the equation: $C_B=(FXD/V_d)(k_a/(k_a-k))(e^{-kt}-e^{-k_at})$ where C_B is the blood concentration (μg/mL) of unchanged BT at time t, F is the fraction of the dose absorbed, D is the dose (μg/kg), V_d is the volume of distribution (mL/kg), k_a is the absorption rate constant (min^{-1}) and k is the elimination rate constant (min^{-1}). This equation represents a one-compartment open pharmacokinetic model with first order absorption and elimination. The computer generated estimates for k_a and k and values for the secondary pharmacokinetic parameters: $t_{(1/2)k}$, F, V_d, Cl and AUC in rats given 30 mg/kg or 20 mg/kg of BT orally are presented in table 8.

Table 8: Pharmacokinetic parameters of β-thujaplicin from rat blood after oral administration

PARAMETER	DOSE	
	30 mg/kg	20 mg/kg
k_a (min^{-1})	0.170	0.651
k (min^{-1})	0.0131	0.0109
$t_{(1/2)k}$ (min)	52.9	63.6
F	0.648	0.687
V_d (mL/kg)	759	627
Cl ($mLkg^{-1}min^{-1}$)	18.5	15.2
AUC (μg/mL)(min)	1050	903

FIG. 6: MEAN TIME COURSE OF UNCHANGED
BT IN THE RAT BLOOD AFTER ORAL ADMINISTRATION OF 30 mg/kg

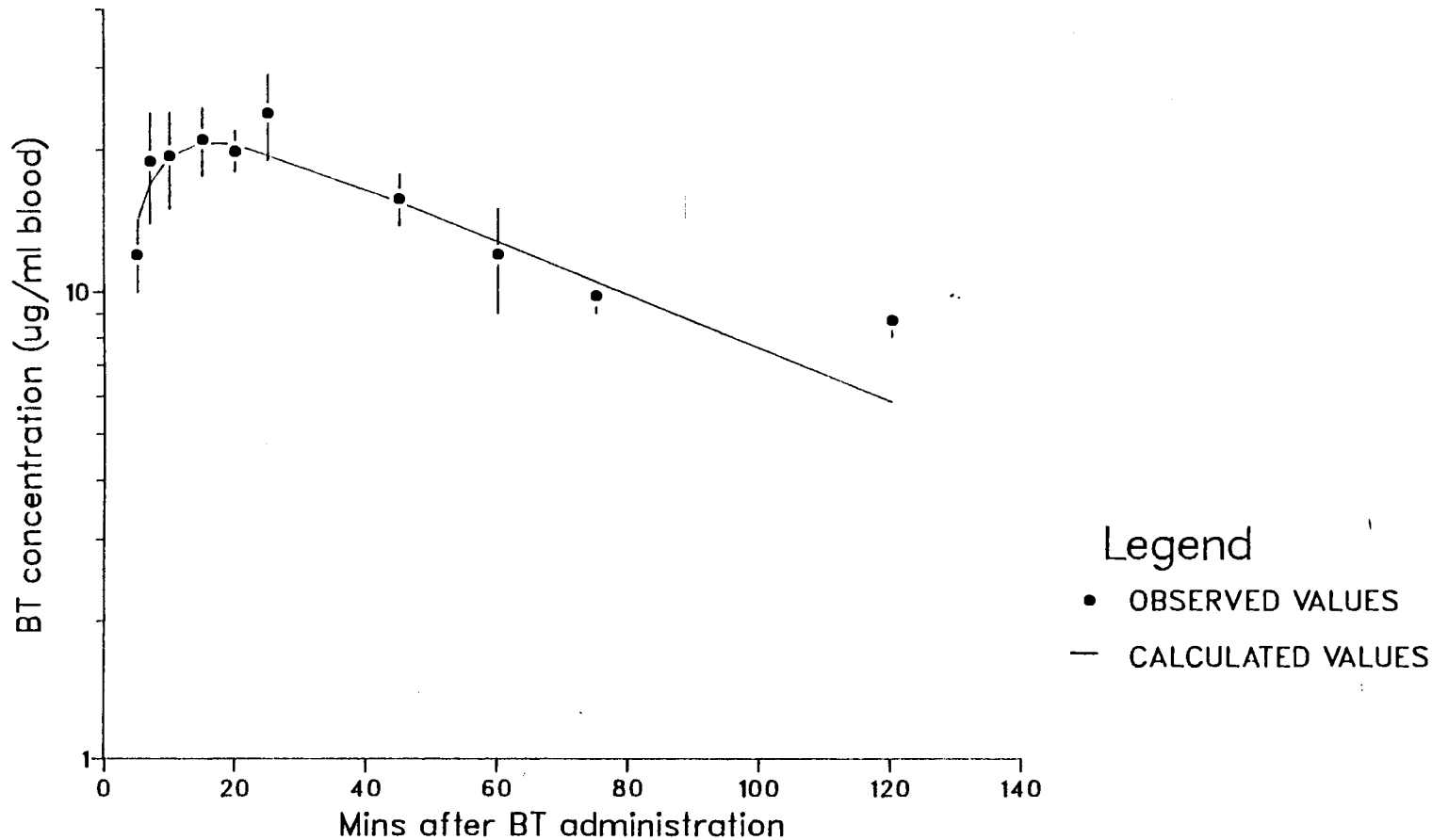


FIG. 7: MEAN TIME COURSE OF UNCHANGED
BT IN THE RAT BLOOD AFTER ORAL ADMINISTRATION OF 20 mg/kg

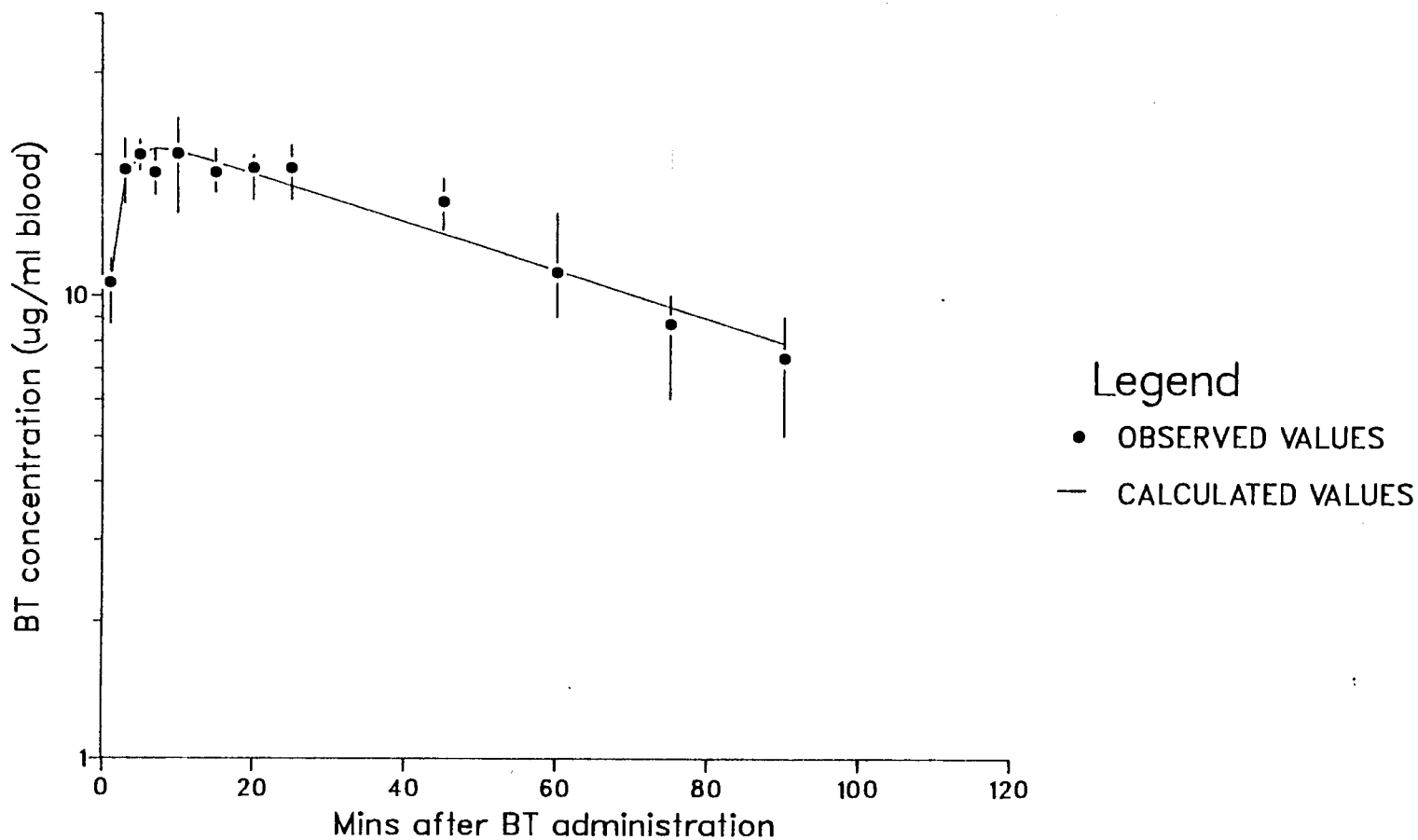
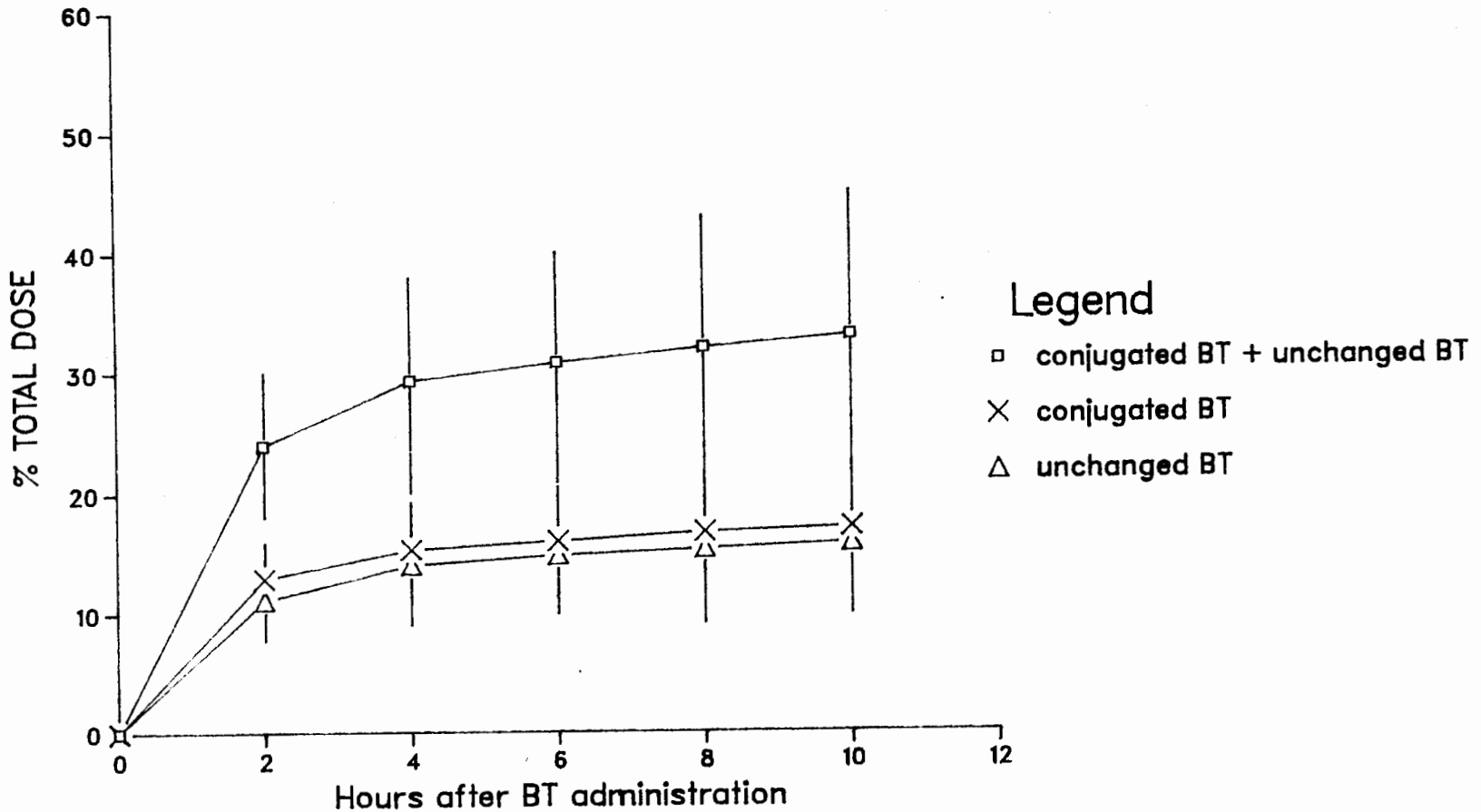


FIG. 8: CUMULATIVE EXCRETION OF CONJUGATED AND UNCHANGED BT IN THE RAT URINE AFTER IV ADMINISTRATION OF 30 mg/kg



Urinary excretion of conjugated and unchanged β-thujaplicin

Fig. 8 shows that BT was rapidly excreted in urine. The mean±SD data of 4 rats are presented. Urinary excretion of BT reached a plateau at 4-6 h after i.v. administration (30 mg/kg). About 26-32% of the administered dose was excreted in the urine in 8-10 h. The amounts of unchanged and conjugated forms of BT were about equal in the urine.

Biliary excretion of conjugated and unchanged β-thujaplicin

Fig. 9 shows the excretion rate of conjugated and unchanged BT in bile after i.v. administration (30 mg/kg). The mean±SD data of 4 rats are presented.

The excretion rates of conjugated BT and unchanged BT in bile could be fitted to a monoexponential equation $C_b=C_0e^{-kt}$, where C_b (μg/min/kg rat) is the amount of unchanged or conjugated BT in bile at time t, C_0 (μg/min/kg rat) the initial amount in bile, k the elimination rate constant (min^{-1}), and t is time (min). The computer generated estimates for C_0 and k are 256.0±89.0 μg/min/kg rat and 0.0258±0.0064 min^{-1}, respectively, for conjugated BT. The computer generated estimates for C_0 and k are 19.7±3.6 μg/min/kg rat and 0.0303±0.00306 min^{-1}, respectively, for unchanged BT.

FIG. 9: MEAN EXCRETION RATE OF CONJUGATED AND UNCHANGED BT IN THE RAT BILE AFTER IV ADMINISTRATION OF 30 mg/kg

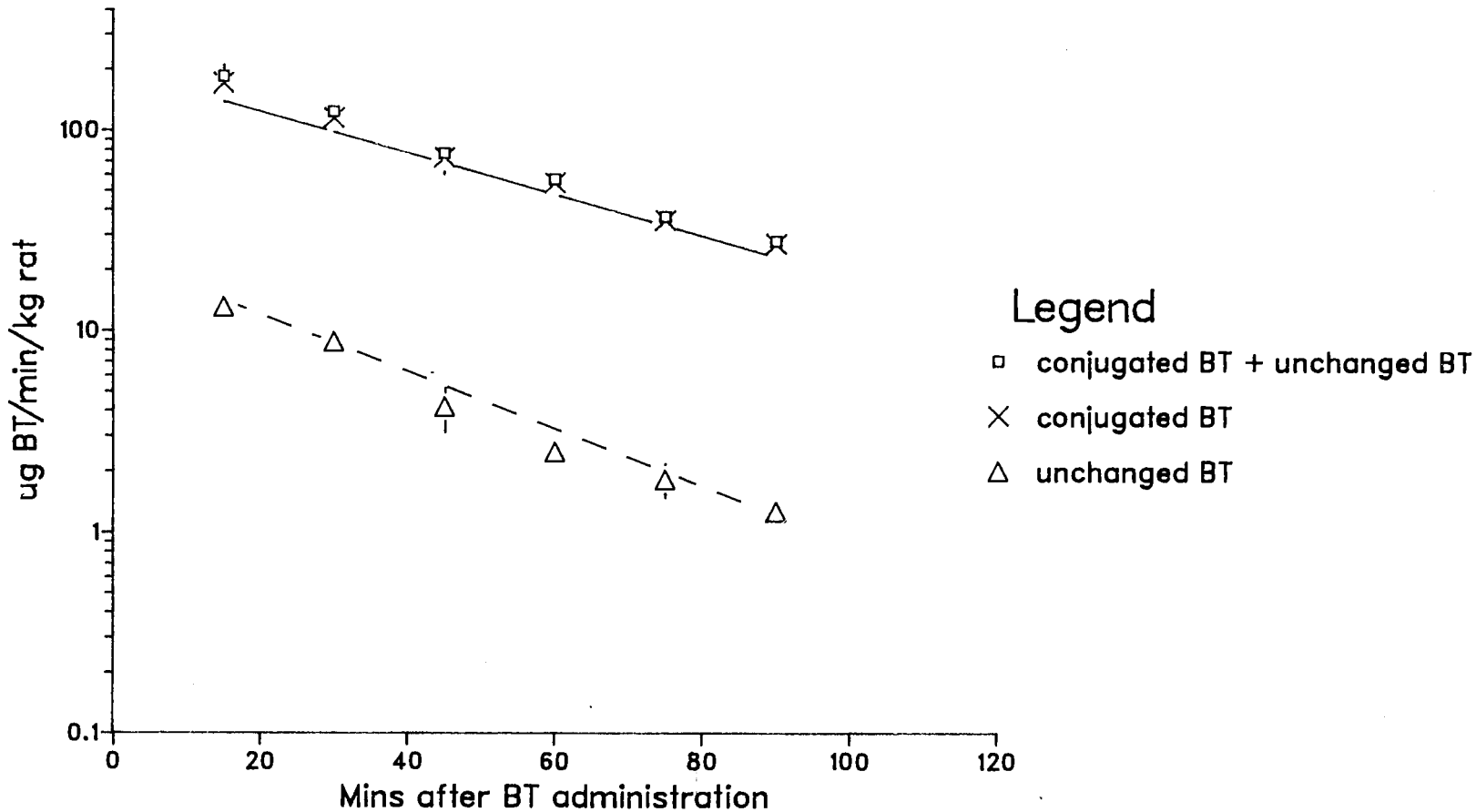


FIG. 10: MEAN BILE FLOW RATE OF FOUR RATS ADMINISTERED IV 30 mg/kg OF BT

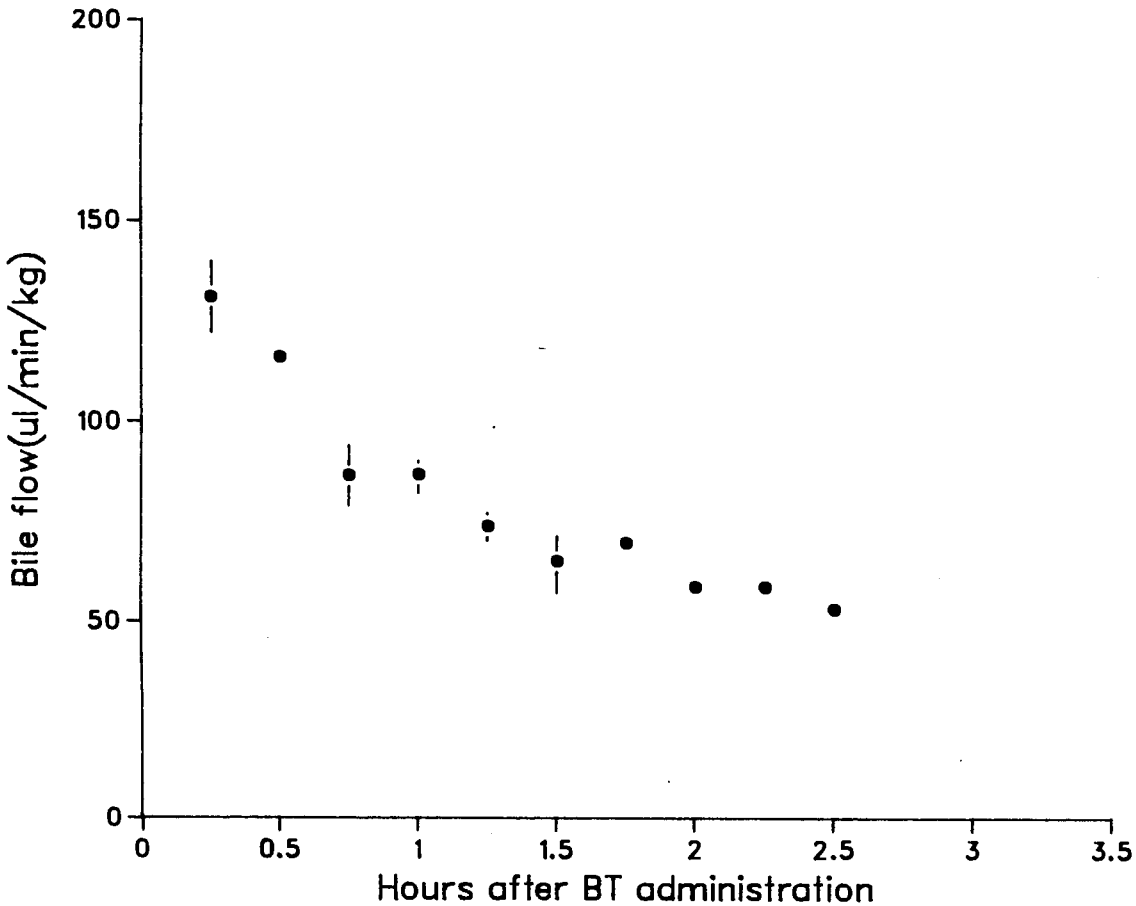


Fig. 10 shows the bile flow rate in treated animals, it decreased rapidly in the first h after BT administration and then leveled off with time. Fig. 11 shows the cumulative excretion of BT in the bile. Approximately 25-28% of the administered dose was excreted in the bile. Most of the BT was excreted within 1.5-2 h after chemical administration. About 85-95% and 5-10% of the BT in the bile were in the conjugated and unchanged forms, respectively.

Enterohepatic recycling of β-thujaplicin

When bile from a rat treated intravenously with BT was given to a recipient rat by gastric gavage, conjugated and unchanged BT was found in the recipient animal's bile (Fig. 12). Biliary excretion of BT peaked at approximately 1.5-2 h after the recipient animal was given donor bile. The percentages of conjugated and unchanged BT in the bile of the recipient rat ranged between 85-90% and 5-10%, respectively.

FIG. 11: CUMULATIVE EXCRETION OF CONJUGATED AND UNCHANGED BT IN THE RAT BILE AFTER IV ADMINISTRATION OF 30 mg/kg

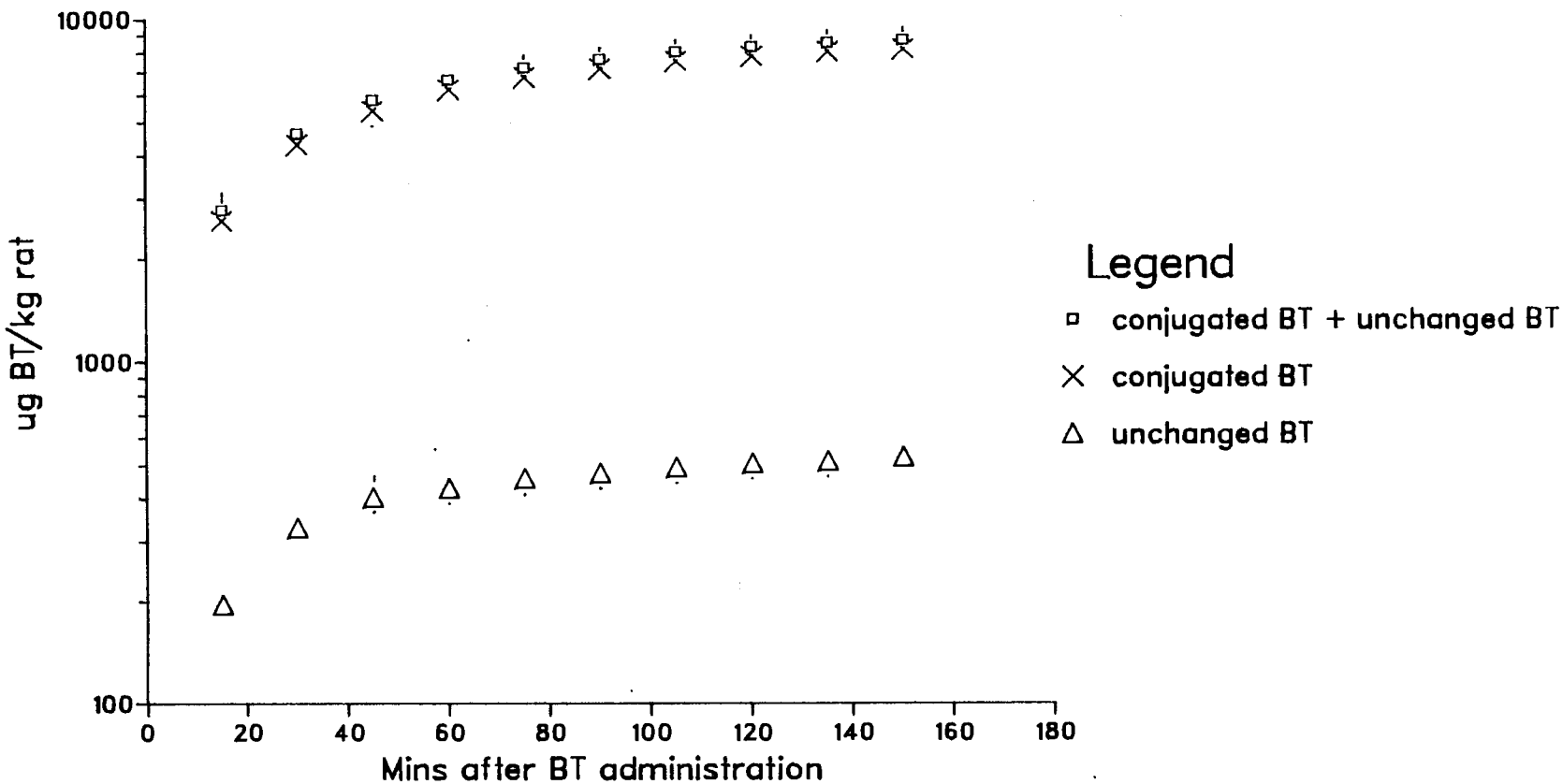
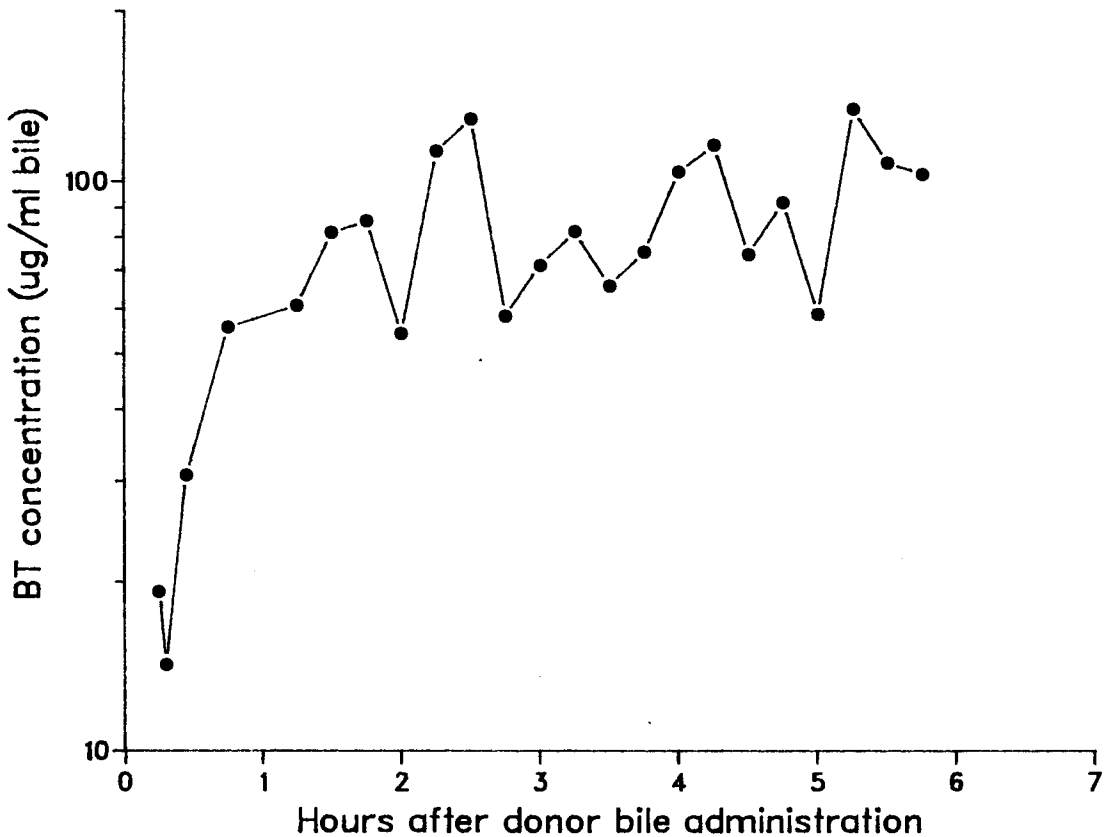


FIG. 12: BT CONCENTRATION IN THE RAT BILE AFTER ORAL ADMINISTRATION OF DONOR BILE



PART D

DISCUSSION

I. ACUTE TOXICITY OF WESTERN RED CEDAR EXTRACTIVES

Based on the results of the LD_{50} studies, the extractives should be classified as practically non-toxic to rats (Gosselin *et al.*, 1976). The high acute dermal LD_{50} of the extractives is consistent with the finding that most aqueous solutions are poorly absorbed through the skin (Renwick, 1982; Klaassen, 1984 and Lien *et al.*, 1973). My results also are consistent with the low toxicity of the individual components of the extractives. For example, γ-thujaplicin, which accounts for 0.0048% of the extractives, has an oral LD_{50} in excess of 100 mg/kg in the rat (Halliday, 1959). Plicatic acid, which accounts for 0.08% of the extractives, is sufficiently non-toxic that low levels are used as food antioxidants (Karchmar and McDonald, 1969; Howard and McIntosh, 1969; Bishov and Henick, 1975). Borax, which accounts for 2% of the extractives, has an oral LD_{50} of 300-700 mg/kg in the rabbit, guinea pig, dog and rat (Verbitskaya, 1975).

The extractives did not cause skin irritation in laboratory animals and should be classified as a non-irritant to the skin (Draize *et al.*, 1944). However, my results appear to differ from the findings of Chan-Yeung *et al.* (1973) who reported the formation of wheals on human skin exposed to Western red cedar extractives. A plausible explanation for the difference in results may be that the human subjects in their studies were sensitized by Western red cedar while working in the forest industry whereas the laboratory animals of my study were not

sensitized to the extractives. In addition, the test mixture in their studies contained 1-5% Western red cedar extractives (Chan-Yeung *et al.*, 1971; Barton and MacDonald, 1971) whereas the solution in my study contained less than 0.1% Western red cedar extractives (Anon, 1982). My result also differs to that of Evans and Nicholls (1974) who showed that Western red cedar dusts (5-100 mg/mL) caused histamine release in human or pig lung tissue cultures. It should be noted, however, that the study of Evans and Nicholls (1974) was performed with *in vitro* tissue incubation and not with whole animals.

Chan-Yeung *et al.* (1980) proposed that the effect of plicatic acid on human skin was caused by the activation of the complement system. This mechanism was confirmed by Giclas (1982) who showed that plicatic acid activates the complement system by the classical pathway. Therefore, plicatic acid may be responsible for wheal formation on human skin exposed to Western red cedar extractives. Since the Western red cedar extractives used in my dermal irritation study contain approximately 0.08% plicatic acid, it is possible that the plicatic acid concentration in the extractives is not high enough to cause skin irritation.

My study indicated that when undiluted Western red cedar extractives were applied to the rabbit eyes, ocular irritation was not detected. This result is consistent with the mild alkalinity (pH 8.5±0.2) of borax solutions which were used as bactericidal eye lotions (Lion Corporation, 1982).

Peters (1976) noted that Western red cedar effluents are relatively toxic to fish. The 96 h LC_{50} of the volatile fraction of Western red cedar effluent for coho salmon is 2.7 mg/L. However, fish have limited predictive value for mammalian toxicity since interactions between chemicals, water and lipid membranes can cause variations in chemical uptake by the fish (Hodson, 1985).

II. SUBCHRONIC ORAL TOXICITY OF WESTERN RED CEDAR EXTRACTIVES IN RATS

No significant difference in the parameters examined was found between the treated and control animals. A transitory change in the external appearance of the rats is a dried red substance accumulating around the eyes, nose and mouth. However, this condition does not persist beyond 3-5 days duration and the affected animal ultimately recovers to its original appearance. Furthermore, this condition appears randomly among the treatment and control groups. This condition may be attributed to stress since the incidence increases with the duration of the study. Similar transitory changes in the facial appearance of rats were reported by Sasmore and coworkers (1983) in their subchronic study of 1,3,5-trichlorobenzene.

Statistically significant differences in mean serum enzyme values for CK and GGT were found at the completion of the subchronic study. However, no toxicological significance could be attributed to such changes since they were not dose related and were within the range of biological variation reported previously by Arnold *et al*. (1977) and Chan *et al*. (1980).

The extractives used in my subchronic study contain water (97.8%), borax (2%), carbohydrate (0.1%), plicatic acid (0.08%) and thujaplicin (0.0048%) (Anon, 1982). Therefore, the potential toxic actions of the extractives most likely are due to the presence of borax, plicatic acid and thujaplicin. Results of the

present study are supported by the findings of previous toxicity studies of the three chemicals:

(1) Borax. Weir and Fisher (1972) studied the subchronic toxicity of borax in rats. Animals fed up to 4630 ppm borax showed no signs of toxicity since 15430 ppm borax was required to cause toxic effects which were primarily reproductive organ degeneration. Weir and Fisher (1972) also reported that 3087 ppm borax fed to rats for 2 years caused no ill-effects. In my subchronic study, the maximum dose was 7% Western red cedar extractives which is equivalent to 1400 ppm borax; a level well below that found to be toxic by Weir and Fisher (1972). My results also are consistent with those of Shaipanich and Anukrahanon (1978) who showed that rats consuming up to 1.5% borax for 3 weeks experienced no mortality.

(2) Plicatic acid. Inhaled plicatic acid is an allergen to humans (Chan-Yeung *et al.*, 1973). It also causes asthmatic attacks in workers exposed occupationally to Western red cedar dust (Chan-Yeung *et al.*, 1980). However, the effects of orally ingested plicatic acid have not been examined. Since plicatic acid is readily soluble in water (Gardner *et al.*, 1959), it is expected to have low toxicity in mammals because it would be excreted readily by animals after oral ingestion. Therefore, low levels of plicatic acid have been used as antioxidants in foods (Karchmar and McDonald, 1969; Bishov and Henick, 1975). Furthermore, due to its low content (0.08%) in the extractives, plicatic acid may contribute little to the toxicological effects of the ingested extractives.

(3) β-Thujaplicin. Although previous studies showed that BT had inhibitory effects on different enzymes (Bohme and coworkers, 1980; Goldstein and coworkers, 1964, 1967; Lyr, 1962, 1966), these studies were carried out with isolated enzymes *in vitro*. In contrast, the present study was carried out in intact animals with BT content less than 0.0048% of the extractives. Moreover, the pharmacokinetics of BT in rats demonstrated that BT is excreted rapidly after oral absorption (see next section).

III. METABOLIC DISPOSITION OF β-THUJAPLICIN IN THE RAT

The present study shows that BT decays rapidly from the rat blood after intravenous administration (Fig. 4 and 5). The half-life of elimination of BT from blood is 18.4-22.4 min. BT declines to undetectable levels within 1.5-2 h after chemical administration. Therefore, my results are consistent with those of Kazuo (1961) who reported that BT levels in rabbit blood decreased to unmeasurable levels within 1 h after i.v. administration.

The time courses of BT concentrations in the blood after i.v. administration of 30 mg/kg and 20 mg/kg to the rat can be described by a two- and one-compartment open pharmacokinetic model, respectively. The pharmacokinetics of BT are not dose dependent since the secondary pharmacokinetic parameters are not affected by the dose (Gibaldi and Perrier, 1975). For example, the $t_{(1/2)k}$ (18.4-22.4 min) and Cl (15.2-18.5 $mLkg^{-1}min^{-1}$) are very similar regardless of the dose given. Furthermore, the similarities in k_{12} (0.307 min^{-1}) and k_{21} (0.266 min^{-1}) show that BT is not appreciably accumulated in the peripheral compartment.

BT concentrations in blood after oral administration of 30 or 20 mg/kg to the rat are best described by a one-compartment open pharmacokinetic model. The pharmacokinetics of BT after oral administration also are dose independent since similar values of $t_{(1/2)k}$, F, V_d and Cl are obtained from rats dosed

with 20 mg/kg or 30 mg/kg of BT.

BT is rapidly excreted from the rat after i.v. administration; urinary excretion peaks at 4-6 h after chemical administration (Fig. 8). About 26-32% of the BT administered is excreted by the urinary route in 8-10 h. My findings agree with those of Hino (1961) who found that urinary excretion of BT in the rabbit after IP administration reached a maximum within 2 h and lasted for 13-19 h.

BT also is rapidly excreted into the bile of rats (Fig. 11). Biliary excretion peaks in 1.5-2 h after chemical administration. About 25-28% of the BT administered is excreted by this route. In contrast, Hino (1961) reported only trace amounts of BT in the bile after IP administration to the rabbit. This difference in results probably is caused by species differences of biliary excretion (Klaassen and Plaa, 1967; Klaassen *et al.*, 1981; Smith, 1966).

BT undergoes enterohepatic recycling in the rat (Fig. 12). Maximum recycling occurs between 1.5-2 h after the rat receives bile from the donor rat. Therefore, the fluctuations observed in the blood concentration-time curves of individual rats (Appendix I-IV) probably are due to enterohepatic recycling of BT since enterohepatic recycling has been shown to alter the shape of blood concentration-time curves (Ritschel, 1980; Molino and Milanese, 1982). Rovei and coworkers (1985) also found that enterohepatic recycling changed the blood concentration-time

curves after intravenous and oral administration of carocainide to man. Wayburn and coworkers (1979) showed that enterohepatic recycling changed the blood concentration-time curves after i.v. administration of phenolpthalein to rats.

IV. SUMMARY

In summary, results of the acute, subchronic and metabolic disposition studies indicate that Western red cedar extractives are relatively non-toxic to laboratory animals. Although these studies were carried out in only a few laboratory animals and may not have been able to detect all potential toxicities of the extractives (Arnold *et al.*, 1977; Garanttini, 1985; Chan *et al.*, 1980), these studies suggest that the Western red cedar extractives used by Canfor are of very low toxicity to mammals.

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APPENDIX I

Time course of unmetabolized BT in the blood after IV administration to rats[1]

TIME(min)	Rat number							
	1	2	3	4	5	6	7	8
1	46.0	73.4	74.3	67.4	18.7	26.9	22.0	22.8
3	50.3	43.4	28.3	65.8	18.1	24.9	29.8	21.5
5	28.3	47.5	40.2	44.0	18.0	28.7	23.8	23.4
7	40.6	21.2	14.2	30.9	17.9	24.0	23.7	18.0
10	n[2]	49.1	23.5	33.5	20.4	20.7	20.5	n
15	29.2	n	31.3	26.1	18.8	24.3	17.2	22.0
20	24.5	16.7	37.2	31.5	17.1	19.0	13.4	18.0
25	32.1	35.8	35.8	13.8	16.1	19.4	14.0	n
30	16.9	n	13.8	47.1	17.3	19.3	-[3]	13.0
45	12.2	17.3	20.2	21.2	12.8	13.0	13.7	13.1
60	9.9	11.0	17.0	7.8	12.9	14.6	11.9	19.9
75	8.1	8.7	9.6	8.7	10.7	11.4	11.1	10.7
90	7.3	7.7	7.0	7.9	-	10.7	10.9	n
120	-	7.5	-	-	-	10.6	-	n
150	-	-	-	-	-	-	-	n

[1] dose of administration, 30 mg/kg

[2] n data not available

[3] - undetectable

APPENDIX II

Time course of unmetabolized BT in the blood after IV administration to rats[1]

TIME(min)	Rat number 1	2	3	4
1	48.9	41.2	43.6	43.6
3	50.3	32.3	63.4	45.4
5	54.3	34.3	35.4	34.9
7	46.4	38.4	33.7	25.0
10	32.3	35.1	43.8	33.9
15	29.1	24.5	26.0	-
20	24.4	42.2	31.5	20.8
25	22.8	26.7	30.9	19.0
30	19.7	22.7	24.5	18.6
45	12.5	18.0	16.5	12.3
60	9.1	13.2	10.9	8.2
75	-[3]	11.2	7.5	7.2
90	-	-	7.0	7.5
120	-	-	-	n[2]
150	-	-	-	-

[1] dose of administration, 20 mg/kg
[2] n data not available
[3] - undetectable

APPENDIX III

Time course of unmetabolized BT in the blood after oral administration to rats[1]

TIME(min)	Rat number							
	1	2	3	4	5	6	7	8
1	7.1	7.5	9.5	7.6	11.0	15.0	17.3	10.1
3	8.9	56.3	19.6	18.4	20.9	40.1	23.7	19.2
5	13.0	8.4	17.5	9.2	22.2	43.6	50.3	27.1
7	n[2]	19.0	29.2	8.3	19.6	34.5	37.6	31.7
10	28.7	11.7	25.2	12.2	15.9	39.0	25.0	22.8
15	26.5	10.0	25.2	22.7	16.5	33.1	26.5	20.7
20	23.7	14.0	22.8	19.1	18.3	43.4	25.4	28.7
25	33.8	18.3	32.1	11.8	14.3	47.0	25.8	30.9
30	n	13.8	n	16.3	15.0	30.5	29.5	20.8
45	17.8	13.8	18.1	n	19.2	22.8	18.8	31.3
60	n	16.6	7.8	7.5	15.3	19.0	14.2	19.9
75	12.4	9.3	-[3]	-	11.9	7.9	10.0	22.6
90	n	11.2	-	-	8.9	7.9	21.5	15.1
120	9.1	8.9	-	-	7.1	7.8	7.4	n
150	8.5	8.4	-	-	-	7.3	-	n

[1] dose of administration, 30 mg/kg

[2] n data not available

[3] - undetectable

APPENDIX IV

Time course of unmetabolized BT in the blood after oral administration to rats[1]

TIME(min)	Rat number 1	2	3	4
1	8.0	14.3	13.9	6.7
3	19.9	25.6	17.1	12.2
5	20.4	23.1	21.0	15.7
7	23.4	17.5	18.3	14.3
10	16.7	19.2	29.5	15.4
15	17.5	19.1	18.8	18.0
20	20.7	20.7	18.3	15.5
25	22.7	18.5	17.4	16.6
30	14.4	17.7	22.1	20.7
45	16.6	13.4	8.2	17.6
60	8.8	9.3	12.4	14.3
75	7.1	7.5	29.0	11.4
90	-[2]	-	7.0	10.3
120	-	-	-	8.2
150	-	-	-	7.5

[1] dose of administration, 20 mg/kg
[2] - undetectable