The Metabolism of Razoxane and Dexrazoxane

In the Sprague-Dawley Rat

BY

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A Thesis

submitted to the Faculty of Graduate Studies

in Partial Fulfillment of the Requirements

for the Degree of

MASTER OF SCIENCE

Faculty of Pharmacy
University of Manitoba
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THE UNIVERSITY OF MANITOBA

FACULTY OF GRADUATE STUDIES

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THE METABOLISM OF RAZOXANE AND DEXRAZOXANE IN THE SPRAGUE-DAWLEY RAT

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RONALD G. AOYAMA

A Thesis/Practicum submitted to the Faculty of Graduate Studies of The University of Manitoba in partial fulfillment of the requirements of the degree of

MASTER OF SCIENCE

Abstract

The objectives of this project were to use a rat model to study the stereoselective metabolism of razoxane and the metabolism of dexrazoxane to its intermediate hydrolysis products. A solid phase method for extraction of razoxane from plasma and a chiral HPLC method for the simultaneous quantitation of razoxane enantiomers were developed. These methods were then used to study the stereoselective metabolism of razoxane in a rat model after i.v. administration. In order to follow the plasma concentrations of the dexrazoxane hydrolysis intermediate products, attempts were made to derivatize dexrazoxane and its hydrolysis intermediates with the fluorescent label 2-bromomethyl-4,5-diphenyloxazole. An HPLC method was used to analyze these fluorescent derivatives. Dexrazoxane was successfully derivatized with the oxazole but the fluorescent derivatives of the hydrolysis intermediates were unable to be identified. This approach was abandoned. Several methods were evaluated for their ability to separate dexrazoxane and the hydrolysis intermediates from buffer and rat plasma. A simple acetonitrile plasma protein precipitation step gave good recoveries of dexrazoxane and its hydrolysis intermediates from rat plasma. An HPLC method was then designed to separate dexrazoxane and its hydrolysis intermediates in rat plasma samples. Post-column derivatization of dexrazoxane and its hydrolysis intermediates with NaOH solution in a reaction bed and detection by UV absorption gave reasonable sensitivities while reducing interferences from endogenous materials in the rat plasma.

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Introduction

CHAPTER 1 INTRODUCTION

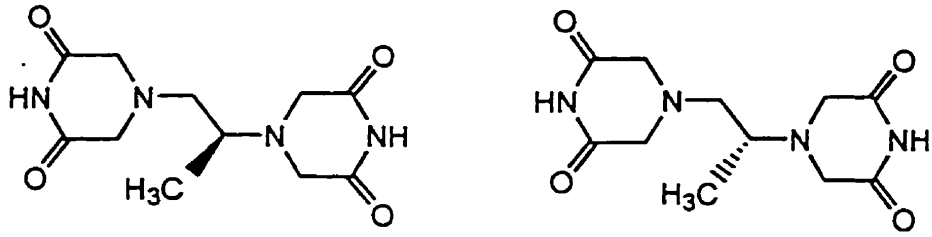
1.0 An Introduction to Dexrazoxane

1.0.1 The Status of Dexrazoxane

Dexrazoxane is the (*S*)-enantiomer of the racemic compound razoxane. Its chemical name is (+)-(*S*)-1,2-bis(3,5-dioxopiperazin-l-yl)propane, and is also known as ADR-529 or ICRF-187. The corresponding (*R*)-enantiomer is known as levrazoxane or ICRF-186. The 2 enantiomers are illustrated in figure 1.1. Dexrazoxane is a cardioprotective antioxidant and received accelerated approval by the United States Food and Drug Administration (US FDA) in the spring of 1995. The US FDA has given this drug a "1A" priority status. Only drugs used to treat AIDS have a higher priority status. This accelerated priority status meant that products used to treat life-threatening or serious diseases do not have to fulfill requirements normally needed for submission of new drug approvals. Dexrazoxane was approved for use in Canada by Health Canada in the spring of 1995. Several clinical trials have shown that dexrazoxane can reduce the incidence and severity of cardiomyopathy in breast cancer patients treated with doxorubicin (Rosenfeld *et al.*, 1992; Speyer *et al.*, 1988). Results from a recent clinical study also showed that dexrazoxane could reduce doxorubicin induced cardiomyopathy in pediatric sarcoma patients (Wexler *et al.*, 1996).

1.0.2 The Compound and its Nature

Dexrazoxane is a small, uncharged compound. Razoxane, the racemic form of dexrazoxane, has the ability to cross cell membranes (Dawson, 1975). Under physiological conditions, dexrazoxane undergoes slow hydrolysis to the one-ring opened intermediates with a reaction half-life of approximately 9.3 hr (Hasinoff, 1993) (see figure 1.2). The final rings-open form ADR-925 is produced with a half life of 23 hr. ADR-925 and its racemate ICRF-198 resemble the strong chelating agent EDTA (Huang *et al.*, 1982). Like EDTA,



Dexrazoxane
(+)-(*S*)-ICRF-187

Levrazoxane
(-)-(*R*)-ICRF-186

Figure 1.1 Structures for Dexrazoxane and Levrazoxane

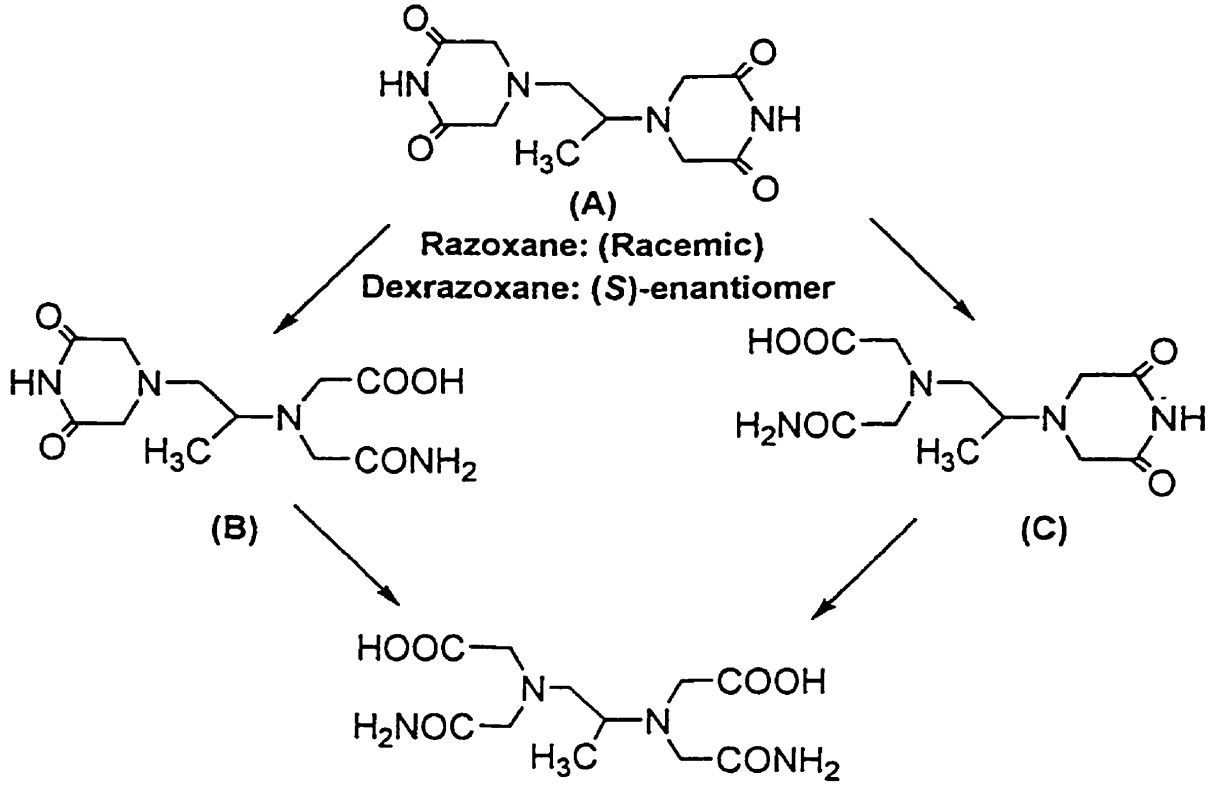


Figure 1.2 Hydrolysis Scheme of Razoxane and Dexrazoxane

razoxane is a strong chelator of divalent transition metal ions such as copper and iron (Huang *et al.*, 1982) and trivalent metals including Fe^{3+} (Hasinoff, 1989). Razoxane (ICRF-159), the racemate of levrazoxane and dexrazoxane, has been shown to diffuse into cultured cells (Dawson, 1975). Razoxane was found to reduce the cardiotoxicities of anthracycline antitumour agents (Herman *et al.*, 1974, Hu *et al.*, 1983) in mice and rats. Razoxane was originally used as an antitumor agent as it exhibited anti-cancer activity against sarcoma 180 and leukemia L1210 cells (Creighton, 1971). In clinical studies, razoxane was found to have activity against Kaposi's sarcoma (Olweny *et al.*, 1976) and non-Hodgkin's lymphoma (Flannery *et al.*, 1978). Razoxane also has limited activity against acute leukemia (Khan and Khan, 1975), breast cancer (Creech *et al.*, 1979), squamous cell carcinoma of the head and neck (Shah *et al.*, 1982), and chronic myeloid leukemia in blast cell crisis (Bakowski *et al.*, 1979), (James and Salsbery, 1974). Razoxane is also used as a radiosensitizer for radiation treatment of tumors (Woodman, 1974).

The clinical use of razoxane is limited by its low solubility in aqueous solutions, about 3 mg/mL. Both razoxane enantiomers, dexrazoxane and levrazoxane have a solubility of 15 mg/mL in water, about 5 times higher than razoxane (Repta *et al.*, 1976). Razoxane's limited solubility is due to a greater degree and number of intermolecular forces acting in its crystal structure (Hempel *et al.*, 1982).

The effectiveness of dexrazoxane was evaluated in preventing anthracycline induced cardiotoxicities in several animal studies. The anthracyclines tested were daunorubicin (Herman *et al.*, 1981), doxorubicin (Herman and Ferrans, 1981) and epirubicin (Dardir *et al.*, 1989). In each study, dexrazoxane was effective in the prevention of anthracycline induced cardiotoxicities.

The results of several human clinical trials found that dexrazoxane was also effective in decreasing doxorubicin-induced cardiotoxicities in patients undergoing cancer chemotherapy (Rosenfeld *et al.*, 1992; Speyer *et al.*, 1988). Findings from a recent clinical study concluded that dexrazoxane could reduce doxorubicin-induced cardiomyopathy in pediatric sarcoma patients (Wexler *et al.*, 1996).

1.1 Doxorubicin-Induced Cardiotoxicity and its Mechanism of Action

The use of the anticancer drug doxorubicin is limited in its usefulness by a unique and potentially fatal cumulative dose dependent cardiotoxicity (Demant and Jensen, 1983; Gianni *et al.*, 1983; Gutteridge, 1984). Generation of hydroxyl free radicals is believed to be the mechanism responsible for the damage inflicted on the heart (Halliwell and Gutteridge, 1985).

Doxorubicin can form hydroxyl radicals in two possible schemes. In the first scheme, enzymes such as xanthine oxidase, cytochrome P450 reductase, b_5 reductase and NADH dehydrogenase (Abdella and Fisher, 1985; Lown, 1985) can reduce doxorubicin by one electron to a semiquinone. The semiquinone can then react with oxygen and ultimately result in the formation of hydroxyl free radicals.

$DOX \rightarrow DOX^{\cdot -}$	(enzymatic reductive activation)	[1]
$DOX^{\cdot -} + O_2 \rightarrow DOX + O_2^{\cdot -}$	(formation of superoxide)	[2]
$2O_2^{\cdot -} + 2H+ \rightarrow H_2O_2 + O_2$	(formation of hydrogen peroxide)	[3]
$2O_2^{\cdot -} + Fe^{3+} \rightarrow Fe^{2+} + O_2$	(reduction of Fe^{3+})	[4]
$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^{\cdot} + OH^{-}$	(Fenton reaction)	[5]

Doxorubicin is a strong metal chelator with a high affinity for Cu(II) and Fe(III) ions and forms a metal-drug complex. Doxorubicin can complex with iron in a 3-drug to one Fe^{3+} arrangement (Gianni *et al.*, 1983). Previous

work has shown that the Fe^{3+}-(doxorubicin)$_3$ complex is responsible for cytochrome *c* oxidase inhibition in purified enzyme (Hasinoff and Davey, 1988a) and heart submitochrondria particles (Hasinoff and Davey, 1988b). The Fe^{3+}-$(DOX)_3$ complex may catalyze hydroxyl radical formation where the iron ion undergoes reduction. The cardiotoxicities of doxorubicin may arise due to damage of cardiac mitochrondria (Gianni *et al.*, 1983) and loss of respiratory enzyme activity (Arena *et al.*, 1975).

$$Fe^{3+}\text{-DOX} \rightarrow Fe^{2+}\text{-DOX}^{\cdot+} \quad [6]$$

$$Fe^{2+}\text{-DOX}^{\cdot+} + DOX \rightarrow Fe^{2+}\text{-DOX}^{\cdot-} + DOX^{\cdot+} \quad [7]$$

$$Fe^{2+}\text{-DOX}^{\cdot+} + O_2 \rightarrow Fe^{3+}\text{-DOX} + O_2^{\cdot-} \quad [8]$$

$$2O_2^{\cdot-} + 2H^+ \rightarrow H_2O_2 + O_2 \quad [9]$$

$$Fe^{2+}\text{-DOX} + H_2O_2 \rightarrow Fe^{3+}\text{-DOX} + OH^{\cdot} + OH^- \quad [10]$$

1.2 The Chemical Nature and Therapeutic Value of Razoxane and its Enantiomers Dexrazoxane and Levrazoxane

1.2.1 The Chemistry of Razoxane and its *S*-Enantiomer Dexrazoxane

In its fully ring-closed form, razoxane has been shown to cross into cultured cells (Dawson, 1975). The complete hydrolysis of the two rings of razoxane results in ICRF-198, a strong metal chelating agent (Huang *et al.*, 1982). The more polar and negatively charged hydrolysis product ICRF-198 probably cannot cross the cell membrane. The corresponding *S*-enantiomer to the metal chelating agent ICRF-198 is ADR-925.

Base catalyzed hydrolysis of dexrazoxane has been followed by both spectrophotometric and HPLC methods (Hasinoff, 1990, Hasinoff, 1994a, Hasinoff, 1994b). The hydrolysis of dexrazoxane has been shown to proceed through the one ring-opened intermediate forms **B** and **C** (See figure 1.2). In Tris buffer at pH 7.4 and 37°C, dexrazoxane was lost from the reaction mixture

with a half life of 9.3 hr. For formation of the fully ring opened compound, ADR-925, the half-life was 23 hr. The intermediate **B** is formed more rapidly than **C** as the ring closest to the methyl group opens 2.1 times faster than the other ring. About 68% of the final hydrolysis product ADR-925 is produced through degradation of the **B** intermediate, and the remaining 32% is produced through the **C** intermediate. Also, the **B** intermediate accumulates in the reaction mixture as its degradation to ADR-925 is slower than **C**. These reactions are illustrated in figure 1.3.

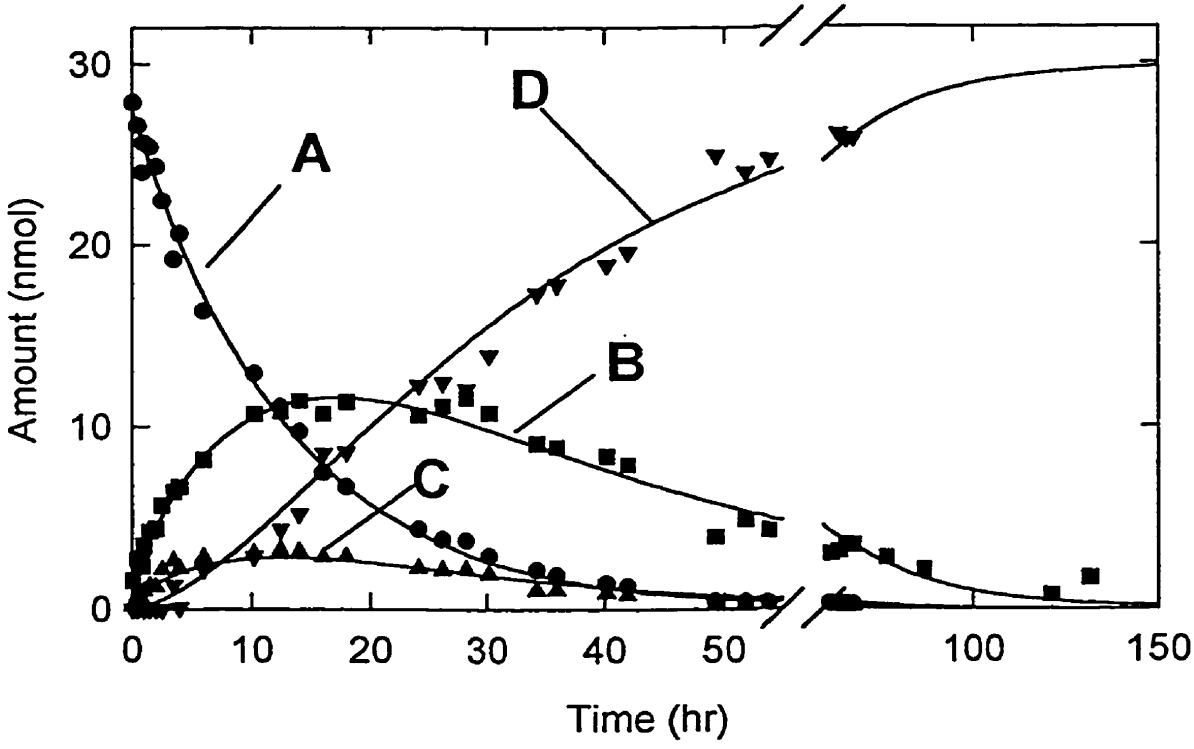


Figure 1.3. Time course for the hydrolysis of dexrazoxane in Tris/NaCl buffer (pH 7.39) at 37°C as quantified by HPLC. The amount is the number of nmol contained in 10 µL of the reaction mixtures applied to the column. **A**) dexrazoxane, **B**) intermediate **B**, **C**) intermediate **C**, **D**) ADR-925. The solid lines are nonlinear least squares calculated for the reaction illustrated in figure 1.2. From Hasinoff (1994b).

Dexrazoxane is also hydrolyzed to its one-ring opened intermediates by incubation in 105,000 *g* supernatant fractions of porcine liver and kidney homogenates. Incubation of dexrazoxane in 105,000 *g* supernatant fractions of porcine heart homogenates resulted in minimal hydrolysis (Hasinoff *et al.*, 1991). Addition of 4-chlorobenzenesulfonamide to liver and kidney homogenates resulted in complete inhibition of hydrolysis of dexrazoxane. The compound 4-chlorobenzenesulfonamide has been shown to be a strong inhibitor of dihydropyrimidine amidohydrolase (DHPase) (Brooks *et al.*, 1983) suggesting that the enzyme DHPase is responsible for dexrazoxane hydrolysis. DHPase is present in the liver and kidneys but not in the heart.

Purified DHPase will open one of the rings of dexrazoxane and its (*R*)-enantiomer levrazoxane (Hasinoff *et al.*, 1991; Hasinoff, 1993; Hasinoff, 1994c). DHPase hydrolyzes dexrazoxane to its one ring-opened form 4 times faster than for hydrolysis of levrazoxane under non-saturating conditions. DHPase hydrolyzes dexrazoxane at a rate comparable to the hydrolysis of the enzyme's natural substrates dihydrouracil and dihydrothymine under non-saturating conditions. DHPase is present in the liver and the kidneys, but not in the heart (Hasinoff *et al.*, 1991). DHPase cannot hydrolyze the one ring-opened forms of dexrazoxane and levrazoxane as they are no longer substrates for the enzyme. Opening of the second ring is probably through base-catalyzed hydrolysis (Hasinoff, 1993). The opening of the bisdioxopiperazine rings is the transition of the dexrazoxane molecule from prodrug to the active metal-chelating form ADR-925.

1.2.2 The Metal-Chelating Chemistry of Dexrazoxane and its Rings-open Hydrolysis Products: Removal of Iron From the Fe^{3+}-(Anthracycline) Complex

In its prodrug form, dexrazoxane displaces iron from the Fe^{3+}-(doxorubicin) complex slowly ($t_{1/2}$ ~ 30 min) (Hasinoff, 1989). The active form of racemic razoxane, ICRF-198 was able to chelate iron from the Fe^{3+}-(doxorubicin) complex, displacing the iron completely and rapidly ($t_{1/2}$ = 1.8 min). Another study examined the ability of dexrazoxane, its one-ring open hydrolysis intermediates and its two-ring open hydrolysis product ADR-925 to remove Fe^{3+} from its complex with doxorubicin, daunorubicin, epirubicin and idarubicin (Buss and Hasinoff, 1993). At pH 7.4, 100 μM of dexrazoxane slowly removed Fe^{3+} from its anthracycline complex with half-times from 230 to 450 min. The one-ring intermediates of dexrazoxane removed Fe^{3+} from its anthracycline complex at a much faster rate, with half-times of 1.7 to 16.7 min. The two-ring open hydrolysis product ADR-925 rapidly and completely removed Fe^{3+} from its anthracycline complex with half-times from 1 to 3 min. In all cases, removal of Fe^{3+} from the daunorubicin complex is the fastest, followed by doxorubicin, epirubicin and idarubicin. The rates at which the various forms of dexrazoxane remove Fe^{3+} from the different Fe^{3+}-(anthracycline) complexes are generally similar except for idarubicin.

Chelation of Fe^{3+} from the Fe^{3+}-(anthracycline) complex is likely to disrupt the formation of the anthracycline-catalyzed production of hydroxyl free radicals. In a study by Hasinoff (1989), both dexrazoxane and ICRF-198 were very effective in protecting against loss of cytochrome *c* oxidase activity of bovine heart submitochrondrial particles by the iron-doxorubicin complex. Within 45 min, ICRF-198 almost completely removed all of Fe^{3+} from the Fe^{3+}-(doxorubicin) complex. Dexrazoxane was slightly less effective than ICRF-198 in protecting against loss of cytochrome *c* oxidase activity. This

apparent reduction in activity may be due to a ring-opening hydrolysis step dexrazoxane must undergo to form its active, metal chelating form ADR-925. It was found that the Fe^{3+}-(doxorubicin) complex promoted the ring-opening hydrolysis of dexrazoxane to a form which is more effective in chelating iron than doxorubicin (Hasinoff, 1989; Hasinoff, 1990). Removal of iron from the doxorubicin-iron complex probably affects production of hydroxyl free radicals by preventing the Fe^{3+}-(doxorubicin) complex from redox cycling as listed in equations [6] to [10] in section 1.1.

1.3 The Differences in Metabolism Between Dexrazoxane and Levrazoxane and its Possible Effects on Cardioprotective Activity

An *in vitro* study found that the isolated and purified enzyme DHPase hydrolyzed the one-ring opening of dexrazoxane and levrazoxane at different rates. Under nonsaturating conditions, DHPase hydrolyzed the ring opening of dexrazoxane 4 times faster than for levrazoxane (Hasinoff, 1993).

The hydrolysis of dexrazoxane and levrazoxane was followed in an isolated rat hepatocytes suspension at 37°C and pH 7.4 (Hasinoff *et al.*, 1994). Each razoxane enantiomer and its one-ringed open intermediates was studied separately by an HPLC method. Dexrazoxane is metabolized by the isolated hepatocytes 1.8 times faster than for levrazoxane. This ratio corresponds to a similar value found for purified DHPase. Addition of the strong DHPase inhibitor 4-chlorobenzenesulfonic acid results in an 82% inhibition of the loss of dexrazoxane. This suggests that DHPase present in hepatocytes catalyzes the ring-opening hydrolysis of dexrazoxane and levrazoxane. The ratios of the rates of formation of the one-ring open intermediates of dexrazoxane and levrazoxane in the hepatocyte suspension were also consistent with DHPase being primarily responsible for metabolism of these drugs.

In an animal study, Herman *et al.*, (1985) measured the protective capacity of a series of structurally related bisdioxopiperazine analogues against acute daunorubicin (DNR) toxicity. Pretreatment of Syrian golden hamsters with dexrazoxane and levrazoxane was shown to reduce the lethal effects of DNR. No difference in efficacy between dexrazoxane and levrazoxane was found, indicating that the protective effects were not stereospecific.

A more recent study by Zhang *et al.*, (1994) compared the effects of dexrazoxane and levrazoxane on doxorubicin-induced toxicities in rats. Three doses of dexrazoxane and levrazoxane were administered. At the lowest dose of 6.25 mg/kg, both drugs had comparable but minimal protective effects. At a dose of 12.5 mg/kg, both drugs were equally effective in protecting against doxorubicin toxicity. At the highest dose of 25 mg/kg, levrazoxane was somewhat less effective in providing protection as compared to dexrazoxane. The researchers felt the difference in the protective activity of the two enantiomers was minor. The researchers theorized that the most likely explanation for the difference in cardioprotection was a difference in the opening of the bisdioxopiperazine rings between the two enantiomers by DHPase.

The results of several *in vitro* studies (Hasinoff *et al.*, 1991; Hasinoff, 1993) suggest that DHPase is responsible for the metabolism of dexrazoxane and levrazoxane to its one ringed-open intermediates. These studies also suggest that DHPase metabolizes dexrazoxane and levrazoxane at different rates. Other studies examining the protective effects of dexrazoxane and levrazoxane against doxorubicin induced cardiotoxicities in animals (Herman *et al.*, 1985; Zhang *et al.*, 1994) found slight differences. These differences in protective effects were probably due to DHPase's stereoselective metabolism of dexrazoxane and levrazoxane. The preferential metabolism of dexrazoxane to

its active state may result in it providing its cardioprotective effects sooner than levrazoxane.

The focus of this study is to study the stereoselective metabolism of dexrazoxane and levrazoxane by administering the racemic drug razoxane in an animal model. Currently there is no published method for simultaneously following the plasma concentrations of dexrazoxane and levrazoxane in either animal or human subjects.

1.4 Pharmacokinetic Studies of Dexrazoxane and Razoxane

There have been relatively few studies of the pharmacokinetics of either razoxane or its (*S*)-enantiomer dexrazoxane. One of the very first pharmacokinetic studies found the plasma half-life of [^{14}C]-razoxane in rats to be 30 min (Field *et al.*, 1971). Another study followed the pharmacokinetics of razoxane in rat plasma, serum and cerebrospinal fluid (Collins *et al.*, 1983). Ultrafiltration was used to treat the plasma and serum samples. Cerebrospinal fluid contains low levels of protein and did not require ultrafiltration prior to analysis. The samples were analyzed with HPLC, using a reversed-phase C-18 column and ultraviolet detection at 206 nm. For i.v. administration of razoxane in rats, a biphasic decay was observed in plasma with an initial half-life of 11.5 min and a terminal half-life of 40.3 min. A similar pattern was found for razoxane in rat serum, with values of 8.1 min and 42.2 min for the initial and terminal half-lives, respectively. Razoxane was also administered intraperitoneally and followed in plasma and cerebrospinal fluid. Peak plasma concentration occurred 15 min, post infusion, with a value of 12 μg/mL. The half-life was 96 min.

A solid phase extraction method was developed to extract the (*S*)-enantiomer of razoxane, dexrazoxane, in dog plasma (Sisco, 1989). Perchloric acid was added to the plasma to prevent further hydrolysis of dexrazoxane.

The samples were mixed with phosphate buffer and eluted through a C-18 solid phase extraction (SPE) cartridge. The cartridge was air dried and washed with hexane to remove interfering compounds. The drug was eluted with acetonitrile and derivatized with the fluorescent compound 2-bromomethyl-4,5-diphenyloxazole. The derivatized samples were analyzed using reversed-phase C-18 HPLC and fluorescent detection. A linear response was observed over the concentration range of 0.01 to 50 μg/mL. Beagle dogs were given 30 mg/kg of dexrazoxane by i.v. administration. The mean plasma terminal half-life was 1.16 hr.

An HPLC method was developed for the determination of dexrazoxane in human plasma and urine (Lewis *et al.*, 1992). The plasma and urine samples are treated with phosphoric acid to prevent further hydrolysis of dexrazoxane. The plasma samples are mixed with pH 7 phosphate buffer and eluted through C-18 SPE cartridges. The C-18 SPE cartridges are placed on top of new C-8 SPE cartridges, the analytes are eluted from the C-18 to the C-8 cartridge with acetonitrile and the C-18 cartridge is discarded. The analytes are eluted off the C-8 cartridge with 2% (vol/vol) trifluoroacetic acid in acetonitrile, evaporated to dryness and reconstituted in acetonitrile. The urine samples are diluted in acetonitrile before injection. The HPLC system uses 2 narrow bore silica columns separated by a switching valve. Endogenous plasma components eluting off the first column are vented to waste. The fraction containing dexrazoxane and the internal standard are switched by the valve to the second column and then the amperometric electrochemical detector. The response was linear for the range of 5 to 500 ng/mL in plasma and 2 to 100 μg/mL in urine. The method was used to determine the pharmacokinetic profile of dexrazoxane in dog plasma after a

single i.v. dose of 10 mg/kg. Dexrazoxane declines in a biphasic pattern with a terminal half-life of 1.3 hr.

The pharmacokinetics of dexrazoxane have been followed in human patients co-administered with doxorubicin (Hochster *et al.*, 1992). Dexrazoxane is administered in doses of 60, 300, 600, 750 and 900 mg/m^2 in a 15 min period starting 30 min before doxorubicin treatment. A fixed doxorubicin dose of 60 mg/m^2 was administered for all doses of dexrazoxane. The plasma levels of dexrazoxane are quantified with solid phase extraction and HPLC analysis with electrochemical detection described by Lewis *et al.* (1992). The distribution phase half-life in plasma is estimated to be 0.46 ± 0.30 hr (± SD). The elimination phase half-life in plasma is estimated to be 4.16 ± 2.94 hr (± SD). Dexrazoxane does not affect the pharmacokinetics of doxorubicin.

Another paper (Jakobsen *et al.*, 1994) studied the pharmacokinetics of dexrazoxane co-administered with epirubicin, cyclophosphamide, 5-fluorouracil and tamoxifen in breast-cancer patients. The patients received i.v. administration of fixed doses of cyclophosphamide (600 mg/m^2), 5-fluorouracil (600 mg/m^2) and tamoxifen (30 mg). The dose of dexrazoxane was varied and ranged from 0 to 1000 mg/m^2. The dose of epirubicin was also varied and was either 60, 80, or 100 mg/m^2. Increasing doses of dexrazoxane do not alter the pharmacokinetic parameters of epirubicin, nor were the pharmacokinetics of dexrazoxane itself affected. Increasing the doses of epirubicin generally does not significantly affect the pharmacokinetics of dexrazoxane, except for when the epirubicin dose increases from 60 to 100 mg/m^2. The plasma clearance of dexrazoxane increases by 30% and the terminal half life decreases by 26%. The authors conclude that this may be due

to the high dose of epirubicin inducing liver microsomal metabolizing enzymes.

1.5 HPLC Methods for the Chiral Analysis of Drugs

High-performance liquid chromatography (HPLC) methods have been developed for the analysis of chiral drugs. These methods include chiral derivatizing reagents, chiral mobile phases and chiral stationary phases. The next section will be a brief review of the methods available.

1.5.1 Chiral Derivatizing Reagents

Derivatizing reagents are an indirect method to resolve enantiomers. The addition of a pure enantiomer of a derivatizing reagent to two enantiomers of a drug will result in the formation of diastereomers. Diastereomers have different physical properties and can be resolved using nonchiral methods. This is an indirect method as the enantiomers themselves are not measured but the converted diastereomers are. The advantage of this method is an existing non-chiral HPLC system can be used with little or no modification.

One disadvantage of using chiral derivatizing reagents is the samples must be treated before analysis. In some cases, drugs can have more than one site at which the reagent can react. If the reaction does not proceed to completion, a mixture of mono and multi-substituted compounds could occur. This will affect the accuracy of the results. Also, other drugs are unreactive towards the derivatizing reagents. The imide nitrogen of razoxane is quite unreactive. Derivatization of razoxane may not occur or only partially proceed to completion.

1.5.2 Chiral Mobile Phases

The second major method for chiral analysis of drugs is to add a chiral selector to the mobile phase. Like the chiral derivatizing reagents, this

method can used on a regular HPLC system with no special modifications. Unlike the chiral derivatizing reagents, pretreatment of the samples before analysis is generally unnecessary.

This technique is known as the inclusion complex method. The enantiomer penetrates or includes into a hollow cavity of a guest molecule, forming a diastereomeric inclusion complex. The host molecule is chiral and the chiral recognition site is usually on the surface of the cavity. Once the inclusion complex forms, a major change in the polarity of the guest molecule occurs.

The major chiral selectors used in the literature are the cyclodextrins. Cyclodextrins are hollow, chiral molecules composed of 6, 7, or 8 glucose subunits. Shaped like a donut, the outer surface is polar due to the presence of hydroxyl groups. The inside cavity is hydrophobic and consists of two rings of C-H groups (Bender and Koriyama, 1978). The enantiomer and cyclodextrins are attracted due to forces of hydrophobic interactions between the cavity and the drug. The diastereomeric complexes are separated due to differences in their relative stabilities due to steric interactions and hydrogen bonding. The cyclodextrins are water soluble and used with aqueous, reverse phase HPLC systems (Szepesi and Gazdag, 1988).

For ß-cyclodextrin to exhibit stereoselectivity, the enantiomer must meet very specific structural requirements. Due to these requirements, the use of cyclodextrin as chiral selectors is limited to a few very specific drugs. If the drug fits the structural requirements and can be enantiomerically resolved, then chiral mobile phases offer many advantages. It is very easy to use with existing reverse phase HPLC systems; no special, expensive columns are required; and no derivatization of the sample is needed.

1.5.3 Chiral Stationary Phases

This HPLC method offers advantages over the other chiral methods. As it is a direct method, derivatization of the drug may not be required. The sample requires no special pretreatment before analysis.

Chiral recognition in chromatography is based on the three-point rule. At least three interactions must occur between the enantiomers or solute, and the chiral stationary phase (CSP). One of these interactions must be stereochemical and the closer the interaction points to the chiral center, the better the selectivity between the enantiomers. As the interaction point moves away from the chiral centre, the conformational differences between the enantiomers decreases. The type of possible interactions are dipole-dipole, electrostatic, hydrogen bonding, hydrophobic or steric. Once the interaction occurs between the enantiomers and the CSP, a temporary diastereomeric complex forms. One of the two complexes is more stable due to a lower free energy. This complex will stay on the column longer, resulting in resolution of the enantiomers (Dalgleish, 1952).

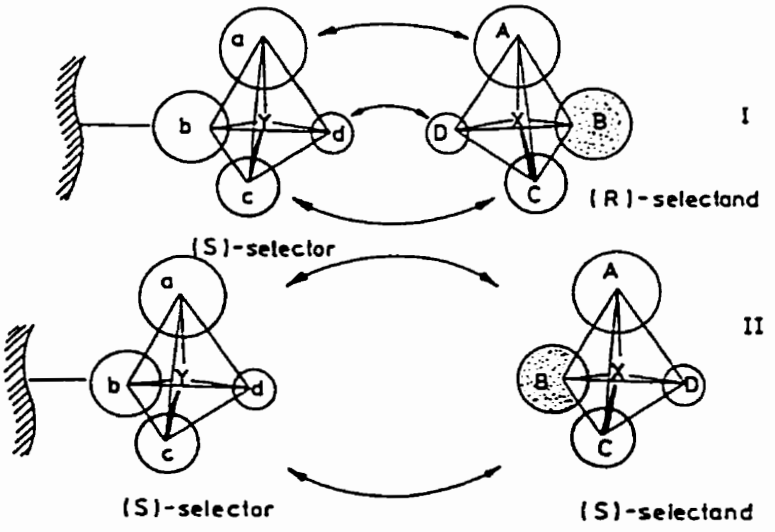


Figure 1.4. Stereoselective binding model between a chiral stationary phase and an enantiomeric solute. I) Ideal fit II)Non-ideal fit.
From Szepesi, 1990.

The types of CSP used have been divided into groups based on the interactions between the CSP and the solute (Wainer, 1993).

1.5.3.1 Type I Chiral Stationary Phases

This is the largest class of CSP and was also the first to be developed for HPLC. The main interactions are of the π-π type, either an aromatic π-acid such as a dinitrobenzyol (DNB)-amino acid derivative stationary phase and an aromatic π-base solute. The reverse can also be true, using a π-base such as a naphthalene amino acid derivative as the CSP and the corresponding aromatic π-acid as the solute. The amide bond between the aromatic group and the amino acid is responsible for the hydrogen bonding and the dipole-dipole interactions needed for the two other points of interaction.

The type I CSP's are limited by the types of functional groups present on the solute. For chiral recognition to occur, aromatic amides, carbamates or ureas must be present. If these groups are not present on the analyte, then naphthyl or dinitrobenzyol (DNB) derivatizing agents can be used to introduce them. If derivatization is required, it may preferable to form derivatives which can be resolved on nonchiral columns. Chiral columns are usually much more expensive than nonchiral columns.

Type I CSPs use mobile phases similar to normal phase silica HPLC. Nonpolar solvents such as hexane and an alcohol such as 2-propanol are generally used.

1.5.3.2 Type II Cellulose Chiral Stationary Phases

These CSPs use cellulose derivatives as the chiral selector. Cellulose is a chiral molecule composed of long chains of D-ß-glucose subunits. Chiral selectivity is due to the enantiomers interacting with chiral cavities formed between the glucose subunits or chiral channels between the cellulose strands. The enantiomers interact by including into these chiral areas.

Selectivity can also occur by attractive forces acting between the enantiomers and the glucose subunits.

Regular cellulose is too fragile to withstand the pressures found in HPLC systems. Newer type II CSP's use cellulose derivatives coated onto silica gel (Okamoto *et al.*, 1986). The enantiomers and CSP form the temporary diastereomeric complexes by either of two major types of interactions. Polar functional groups such as amides bind through attractive interactions by dipole-dipole interactions, hydrogen bonding, and π-π interactions. The chiral recognition mechanism is a differential fit between the two enantiomers into the chiral cavities of cellulose CSP. For aromatic compounds such as aromatic alcohols, the formation of the enantiomer-CSP complex occurs through inclusion of the phenyl group into a chiral cavity (Wainer and Alembik, 1986).

Generally most of the enantiomers resolved on cellulose-silica CSPs required the presence of one or more aromatic rings, or polar groups such as carbonyl, nitro or sulfinyl functional groups. This is consistent with the mechanisms postulated by Wainer. Aliphatic alcohols which lacked these constituents had to derivatized before resolution of the enantiomers occurred (Stevenson and Wilson, 1990).

Mobile phases used for cellulose-silica CSPs are generally nonpolar solvents such as hexane and a polar modifier, usually 2-propanol or ethanol. Addition of diethylamine can improve peak symmetry and separation (Krstulovic *et al.*, 1988). Hollenhorst and Blaschke (1991) found that replacing diethylamine with acetic acid improved the resolution of propafenone, dipropafenone, 5-hydroxypropafenone, and 5-hydroxydipropafenone.

1.5.3.3 Type III Chiral Stationary Phases

Cyclodextrins have been successfully used as chiral selectors in mobile phases. Cyclodextrins can also be used as chiral stationary phases by being bound to a solid support of silica gel. Cyclodextrin mobile phases are usually less efficient at separations than the equivalent stationary phase (Armstrong *et al.*, 1986).

The principle of chiral selection is the same for both methods. The enantiomer and cyclodextrins are attracted due to forces of hydrophobic interactions between the cavity and the drug. Enantiomers fit into chiral cavities of the cyclodextrin and form inclusion complexes. These diastereomeric complexes are separated due to differences in their relative stabilities caused by steric interactions and hydrogen bonding.

Generally for separation of enantiomers to occur, certain requirements must be met. These requirements were reviewed in the section on chiral mobile phases (refer to section 1.5.2). The analyte should fit tightly inside the cyclodextrin cavity, with aromatic ring systems being optimal. The ring should be next to the chiral carbon for optimum chiral selectivity (Fujimura *et al.*, 1983). The cyclodextrin CSPs are available in the α, ß and γ forms of cyclodextrin. One ring compounds will fit into α-cyclodextrin bonded CSPs. The ß-cyclodextrin CSPs required 1 or 2 rings for a good fit and fused or multiple fused systems have the best fit in the larger γ-cyclodextrin CSP. Of the 3 cyclodextrin classes of CSPs, the ß-form was the most widely used (Menges and Armstrong, 1991). The cyclodextrin CSP's are generally referred to as the type III CSP's and use aqueous mobile phases. The organic modifiers are methanol, ethanol and acetonitrile. Greater stereoselectivity and retention occur with a larger aqueous content.

1.5.3.4 Type IV Chiral Stationary Phases

These types of CSP's involve the use of chiral ligand exchange chromatography. An enantiomer of a chiral amino acid is bound to a solid support. In the presence of a transition metal, a ternary diastereomeric complex forms with the enantiomers of the analyte. Generally the less stable complex elutes first. The cyclic amino acids proline and hydroxyproline were the best chiral selectors. Copper(II) formed the most stable complexes with proline and the analyte (Gubitz *et al.*, 1982).

Mobile phases generally consist of an aqueous buffer and copper sulphate. As the concentration of copper sulphate increases, the retention time of the analyte decreases. Organic modifiers such as methanol and acetonitrile can also be used to modify retention time. Unlike reverse phase systems, increasing concentration of the organic component increases the retention time.

As the analyte must be able to form a complex with the metal and the amino acid, the types of compounds that can be separated are limited. Generally, the analytes are α-amino acids or mono or dicarboxylic acids with α-hydroxyl groups.

1.5.3.5 Type V Chiral Stationary Phases

Proteins can bind with small molecules, often stereospecifically. This ability is used to develop the type V class of CSP's. The interactions responsible for binding between protein based CSPs and enantiomers seem to involve a combination of electrostatic interactions, hydrogen bonding and hydrophobic interactions. At present, a clear model of the chiral recognition mechanisms has not been determined (Krstulovic, 1989). The major types of proteins used in these type of CSP's are bovine and human serum albumin (BSA, HSA) and α_1-acid glycoprotein (AGP).

BSA and HSA are very similar in structure and have correspondingly similar chromatographic properties. The analytes which can be separated are anionic and neutral compounds (Domenici *et al.*, 1991). The presence of functional groups such as aromatic and polar groups enhances separation (Allenmark *et al.*, 1984). Drugs which have high levels of protein binding are most suitable for use with these types of columns. Mobile phases for the albumin CSP's are aqueous phosphate buffers and can have either organic or inorganic modifiers.

The other major type V CSP is the α_1-acid glycoprotein (AGP). Unlike the serum albumin (SA) CSP, the AGP-CSP's can also analyze cationic compounds. Mobile phases for the AGP type CSP's are generally aqueous phosphate buffers. A wide variety of modifiers can be used to change the retention. Common modifiers include uncharged solvents 1-propanol, 2-propanol, ethanol, ethylene and propylene glycol, cationic compounds tetrabutylammonium bromide and anionic compounds such as butyric, decanoic, and octanoic acid (Wainer, 1993). The AGP-CSP has a relatively wide enantiospecificity and is widely used for chiral separations in pharmacokinetic studies.

1.6 The Stereoselective Metabolism of Razoxane; and the *In Vivo* Metabolism of Dexrazoxane to its One-ring Open Hydrolysis Products: Research Reported on in This Thesis

The topics covered in this project examine aspects of dexrazoxane and razoxane metabolism. Nothing is known about the relative *in vivo* metabolism of the enantiomers of razoxane. The goal of this project was to study the stereoselective metabolism of razoxane in a rat model, and to study the metabolism of dexrazoxane to its intermediate hydrolysis products in a rat model.

Chapter 2 details the development of a solid phase extraction method for extraction of razoxane from plasma and a chiral HPLC method for the simultaneous quantitation of razoxane enantiomers. These methods were then used to study the stereoselective metabolism of razoxane in a rat model after i.v. administration.

Chapter 3 details the attempted development of a method to derivatize dexrazoxane and its hydrolysis intermediates with the fluorescent label 2-bromomethyl-4,5-diphenyloxazole and an HPLC method to quantify these derivatives. Dexrazoxane was successfully derivatized with the oxazole and a linear calibration plot was prepared. Unfortunately the fluorescent derivatives of the hydrolysis intermediates could not be identified using HPLC methods. This approach was abandoned and new approaches were tried in chapter 4.

Chapter 4 lists the attempts made to extract dexrazoxane and the hydrolysis intermediates from buffer and rat plasma using solid phase extraction techniques. Poor recoveries of the hydrolysis intermediates from rat plasma lead to the abandonment of this approach. Some protein precipitation methods were tried in an effort to separate the drug from the plasma. A simple acetonitrile plasma protein precipitation step gave good

recoveries of dexrazoxane and its hydrolysis intermediates from rat plasma. After this method had been developed, an HPLC method was then designed to analyze rat plasma samples containing dexrazoxane and its hydrolysis intermediates. Post-column derivatization of dexrazoxane and its hydrolysis intermediates with NaOH solution in a reaction bed and detection by UV absorption gave reasonable sensitivities, with generally good separations between the compounds and endogenous materials in the rat plasma. Despite all attempts, there was some co-elution between intermediate **B** (Fig. 1.2) and an endogenous plasma peak. The calibration plot for intermediate **B** still gave good linear results.

Chapter 5 details the study of dexrazoxane metabolism to its hydrolysis intermediates in a rat model after i.v. administration. The methods developed in chapter 4 were used to separate dexrazoxane and its hydrolysis intermediates from rat plasma and then to quantitate these compounds.

Chapter 2

Stereoselective Metabolism of Razoxane in the Rat

2.0 INTRODUCTION

2.0.1 Stereoselective Metabolism Experiments of Razoxane

The experiments described in this chapter involve the study of the stereoselective metabolism of the racemic drug razoxane. Two chiral HPLC methods were evaluated for their ability to resolve the enantiomers of razoxane. A chiral mobile phase containing ß-cyclodextrin and the use of a type II chiral cellulose column were evaluated.

An *in vivo* study was conducted with the i.v. administration of razoxane to Sprague-Dawley rats. Solid phase extraction was used to separate razoxane from plasma components. A chiral cellulose column simultaneously separated the razoxane enantiomers dexrazoxane and levrazoxane.

The plasma concentration of dexrazoxane and levrazoxane were studied over the course of 150 min. The R/S ratios of levrazoxane and dexrazoxane were also calculated over time to see if differences in the metabolism of the two razoxane enantiomers occurred.

Two *in vitro* studies were also carried out. Razoxane was incubated in rat plasma at pH 7.4 and 37°C to determine if plasma components may be involved in the stereoselective metabolism process. Razoxane was also incubated in rat liver supernatant extract at pH 7.4 and 37°C. Previous work by Hasinoff *et al.*, (1991) identified an enzyme present in rat liver supernatant as the agent responsible for the enzymatic hydrolysis of dexrazoxane and levrazoxane. The enzyme identified, dihydropyrimidine amidohydrolase (DHPase), is strongly inhibited by the compound 4-chlorobenzenesulfonamide. This compound was also added to rat liver supernatant containing razoxane to determine if hydrolyis of the rings still occurred.

2.1 EXPERIMENTAL

2.1.1 Materials

2.1.1.1 Chemicals

Razoxane was a gift from K. Hellman (London, England). Dexrazoxane and levrazoxane were gifts from Pharmacia & Upjohn (Columbus, OH). HPLC grade reagent alcohol containing 95% ethanol, 5% methanol and 5% isopropanol by volume, concentrated phosphoric acid, potassium hydroxide and potassium phosphate were obtained from Fisher Scientific (Nepean, Canada). HPLC grade acetonitrile and hexanes, containing not less than 95% by volume *n*-hexane, volumetric grade hydrochloric acid and sodium hydroxide solutions were obtained from Mallinckrodt (Mississauga, Canada). Saline solution, (0.9% wt/vol sodium chloride USP), was obtained from Astra Pharmaceuticals, Inc. (Mississauga, Canada). ß-cyclodextrin, heparin, the protein determination kit (690-A), Sigma Ultra grade Tris base and Tris hydrochloride were from Sigma Chemical Co. (St. Louis, MO). The 4-chlorobenzenesulfonamide was from Aldrich Chemical Co. (Milwaukee, WI). Ketamine, pentobarbital sodium salt and xylazine were obtained from Central Animal Care Services, University of Manitoba. Lidocaine HCl 2% (wt/vol) solution with 0.05% (wt/vol) epinephrine was obtained from MTC Pharmaceuticals (Cambridge, ON).

2.1.1.2 Animals

Eight male Sprague-Dawley rats were obtained from the Central Animal Care Services, University of Manitoba. Rats with weights between 350 and 400 grams were used in the i.v. administration study. The rats were allowed food and water *ad libitum* prior to the study. The animals were transported from the animal care facility to the Faculty of Pharmacy immediately before the start of the study. The animals were not acclimatized before the study was started.

2.1.1.3 Instrumentation

2.1.1.3.1 Chromatographic

The HPLC system consisted of a Varian 9010 pump, a 9050 programmable wavelength detector, Varian Star integration software, and a Rheodyne injector. For the HPLC analysis with a chiral mobile phase, a μBondapak 3.9 mm I.D. X 300 mm reversed-phase C-18 column with a particle size of 10 μm (Waters Chromatography, Mississauga, ON) was used. The sample loop volume was 10 μL and the detection wavelength was 207 nm. For the chiral assay, the system was fitted with a Brownlee Labs NewGuard column containing an 1.5 cm X 3.2 mm I.D. silica cartridge (Applied Biosystems, Santa Clara, CA, USA). The analytical column was a 4.6 mm I.D. X 250 mm Chiralcel OD chiral cellulose tris(3,5-dimethylphenylcarbamate) column with a particle size of 10 μm (Chiral Technologies, Exton, PA). The sample loop volume was 50 μL and the detection wavelength was 205 nm.

Peak areas were calculated manually using the Varian Star integration software. Manual integration was used to give more consistent results between chromatograms. The start of the levrazoxane peak was defined as starting 0.70 min before the retention time of 14.7 min for the maximum amplitude of the peak. When the peaks eluted closely together, the stop of the levrazoxane peak and start of the dexrazoxane peak was the time where the amplitude was at the lowest point in the valley. The end time for the dexrazoxane peak was was defined as starting 0.70 min after the retention time of 16.5 min for the maximum amplitude of the peak.

2.1.1.4 Statistical Analysis

Statistical analysis of the animal, plasma and liver homogenate supernatant incubation data was performed using SigmaStat for Windows

(Jandel Scientific, 1994). Paired one-tailed *t*-tests, unpaired two-tailed *t*-tests and one-way repeated measures analysis of variance (ANOVA) were used to analyze the data.

2.1.2 Methods

2.1.2.1 Surgical Procedure: Femoral Vein Cannulation

The rat was anesthetized with a combination of ketamine (90 mg/kg) and xylazine (10 mg/kg) given i.p. The degree of anesthesia was checked by gently stretching out the front limb and checking for retraction of the limb. The blink response was also examined by gently touching the eye with the tip of a blunt probe. Surgery was performed only after the rat's response to both stimuli was negative. Throughout the study, the rat was kept warm by illumination with a 60 watt lamp 20 cm from the rat. Breathing and response to stimuli was monitored throughout the study.

A 4 cm transverse incision was made 2 cm below the rib cage to the right leg to penetrate the skin. The skin and fat were gently separated from the muscle surface. Saline (0.9% wt/vol NaCl) was used to moisten the surface. The femoral artery and vein were located and separated with the tip of a 6 cm hemostat. Two pieces of 3-O surgical silk were loosely tied around the vein 1 cm apart. A 0.5 x 3 cm piece of cardboard was placed underneath the isolated femoral vein between the surgical silk. Several drops of lidocaine solution were applied to the vein and surrounding area. Gentle tension was applied on the front piece of silk and a 5/8" 24 gauge Jelco winged catheter (Critikon Inc., Tampa FL) was slowly inserted into the vein until it stopped. The internal wire guide was removed, and blood flow was observed. To prevent clotting, 100 µL of saline containing heparin (20 units/mL) was injected through the cannula before it was closed.

2.1.2.2 Dosing Protocol: Intravenous Administration of Razoxane

The dosage for each rat was 20 mg/kg in a 1 mg/mL solution of razoxane dissolved in 0.9% (wt/vol) saline solution. The razoxane solution was administered via an i.v. infusion through the femoral vein cannula. For a 400 g rat, the volume of drug infused was 8.0 mL. The infusion occurred over a period of approximately 3 min. After the infusion, 100 µL of saline containing heparin (20 units/mL) was injected through the cannula before it was closed to prevent clotting.

2.1.2.3 Blood Collection

A 500 µL sample of blood was taken through the femoral vein cannula prior to the administration of razoxane. This sample was used as a blank to ensure that no endogenous compounds eluted at the same time as the enantiomers of razoxane. For the first group of four rats, 500 µL of blood were removed at 10, 20, 30, and 40 min after the infusion of razoxane. Catheter deadspace was compensated for all blood samplings. The last sample of 2.5 mL of blood was removed at 60 min post infusion. At the end of this study, the rats did not appear to be in hypovolemic shock. All rats were still alive at the end of the study. For a second group of 4 rats, 500 µL of blood was removed at 10 min post infusion. A 3 mL aliquot of blood was removed at 120 and 150 min post infusion. Larger blood volumes were required at later timepoints due to the decreased concentration of razoxane in the plasma and the relatively low sensitivity of the HPLC assay. Blood samples were added to 1.5 mL polyethylene microcentrifuge tubes containing 15 µL of heparin in saline (1000 units/mL). The blood was immediately centrifuged at 11,750 *g* for 2 min. The plasma was removed and treated with 50 µL of 42.5% (wt/vol) phosphoric acid per mL of plasma to stop further hydrolysis of razoxane. The treated plasma samples were then stored at -80°C. After each blood collection,

100 µL of saline containing heparin (20 units/mL) was injected through the cannula before it was closed to prevent clotting. After the last blood collection, the animals were euthanized with pentobarbital sodium salt at a dosage of 22 mg/kg, administered by i.v. through the femoral cannula.

2.1.2.4 Incubation of Razoxane in Rat Plasma

An aliquot of 2.33 mL rat plasma was added to a 10 mL disposable glass culture tube and the pH was adjusted to 7.4 at 37°C with 125 µL 1.0 M Tris base. The mixture was vortex mixed for 10 s. The pH of unbuffered rat plasma at 37°C was found to be pH 8.5. Razoxane has been shown to be sensitive to hydrolysis at elevated pH and temperature. To the buffered plasma (pH 7.4), 50 µL of 1.0 mg/mL razoxane solution was added and the mixture was vortex mixed for 10 s. The final concentration of razoxane was 40 µg/mL. One 260 µL aliquot was removed, and mixed with 3 µL of 1 mM 4-chlorobenzenesulfonamide dissolved in ethanol. The final concentration of 4-chlorobenzenesulfonamide in the sample was 115 µM. This concentration of 4-chlorobenzenesulfonamide has been shown to inhibit the enzyme dihydropyrimidine amidohydrolase (DHPase). The sample was incubated at 37°C for 150 min. The remaining plasma was incubated at 37°C and aliquots (260 µL) of plasma were removed at 0, 30, 60 and 150 min. All aliquots were treated with 13 µL of 42.5% (wt/vol) phosphoric acid to stop further hydrolysis. The samples were then extracted immediately, as described in section 2.1.2.8.

2.1.2.5 Preparation of Dexrazoxane and Levrazoxane Standard Solutions

To determine the elution order of the 2 razoxane enantiomers, solutions containing either 30 µg/mL of dexrazoxane or levrazoxane were dissolved in reagent alcohol. For long-term storage, samples were kept at -80°C. Aliquots of 60 µL were injected into the HPLC.

2.1.2.6 Preparation of Razoxane Standard Solutions

Solutions of razoxane at various concentrations (32.5 μg/mL to 650 μg/mL) were prepared by adding the appropriate volume of 1.0 mg/mL razoxane stock solution and making up to 1.0 mL with 10 mM HCl. The razoxane stock solution was prepared by dissolving 1.0 mg of razoxane in 1 mL of 10 mM HCl.

2.1.2.7 Preparation of the Calibration Plot

Aliquots (20 μL) of razoxane solution were added to 260 μL volumes of acidified (50 μL of 42.5% (wt/vol) phosphoric acid per mL of plasma) blank plasma. The plasma calibration samples were extracted by the same procedure for the plasma samples from the animal study, as described in section 2.1.2.8. The final razoxane concentration in plasma after extraction and reconstitution was from 5 μg/mL to 100 μg/mL. A blank plasma sample was also prepared and extracted using plasma free of razoxane and treated with 50 μL of 42.5% (wt/vol) phosphoric acid per mL of plasma.

2.1.2.8 Extraction of Razoxane from Plasma and Rat Liver Homogenate Supernatant

Both the plasma and rat liver homogenate supernatant samples were processed by the same procedure. The plasma samples were thawed at room temperature before use. The rat liver supernatant samples were extracted immediately after completion of the incubation study and treated with 13 μL of 42.5% (wt/vol) phosphoric acid per 260 μL of rat liver supernatant. In a 1.5 mL microcentrifuge tube, 70 μL of 6 M HCl was added to 260 μL of acidified plasma or rat liver supernatant. The HCl precipitated any proteins present in the sample. Samples were vortexed at medium speed, centrifuged and the supernatant was removed. The pellet was rinsed three times with 250 μL aliquots of 10 mM HCl and the washings transferred to the supernatant that

had been removed. The pellet was then discarded. To the supernatant, 1.5 mL of 0.2 M phosphate buffer (pH 7.0) was added, and adjusted to a pH of 6.0 with 80 µL of 5 M NaOH. The buffer solution was then transferred to conditioned Sep-Pak Plus C-18 solid phase extraction cartridges (Waters Chromatography, Milford, MA, USA). The cartridges were conditioned by eluting through 5 mL of acetonitrile followed by 5 mL of double distilled water under vacuum (5 mm Hg). The buffer solution was passed through the cartridges under vacuum (5 mm Hg). The cartridges were then washed with 5 mL of double-distilled water and dried under vacuum (15 mm Hg) for 5 min. The cartridges were washed with 1 mL of hexanes under low vacuum (< 2 mm Hg) and the hexane discarded. The cartridges were then eluted with a 5 mL portion of acetonitrile and then a 2.5 mL portion of acetonitrile. The acetonitrile samples were combined in a 10 mL disposable glass culture tube and evaporated to dryness under a stream of argon gas in a 40°C water bath. Samples were sealed with disposable plastic caps and stored at -80°C. Samples were allowed to warm to room temperature and then reconstituted in 130 µL of HPLC reagent alcohol used in the mobile phase, just prior to analysis.

2.1.2.9 Hydrolysis of Razoxane in Rat Liver Homogenate Supernatant

2.1.2.9.1 Preparation of Rat Liver Supernatant Homogenate Fraction

Approximately 6 g of rat liver were homogenized by hand in an glass Potter-Elvehjem tissue grinder in 2 volumes (12 mL) of 50 mM cold (4°C) Tris buffer (pH 7.6 at 4°C). The homogenate was transferred to a 50 mL polycarbonate centrifuge tube and centrifuged at 35,000 *g* for 2 hours at 4°C. About 10 mL of the supernatant wertt removed and transferred to dialysis tubing with a molecular cutoff of 50,000 daltons (Spectrum Medical Industries, Inc., Los Angeles, CA). The supernatant was dialyzed overnight in

1 L of 50 mM Tris buffer (pH 7.6) at 4°C. During dialysis, the Tris buffer was changed 3 times.

After dialysis, the sample was removed from the tubing, and transferred to a 50 mL polycarbonate centrifuge tube. The sample was then centrifuged at 13,000 *g* for 5 min at 4°C and then stored at -20°C.

2.1.2.9.2 Protein Analysis of Rat Liver Homogenate Supernatant

The rat liver supernatant fraction was analyzed for protein content with a microprotein determination kit (Sigma 690-A). The method suggested by the manufacturer was modified to use 50% of the volume of reactants.

The supernatant was diluted with 0.85% (wt/vol) NaCl solution to give 1/50 and 1/100 test solutions. Aliquots of 100 µL of the supernatant test solutions were pipetted into 10 mL disposable glass culture tubes. A protein stock solution with a concentration of 1 mg/mL was prepared by diluting the supplied bovine serum albumin (BSA) protein standard with 0.85% (wt/vol) NaCl solution. In 10 mL disposable glass culture tubes, various volumes of the BSA stock solution were diluted with 0.85% (wt/vol) NaCl solution to give a final sample volume of 100 µL. The concentration range was from 0.25 to 1.0 mg/mL. A blank was also prepared using 0.85% (wt/vol) NaCl solution. To 100 µL aliquots of the samples, 1.1 mL of the Biuret reagent was added, vortexed-mixed for 15 s and allowed to stand for 10 min at room temperature. To each tube, 100 µL each of Folin and Ciocalteu's Phenol reagent were added, vortex-mixed for 15 s and allowed to stand at room temperature for 30 min. The samples were transferred to 1 cm disposable plastic cuvettes and read at 720 nm. A calibration curve was prepared by plotting the absorbance values for each protein sample against their concentration.

2.1.2.9.3 Incubation of Razoxane in Rat Liver Supernatant Fraction

In a 10 mL disposable glass culture tube, the pH of 220 µL rat liver homogenate supernatant fraction was adjusted to 7.4 at 37°C with 3.7 mL of 150 mM KCl/50 mM Tris buffer. The mixture was vortex mixed for 10 s and 80 µL of 1.0 mg/mL razoxane solution were added. The diluted concentration of razoxane was 20 µg/mL and the protein concentration of the solution was 2 mg/mL. The mixture was vortex mixed again for 10 s. One 260 µL aliquot was removed, and mixed with 3 µL of 1 mM 4-chlorobenzenesulfonamide dissolved in ethanol. The final concentration of 4-chlorobenzenesulfonamide in the sample was 115 µM. This sample was incubated at 37°C for 150 min. The remaining rat liver supernatant sample was incubated at 37°C and 260 µL aliquots were removed at 0, 30, 60 and 150 min. All aliquots were treated with 13 µL of 42.5% (wt/vol) phosphoric acid to stop further hydrolysis of razoxane. The samples were then extracted immediately as described in section 2.1.2.8.

2.1.2.10 HPLC Method for Chiral Analysis of Razoxane

2.1.2.10.1 Addition of the Chiral Selector ß-cyclodextrin to the Mobile Phase

2.1.2.10.1.1 Preparation of Na_2EDTA and ß-cyclodextrin Solutions

A 500 µM Na_2EDTA solution (pH 4.5) was made by dissolving 0.186 g of disodium Na_2EDTA in 1 L of double distilled water. A 15 mM ß-cyclodextrin solution was made by dissolving 4.26 g of ß-cyclodextrin in 250 mL of 500 µM Na_2EDTA solution.

Mobile phase 1 was ß-cyclodextrin/Na_2EDTA/MeOH solution (75/20/5 v/v). Mobile phase 2 was ß-cyclodextrin/Na_2EDTA/MeOH solution (75/22/3 v/v). Mobile phase 3 was ß-cyclodextrin/Na_2EDTA (75/25 v/v) solution. Mobile phase 4 was ß-cyclodextrin solution (100%).

2.1.2.10.2 Chiralcel Chiral Column

Standard solutions of dexrazoxane and levrazoxane dissolved in reagent alcohol (90% ethanol, 5% methanol and 5% isopropanol, by volume) were analyzed on the Chiralcel OD cellulose tris(3,5-dimethylphenyl carbamate) chiral column. The mobile phase used was 100% reagent alcohol at a flow rate of 0.5 mL/min. Each sample was run to determine enantiomer elution times.

Several mobile phases were tried for the chiral cellulose column. Mobile phase 1 was 50 parts reagent alcohol and 50 parts hexanes, by volume. Mobile phase 2 was 70/30 (v/v) reagent alcohol and hexanes. Mobile phase 3 was 80/20 (v/v), mobile phase 4 was 85/15 (v/v) and mobile phase 5 was 90/10 (v/v) reagent alcohol and hexanes.

The organic modifier diethylamine was added to 2 mobile phases in an attempt to reduce tailing. Mobile phase 6 was 79.925/20/0.075 (v/v/v) reagent alcohol, hexanes and diethylamine. Mobile phase 7 was 79.9/20/0.1 (v/v/v) reagent alcohol, hexanes and diethylamine.

The mobile phase used for the analysis of the plasma calibration plots, animal study, plasma incubation study and rat liver extract incubation study was 85/15 (v/v) reagent alcohol and hexanes. Aliquots of 60 µL of the reconstituted samples were injected into the HPLC. The 50 µL sample loop was rinsed between injections with 1.0 mL of reagent alcohol.

2.2 RESULTS

2.2.1 HPLC Assay

2.2.1.1 Addition of the Chiral Selector ß-cyclodextrin to the Mobile Phase

A chiral selector, ß-cyclodextrin, was added to a mobile phase in an effort to resolve the two enantiomers of razoxane. A regular, reversed-phase C-18 column was used with all the chiral mobile phases. Five mobile phases,

were used, with 3 containing 11.75 mM ß-cyclodextrin and methanol concentrations of either 3, 5 and 8% (v/v). The other two mobile phases consisted of 12.75 and 15 mM ß-cyclodextrin. The enantiomers were not resolved with any of the chiral ß-cyclodextrin mobile phases listed in section 2.1.2.10.2.

2.2.1.2 Chiralcel Chiral Column

Standard solutions of dexrazoxane and levrazoxane dissolved in reagent alcohol were analyzed to determine the enantiomer elution order. Levrazoxane eluted first on the chiral cellulose column and was followed by dexrazoxane. The dexrazoxane sample was found to contain a small amount of levrazoxane, less than 0.5%. No dexrazoxane contamination was detected in the levrazoxane sample. With a mobile phase of 100% reagent alcohol delivered at a flow rate of 0.5 mL/min, the retention time of levrazoxane was 15.1 min and the retention time for dexrazoxane was 16.6 min.

Razoxane standard samples dissolved in reagent alcohol were analyzed using mobile phases consisting of various proportions of hexane and reagent alcohol. All mobile phases used with the chiral cellulose column were delivered isocratically at a flow rate of 0.5 mL/min.

The resolution or R value was calculated for each mobile phase using the equation listed below. The peak width used was at the baseline. The resolution values are listed in Table 2.1.

$$\text{R value} = \frac{2(t_{r\,\text{dexrazoxane}} - t_{r\,\text{levrazoxane}})}{(w_{\text{dexrazoxane}} + w_{\text{levrazoxane}})}$$

where: $t_{r\,\text{dexrazoxane}}$ = retention time for dexrazoxane

$t_{r\,\text{levrazoxane}}$ = retention time for levrazoxane

$w_{\text{dexrazoxane}}$ = peak width at the baseline for dexrazoxane

$w_{\text{levrazoxane}}$ = peak width at the baseline for levrazoxane

All mobile phases gave peaks which had signs of tailing. In an attempt to reduce tailing, diethylamine was added to mobile phases 6 and 7. Mobile phase 6 was 79.9/20/0.1 (v/v/v) reagent alcohol, hexanes and diethylamine. Mobile phase 7 was 79.875/20/0.125 (v/v/v) reagent alcohol, hexanes and diethylamine. Tailing was not reduced for either mobile phase containing diethylamine and the background absorbance was substantially increased.

Table 2.1. Resolution (R) Values for Various Mobile Phases used with a Chiralcel OD Column

	Mobile phase (reagent alcohol/hexanes (v/v))				
	50/50	70/30	80/20	85/15	90/10
R-value	2.07	1.81	1.69	1.51	1.36
t_r Levrazoxane (min)	21.5	19.7	17.6	16.5	15.6
t_r Dexrazoxane (min)	23.8	21.2	19.7	18.5	17.4

2.2.1.3 HPLC Chromatograms

Representative HPLC chromatograms for the enantiomers of razoxane are shown in figures 2.1 to 2.3. Figure 2.1 is the chromatogram for a blank plasma sample from rat 5. Figure 2.2 is the chromatogram for a plasma sample from rat 5 at 10 min post i.v. infusion of 20 mg/kg razoxane. The plasma volume for both samples was 260 µL, processed and reconstituted in 130 µL of reagent alcohol. The chromatogram for a plasma sample from rat 1 at 150 min post i.v. infusion of 20 mg/kg razoxane is illustrated in figure 2.3. The plasma volume for the sample was 2.08 mL, processed and reconstituted in 130 µL of reagent alcohol. Retention times of levrazoxane and dexrazoxane were 14.7 and 16.5 min respectively for the mobile phase of reagent alcohol/hexanes (85/15 v/v). There were no interfering peaks in the blank plasma samples in the regions where levrazoxane and dexrazoxane eluted.

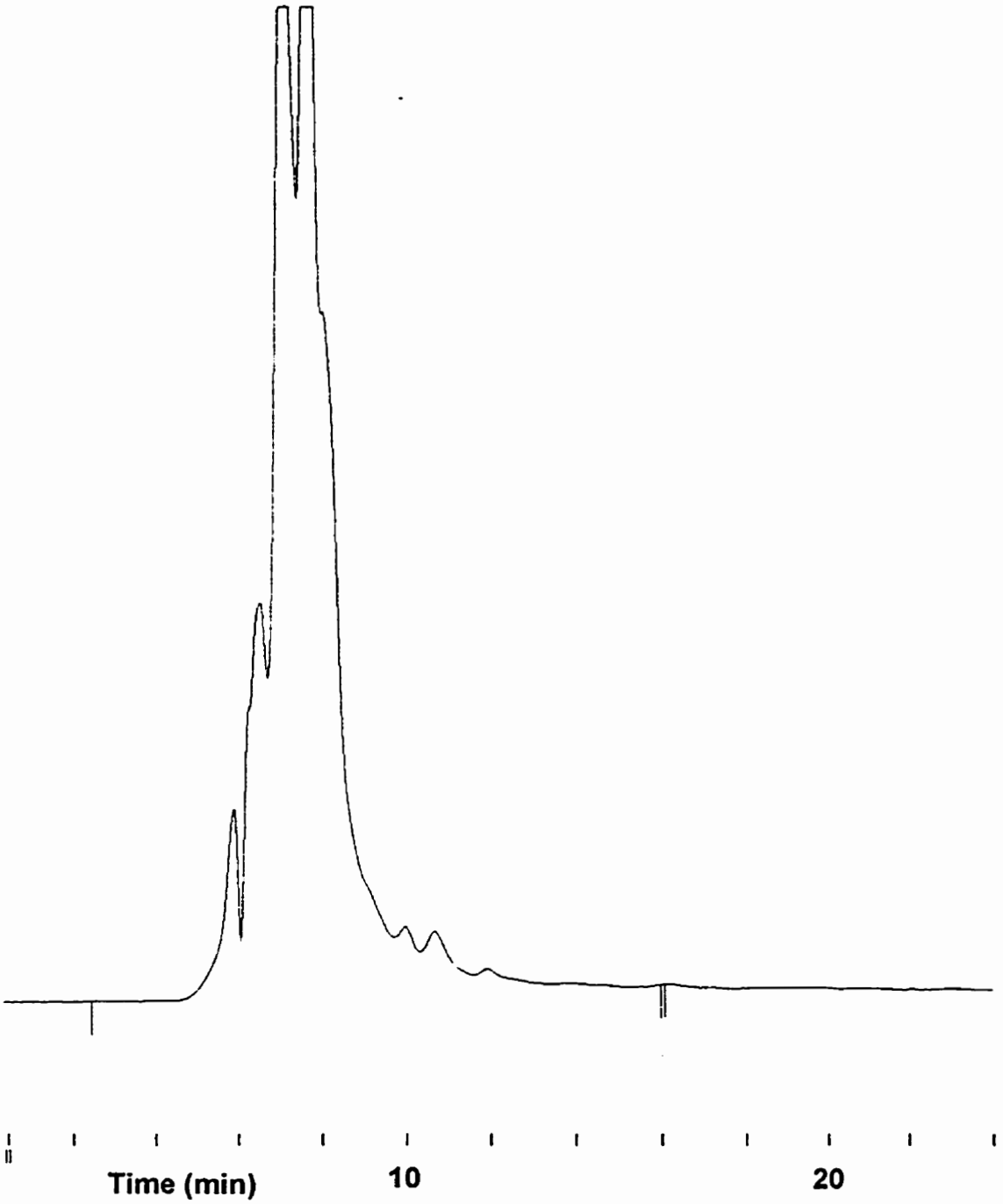


Figure 2.1. Chromatogram for a blank plasma sample from Rat 1, before i.v. administration of razoxane. The volume of the plasma sample was 260 µL, processed and reconstituted in 130 µL of reagent alcohol.
HPLC conditions were reagent alcohol/hexanes (85/15 v/v) at a flow rate of 0.5 mL/min. A Chiralcel OD cellulose column, 4.6 mm I.D. X 250 mm with a particle size of 10 µm was used for the chiral separation of levrazoxane and dexrazoxane. UV detection wavelength was 207 nm.

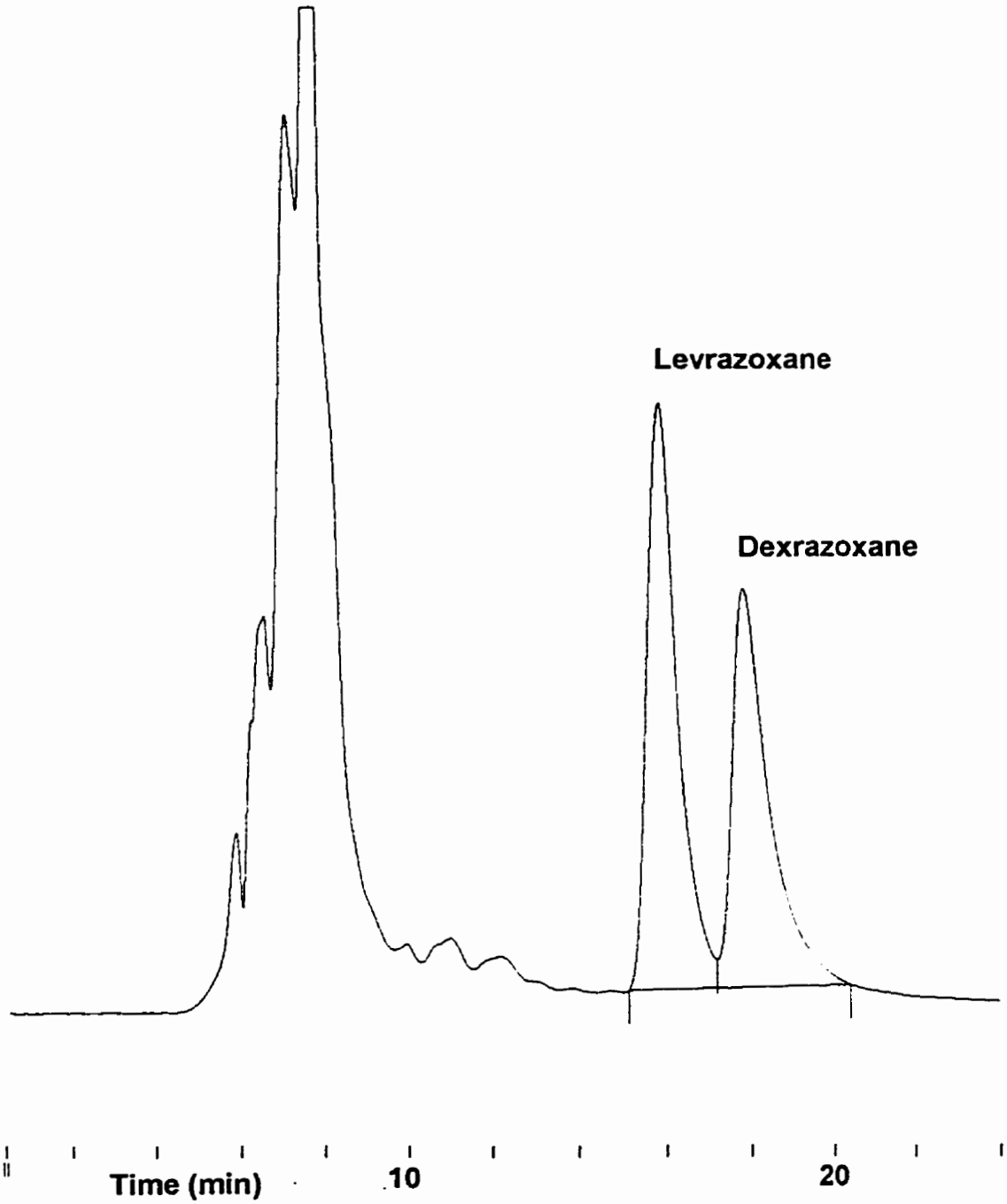


Figure 2.2. Chromatogram for a plasma sample from Rat 1, 10 min post i.v. administration of 20 mg/kg razoxane. The volume of the plasma sample was 260 µL, processed and reconstituted in 130 µL of reagent alcohol.
HPLC conditions were reagent alcohol/hexanes (85/15 v/v) at a flow rate of 0.5 mL/min. A Chiralcel OD cellulose column, 4.6 mm I.D. X 250 mm with a particle size of 10 µm was used for the chiral separation of levrazoxane and dexrazoxane. UV detection wavelength was 207 nm.

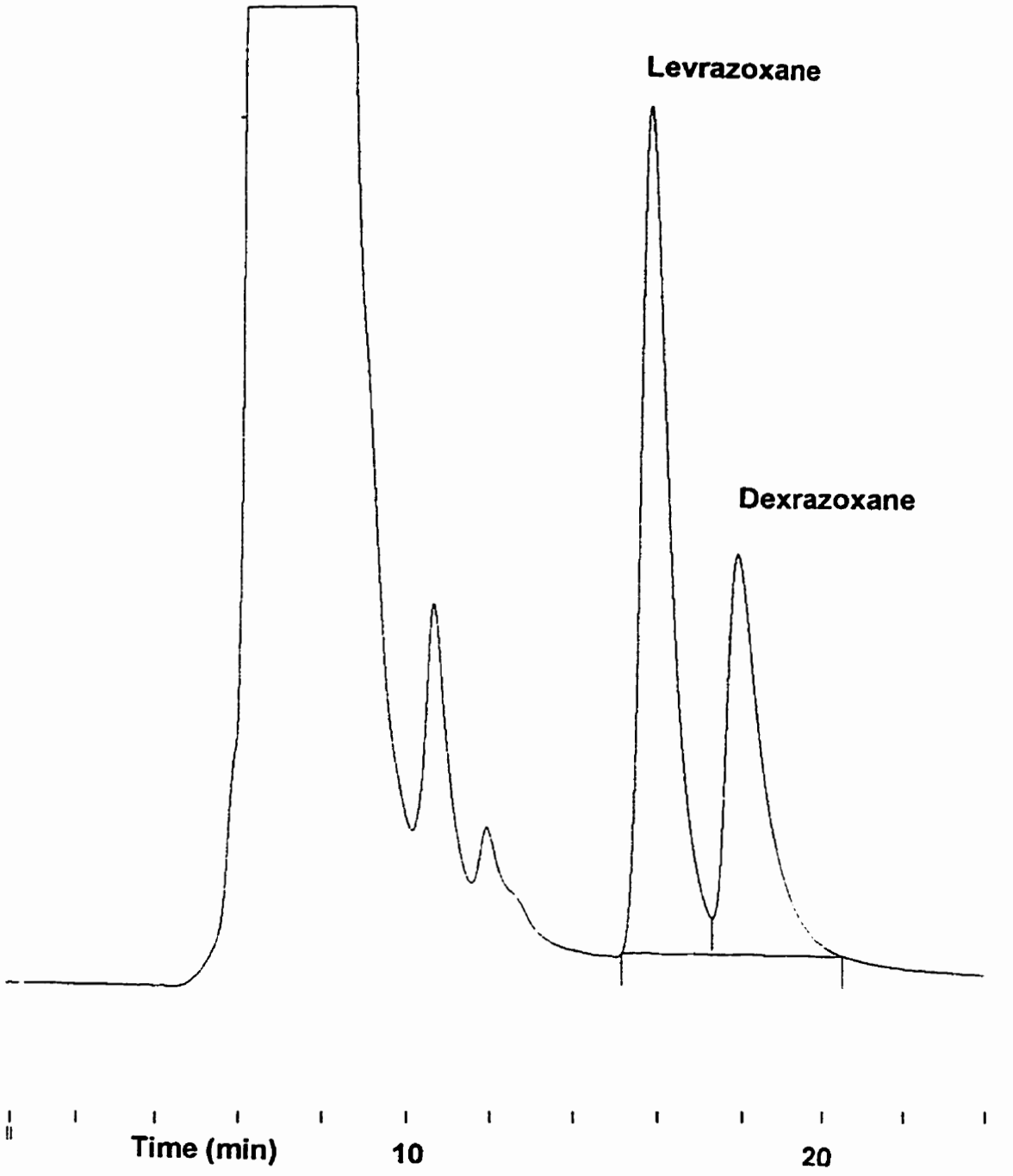


Figure 2.3. Chromatogram for a plasma sample from Rat 1, 150 min post i.v. administration of 20 mg/kg razoxane. The volume of the plasma sample was 2080 µL, processed and reconstituted in 130 µL of reagent alcohol.
HPLC conditions were reagent alcohol/hexanes (85/15 v/v) at a flow rate of 0.5 mL/min. A Chiralcel OD cellulose column, 4.6 mm I.D. X 250 mm with a particle size of 10 µm was used for the chiral separation of levrazoxane and dexrazoxane. UV-detection wavelength was 207 nm.

2.2.1.4 Calibration Plots

The calibration plots for each enantiomer were constructed by plotting enantiomer peak areas against the enantiomer concentrations. The data for the calibration plots are listed in Table 2.2. The calibration plots are shown in figure 2.4 and the concentration range of dexrazoxane and levrazoxane was from 5.0 to 100 μg/mL. The calibration plots were linear with r^2 values of over 0.99 for this concentration range. The linear regression data for the plots of each enantiomer are listed in Table 2.3.

Table 2.2. Peak Areas of Dexrazoxane and Levrazoxane in Rat Plasma vs. Concentration

Drug Concentration (μg/mL)	Average Peak Area of Levrazoxane ± SEM (arbitrary units)	Average Peak Area of Dexrazoxane ± SEM (arbitrary units)
5	230,315 ± 2,494	191,176 ± 2,252
10	499,737 ± 4,140	437,629 ± 6,214
20	962,024 ± 36,165	882,340 ± 22,698
40	2,168,753 ± 31,594	1,969,564 ± 36,771
80	4,607,753 ± 45,379	4,464,874 ± 86,561
100	5,574,983 ± 100,184	5,535,637 ± 103,870

Table 2.3. Linear Regression Data for the Calibration Plots of Dexrazoxane and Levrazoxane Concentrations in Plasma

	Levrazoxane	Dexrazoxane
Slope ± SEM $(\mu g/mL)^{-1}$	57,418 ± 959	57,178 ± 1138
Y-intercept ± SEM	-99,702 ± 53,293	-183,236 ± 63,236
r^2-value	0.999	0.998

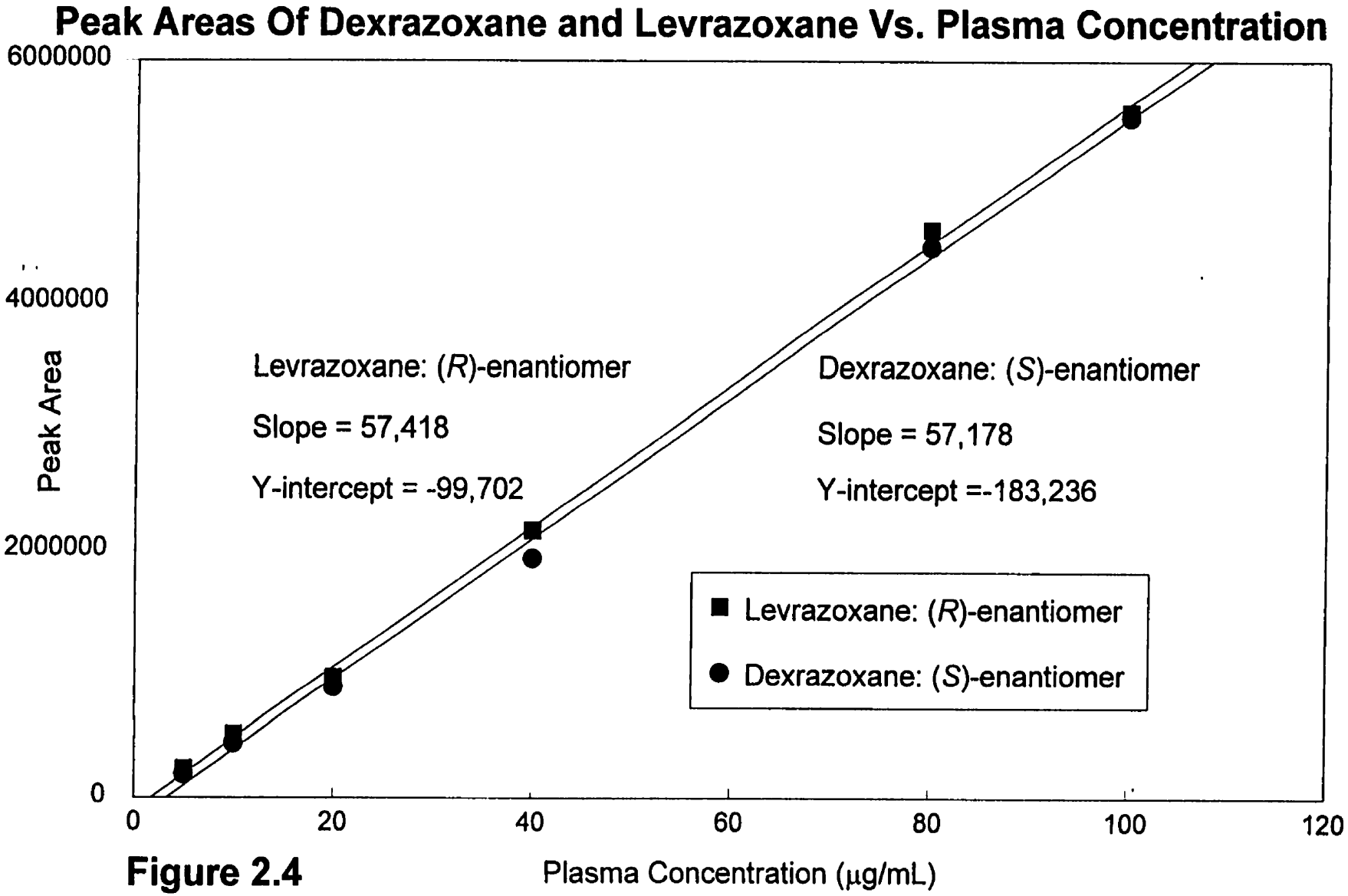

Peak Areas Of Dexrazoxane and Levrazoxane Vs. Plasma Concentration
6000000
4000000
2000000
0
Peak Area
Levrazoxane: (R)-enantiomer
Slope = 57,418
Y-intercept = -99,702
Dexrazoxane: (S)-enantiomer
Slope = 57,178
Y-intercept =-183,236
Levrazoxane: (R)-enantiomer
Dexrazoxane: (S)-enantiomer
0
20
40
60
80
100
120
Plasma Concentration (µg/mL)

Figure 2.4

2.2.2 Animal Study

Plasma samples were taken from 4 rats at 10, 20, 30, 40 and 60 min. Plasma samples were also taken from a second group of 4 additional rats at 10, 120 and 150 min. Table 2.4 lists the plasma concentrations of levrazoxane and dexrazoxane. These concentrations were plotted against time and are shown in figures 2.5 through 2.12.

The average plasma concentrations of levrazoxane and dexrazoxane are listed in Table 2.5 and plotted against time in figure 2.13. Peak concentration of razoxane was at 10 min with 27.97 μg/mL for levrazoxane and 25.40 μg/mL for dexrazoxane.

Table 2.4. Plasma Concentration of Dexrazoxane and Levrazoxane in Rats After i.v. Administration of 20 mg/kg Razoxane

	Animal 1		Animal 2	
Time (min.)	Levrazoxane (μg/mL)	Dexrazoxane (μg/mL)	Levrazoxane (μg/mL)	Dexrazoxane (μg/mL)
10	24.60	20.02	30.52	27.74
20	16.36	12.78	15.62	12.87
30	13.75	9.77	14.07	11.36
40	9.84	7.66	10.38	7.78
60	4.26	2.69	8.03	6.17

	Animal 3		Animal 4	
Time (min.)	Levrazoxane (µg/mL)	Dexrazoxane (µg/mL)	Levrazoxane (µg/mL)	Dexrazoxane (µg/mL)
10	32.10	29.58	37.34	34.20
20	15.01	12.85	16.20	12.95
30	11.23	9.68	12.08	10.10
40	8.35	7.13	11.79	9.08
60	6.57	5.13	5.31	3.73

	Animal 5		Animal 6	
Time (min.)	Levrazoxane (µg/mL)	Dexrazoxane (µg/mL)	Levrazoxane (µg/mL)	Dexrazoxane (µg/mL)
10	29.89	26.88	24.17	21.55
120	n/a	n/a	3.19	2.05
150	5.38	3.38	2.33	1.55

*n/a: sample not available for analysis

	Animal 7		Animal 8	
Time (min.)	Levrazoxane (µg/mL)	Dexrazoxane (µg/mL)	Levrazoxane (µg/mL)	Dexrazoxane (µg/mL)
10	17.15	15.34	n/a	n/a
120	2.46	1.77	2.55	1.86
150	2.01	1.43	2.41	1.65

*n/a: sample not available for analysis

Table 2.5. Average Plasma Concentration of Dexrazoxane and Levrazoxane in Rats After i.v. Administration of 20 mg/kg Razoxane

Time (min)	Levrazoxane (μg/mL)	Standard Error	Dexrazoxane (μg/mL)	Standard Error
10	27.97	2.48	25.04	2.42
20	15.80	0.31	12.86	0.03
30	12.78	0.68	10.22	0.39
40	10.09	0.71	7.91	0.41
60	6.04	0.81	4.43	0.77
120	2.73	0.23	1.89	0.08
150	3.03	0.79	2.00	0.46

Paired one-tailed *t*-tests were performed on the plasma concentrations of dexrazoxane and levrazoxane at each timepoint using the program Sigmastat. The results of the paired *t*-tests are listed in Table 2.6. A significant difference between the plasma concentration of dexrazoxane and levrazoxane was found for the 10, 20, 30, 40, 60 and 120 min timepoints. As noted in section 2.1.2.3, all timepoints were at post-infusion. Time t = 0 was started at the end of the infusion. For the 150 min timepoint, the difference between the two plasma concentrations was not large enough to exclude the possibility that this difference may be due to chance. The *p*-value at 10 min was < 0.0001 with 6 degrees of freedom. There were 3 degrees of freedom for the 20, 30, 40, and 150 min timepoints. At 120 min, there were 2 degrees of freedom. The *p*-value at 20 min was 0.0025, 0.0168 at 30 min, 0.0075 at 40 min, 0.0004 min at 60 min, 0.0304 at 120 min and 0.0511 min at 150 min. In this study, $p < 0.05$, with less than a 5% probability of incorrectly concluding a difference between 2

groups. The degrees of freedom is a reflection of sample size and is usually n-1, where n is the sample size.

Table 2.6. Paired t-test Between Dexrazoxane and Levrazoxane Rat Plasma Concentrations

Time (min)	p-value	degrees of freedom
10	< 0.0001	6
20	0.0025	3
30	0.0168	3
40	0.0075	3
60	0.0004	3
120	0.0304	2
150	0.0511	3

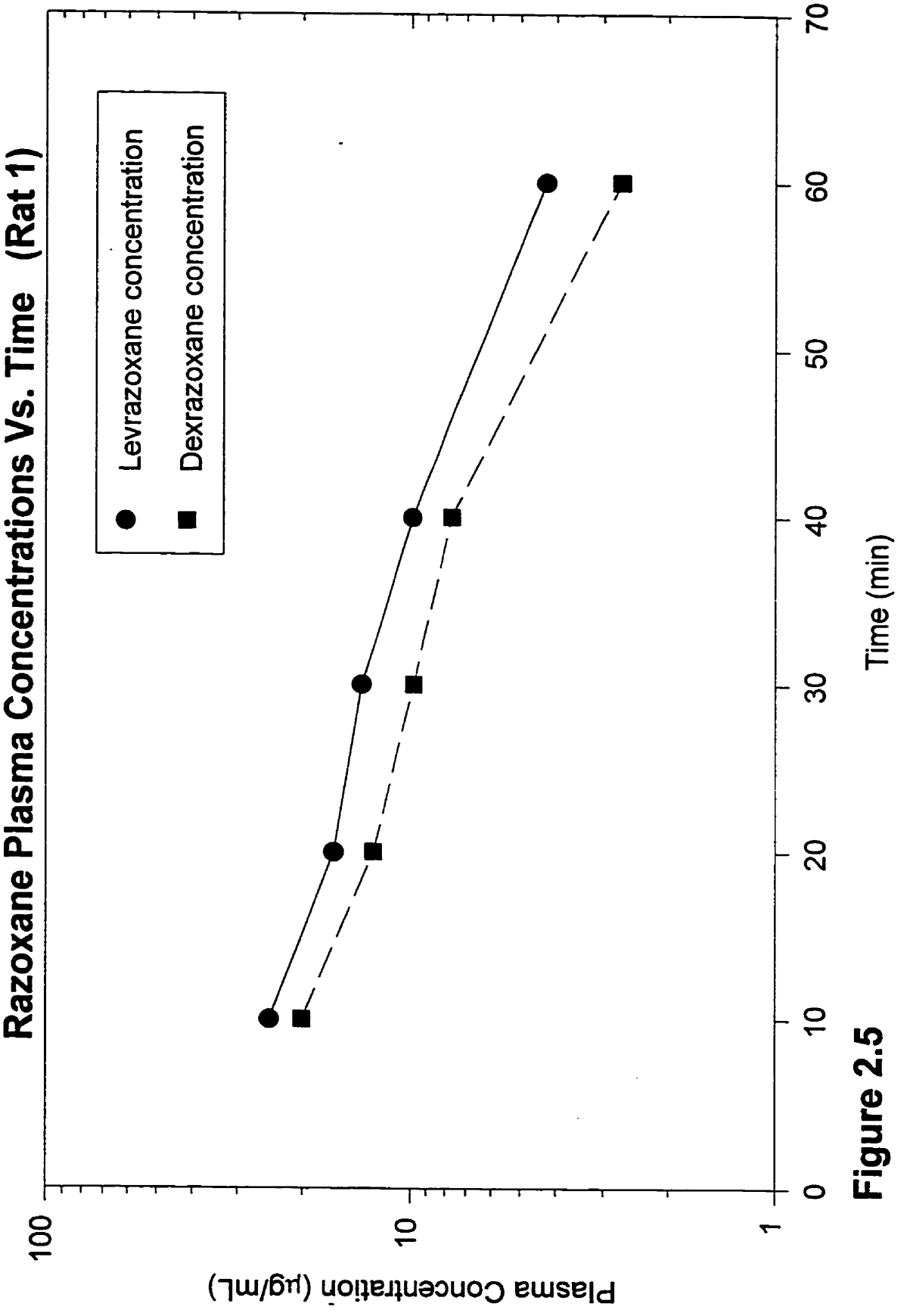

Razoxane Plasma Concentrations Vs. Time (Rat 1)
Levrazoxane concentration
Dexrazoxane concentration
Plasma Concentration (µg/mL)
100
10
1
0
10
20
30
40
50
60
70
Time (min)

Figure 2.5

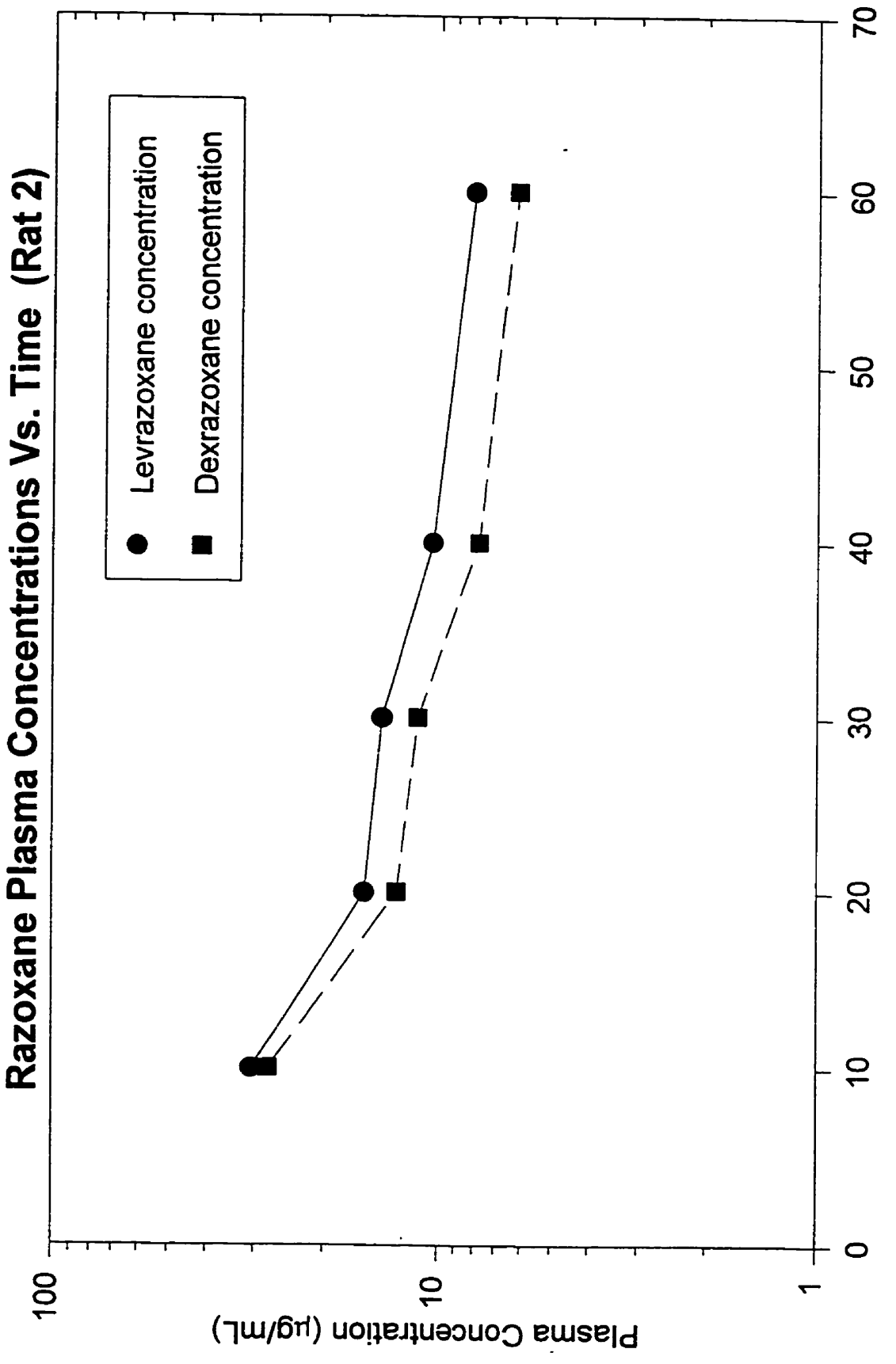

Razoxane Plasma Concentrations Vs. Time (Rat 2)
Levrazoxane concentration
Dexrazoxane concentration
Plasma Concentration (µg/mL)
100
10
1
0
10
20
30
40
50
60
70
Time (min)

Figure 2.6

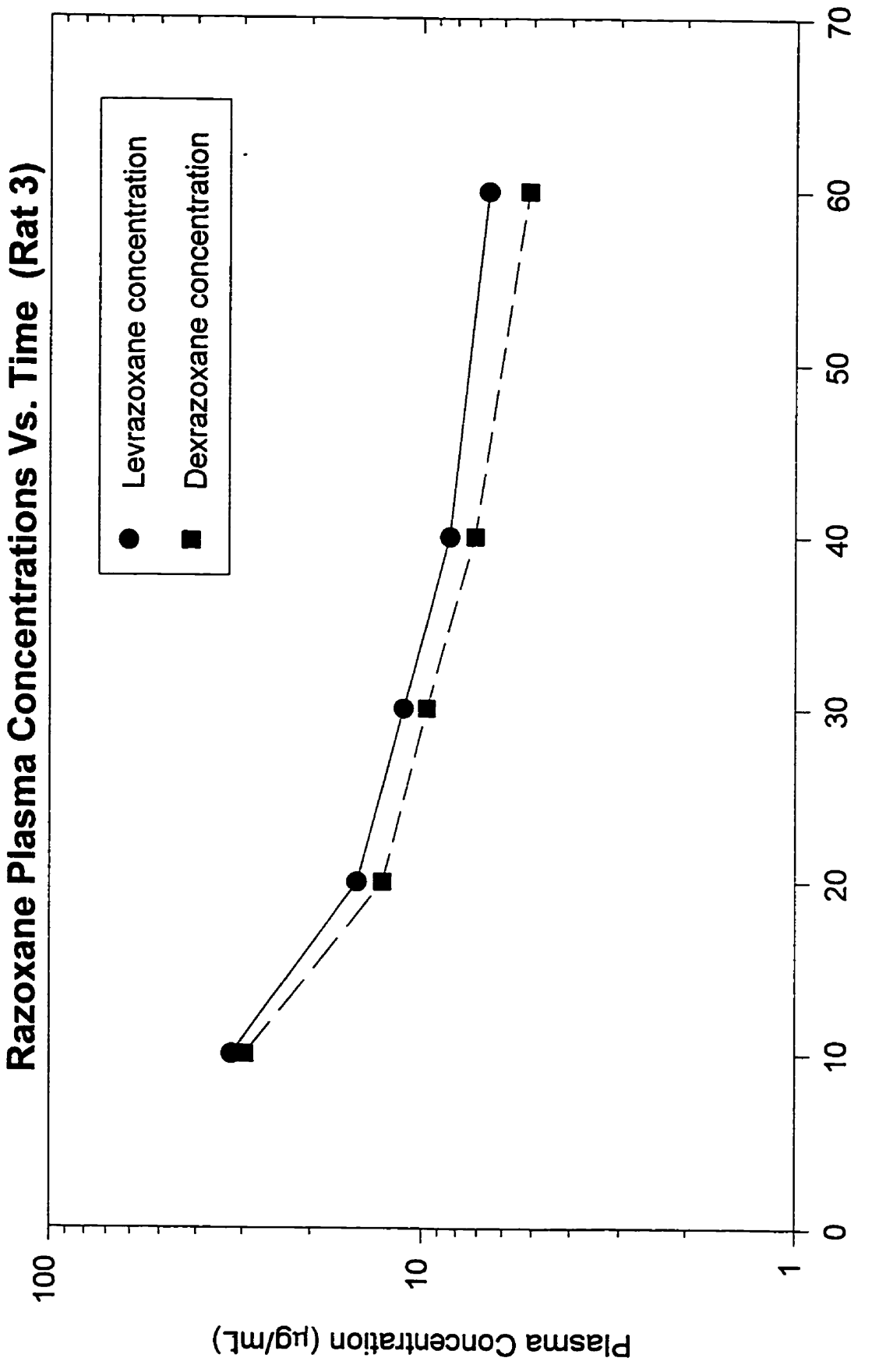

Razoxane Plasma Concentrations Vs. Time (Rat 3)
Levrazoxane concentration
Dexrazoxane concentration
Plasma Concentration (µg/mL)
100
10
1
0
10
20
30
40
50
60
70
Time (min)

Figure 2.7

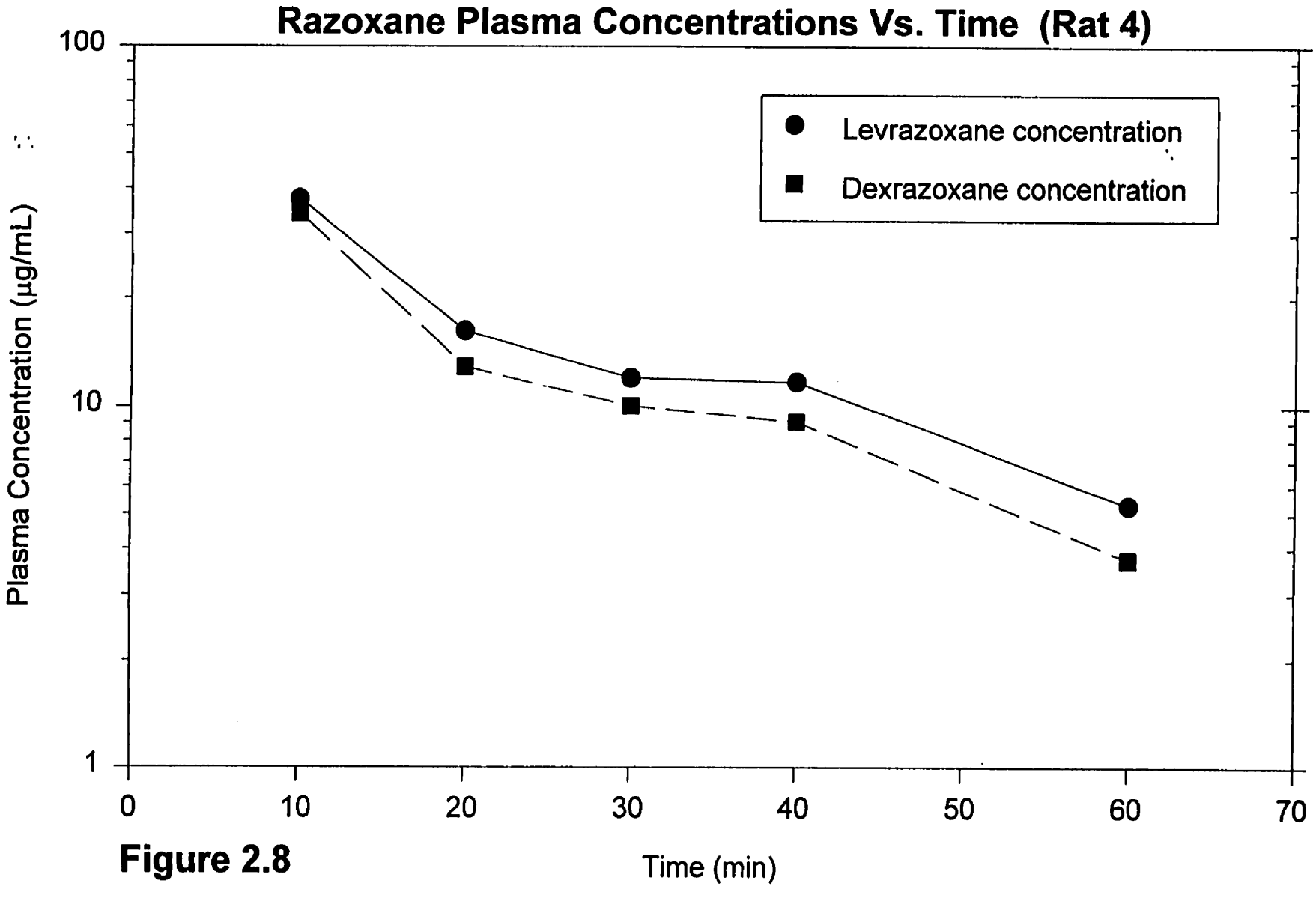

Razoxane Plasma Concentrations Vs. Time (Rat 4)
Levrazoxane concentration
Dexrazoxane concentration
Plasma Concentration (µg/mL)
100
10
1
0
10
20
30
40
50
60
70
Time (min)

Figure 2.8

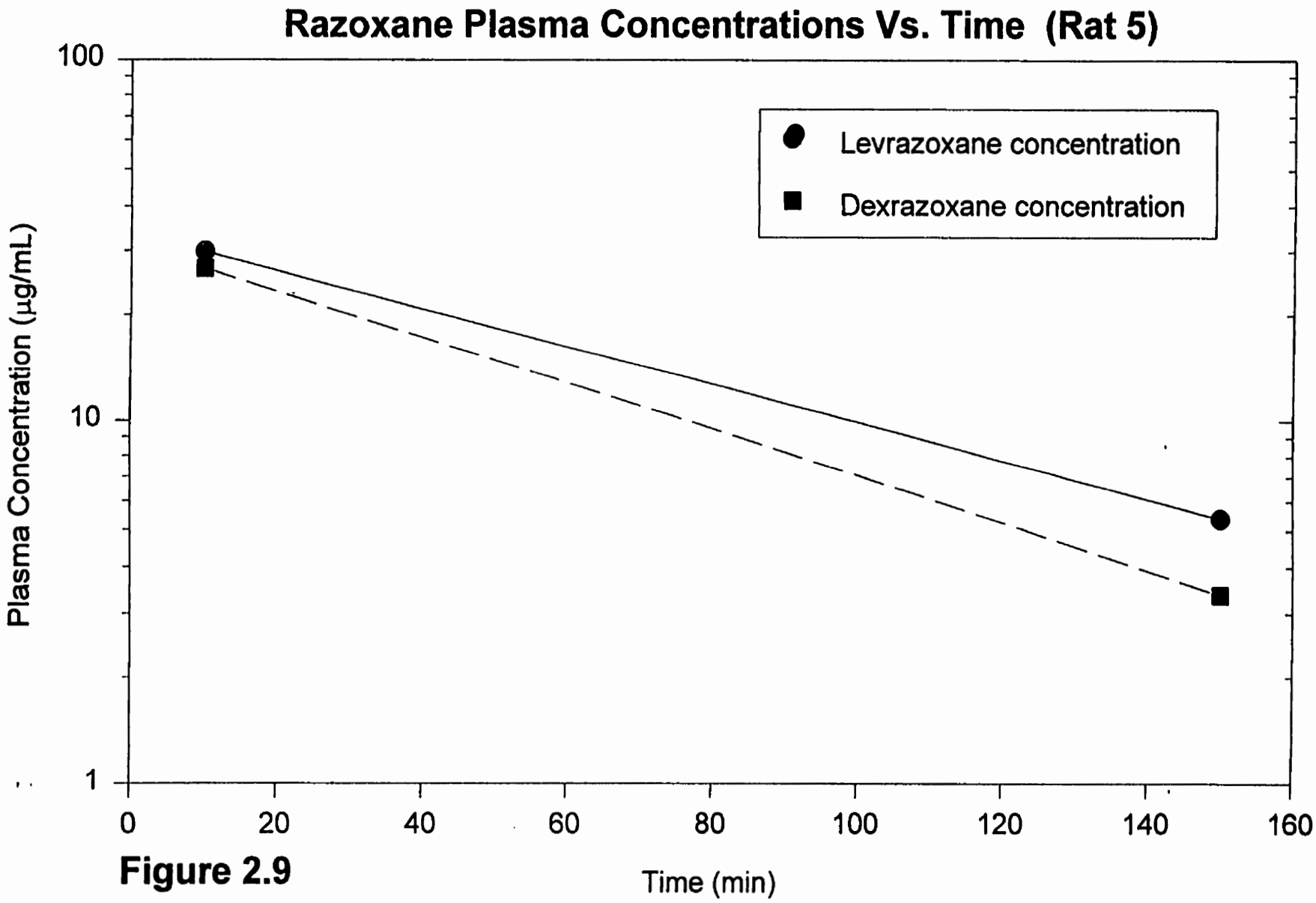

Razoxane Plasma Concentrations Vs. Time (Rat 5)
100
10
1
Plasma Concentration (µg/mL)
Levrazoxane concentration
Dexrazoxane concentration
0
20
40
60
80
100
120
140
160
Time (min)

Figure 2.9

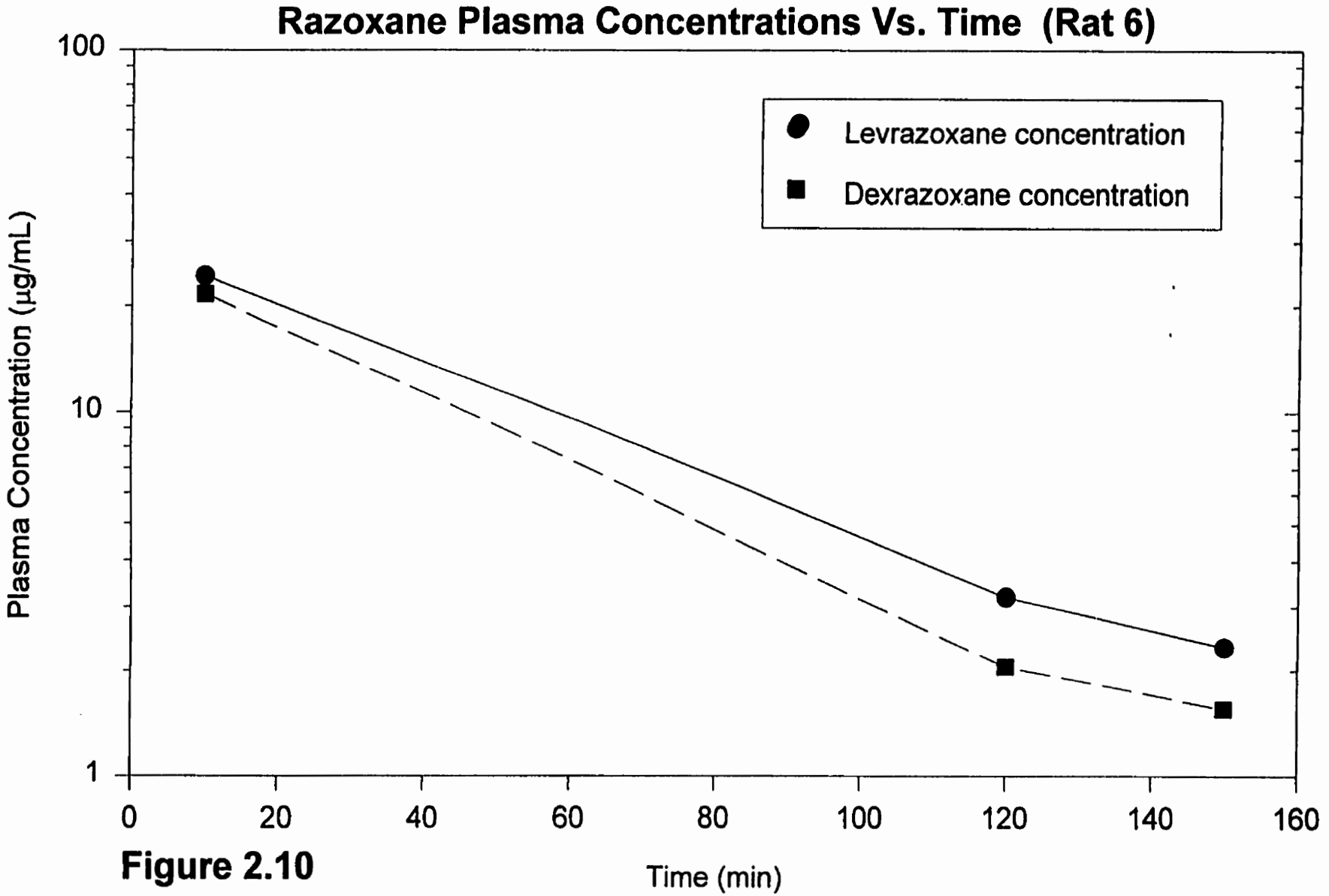

Razoxane Plasma Concentrations Vs. Time (Rat 6)
100
10
1
Plasma Concentration (µg/mL)
0
20
40
60
80
100
120
140
160
Time (min)
Levrazoxane concentration
Dexrazoxane concentration

Figure 2.10

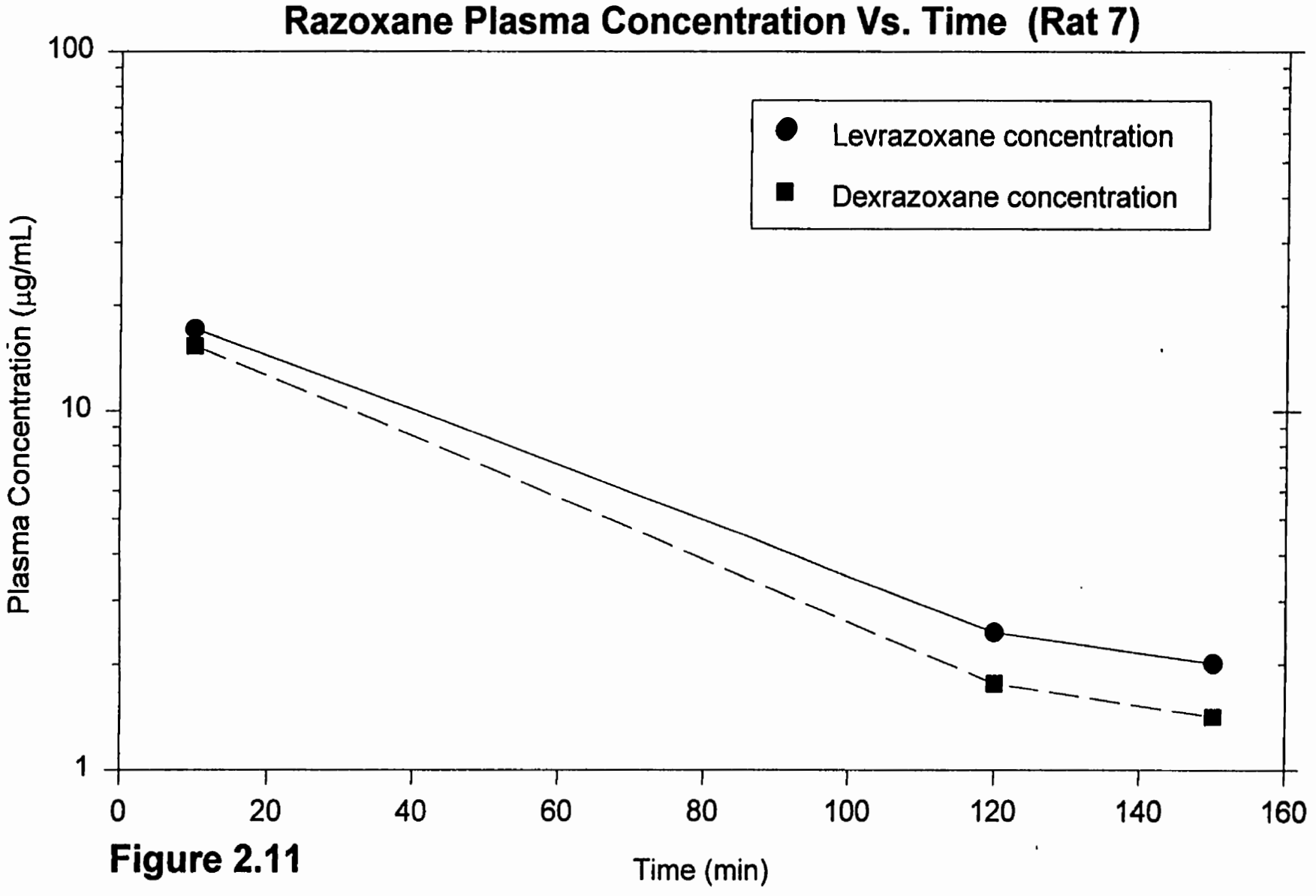

Razoxane Plasma Concentration Vs. Time (Rat 7)
100
10
1
Plasma Concentration (µg/mL)
Levrazoxane concentration
Dexrazoxane concentration
0
20
40
60
80
100
120
140
160
Time (min)

Figure 2.11

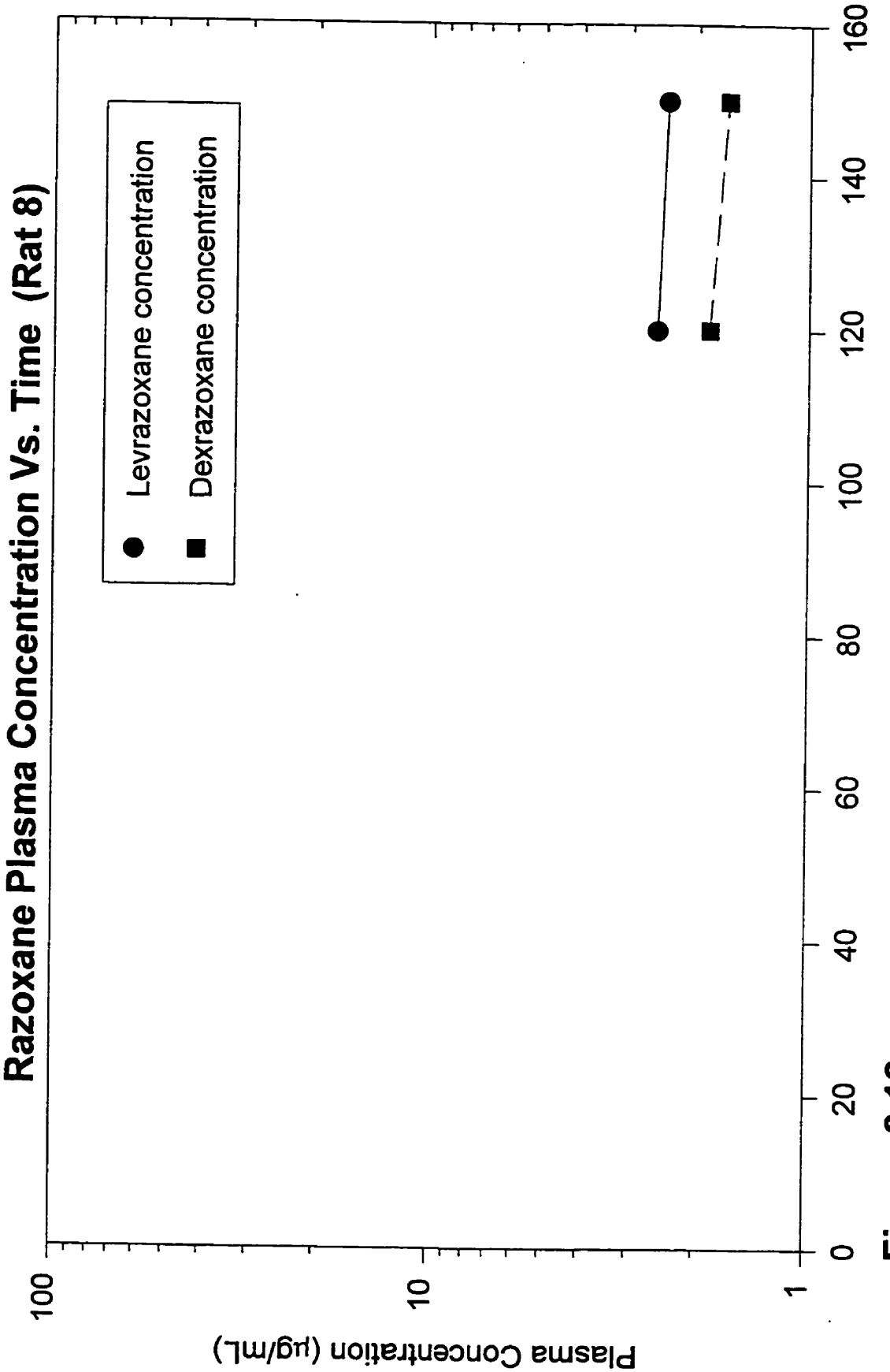

Razoxane Plasma Concentration Vs. Time (Rat 8)
Levrazoxane concentration
Dexrazoxane concentration
Plasma Concentration (µg/mL)
100
10
1
0
20
40
60
80
100
120
140
160
Time (min)

Figure 2.12

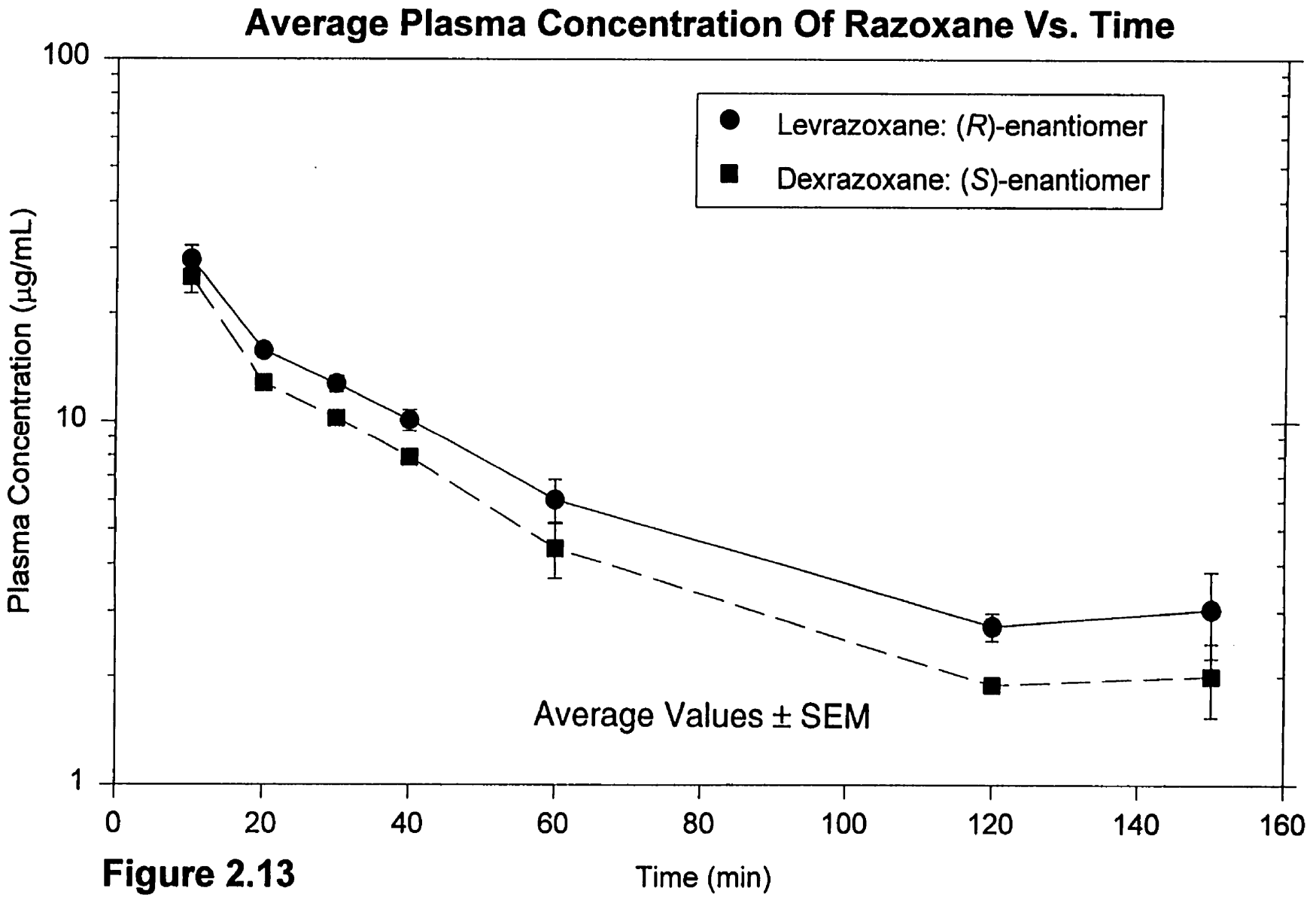

Average Plasma Concentration Of Razoxane Vs. Time
100
10
1
Plasma Concentration (µg/mL)
Levrazoxane: (R)-enantiomer
Dexrazoxane: (S)-enantiomer
Average Values ± SEM
0
20
40
60
80
100
120
140
160
Time (min)

Figure 2.13

The *R/S* ratios were calculated by dividing the plasma concentrations of the *R*-enantiomer levrazoxane by the plasma concentrations of the *S*-enantiomer dexrazoxane. Table 2.7 summarizes the *R/S* ratios for each animal.

The average *R/S* ratios are listed in Table 2.8. The average *R/S* ratios were plotted versus time and shown in figure 2.14. The *R/S* ratio increased over time and had a maximum value of 1.49 at 150 min.

Table 2.7. Ratio of *R/S* Plasma Concentrations in Rats After i.v. Administration of 20 mg/kg Razoxane

	Animal 1	Animal 2	Animal 3	Animal 4
Time (min)	*R/S* ratio	*R/S* ratio	*R/S* ratio	*R/S* ratio
10	1.23	1.10	1.09	1.09
20	1.28	1.21	1.16	1.25
30	1.41	1.24	1.16	1.20
40	1.28	1.33	1.17	1.30
60	1.59	1.30	1.28	1.42

	Animal 5	Animal 6	Animal 7	Animal 8
Time (min)	*R/S* ratio	*R/S* ratio	*R/S* ratio	*R/S* ratio
10	1.11	1.12	1.12	n/a
120	n/a	1.56	1.39	1.38
150	1.59	1.50	1.41	1.46

n/a: sample not available for analysis

Table 2.8. Average Ratio of *R*/*S* Plasma Concentrations in Rats After i.v. Administration of 20 mg/kg Razoxane

Time (min)	Average of *R*/*S* ratios	SEM	Sample Size *n*
10	1.12	0.02	7
20	1.23	0.02	4
30	1.25	0.05	4
40	1.27	0.04	4
60	1.40	0.07	4
120	1.44	0.06	3
150	1.49	0.04	3

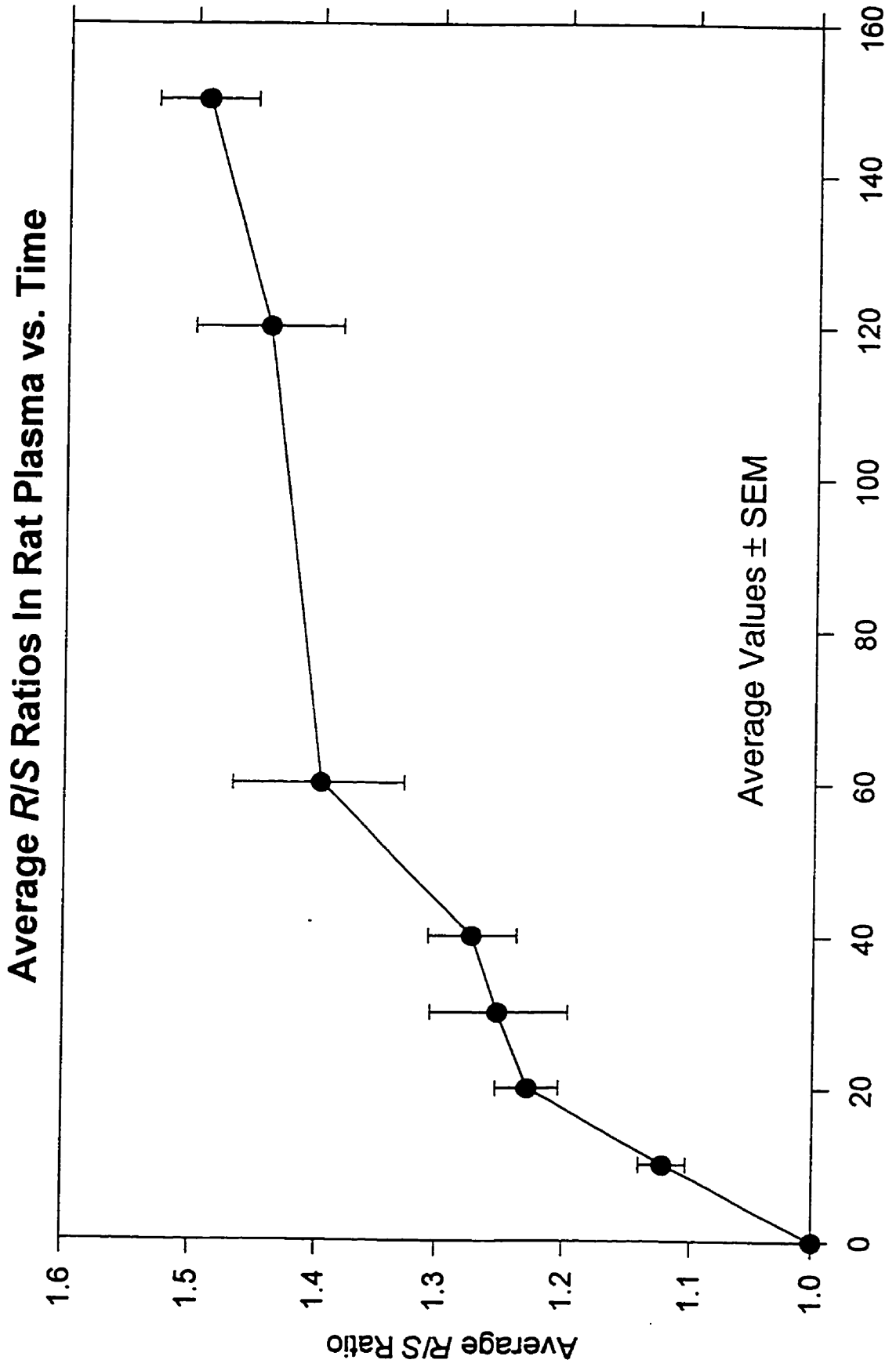

Average R/S Ratios In Rat Plasma vs. Time
Average R/S Ratio
1.0
1.1
1.2
1.3
1.4
1.5
1.6
0
20
40
60
80
100
120
140
160
Time (min)
Average Values ± SEM

Figure 2.14

Statistical analysis of the R/S ratios was performed using SigmaStat. The values were divided into 2 groups with the first set of animals sampled at 10, 20, 30, 40 and 60 min. The second set had timepoints at 10, 120 and 150 min.

A one-way repeated measured analysis of variance (ANOVA) was used to analyze group 1 of animals and the results are listed in Table 2.9. The difference in the mean R/S ratios among the 10, 20, 30, 40 and 60 min timepoints is larger than would be expected by chance. To isolate the groups that differ from the others, a multiple comparion procedure was used and the results are listed in Table 2.10. A significant difference was found between the R/S ratios for the 10 and 40, 10 and 60, 20 and 60, and the 30 and 60 min timepoints. The difference in the R/S ratios for the 10 and 20, 10 and 30, 20 and 30, 20 and 40, 30 and 40, and the 40 and 60 min timepoints was not large enough to rule out the possibility that it was due to chance.

For the second set of animals, a sample from one animal was not collected at 10 min and a sample from a different animal was not collected at 120 min, due to problems in the sampling procedure. A comparison could not be made between the 10 and 120 min timepoints. Only the 10 and 150 min timepoints could be compared. Since only 2 groups were compared, a paired t-test was used rather than an ANOVA to analyze the mean R/S values and the results listed in Table 2.11. The mean values were significantly different than would be expected by chance. The difference between means of the two groups was statistically significant with $p = 0.019$ and 2 degrees of freedom.

The p-value is the probability of being wrong in concluding that there is a true difference in the 2 groups. In this study, $p < 0.05$, which means 1 assertion in 20 trials will be incorrect. The degrees of freedom reflects sample size and is usually n-1, where n is the sample size.

Table 2.9. Analysis of Variance for R/S ratios Between 10 min and Other Timepoints for Animals in Group 1 of the Rat Study

Source of Variance	SS	DF	MS	F	P
Between Subjects	0.0871	3.00	0.02902		
Between Treatments	0.1518	4.00	0.03796	10.6	0.000647
Residual	0.0429	12.00	0.00357		
Total	0.2818	19.00			

Where: SS = Sum of Squares
DF = Degrees of Freedom
MS = Mean of Squares
F = F Ratio (MS_B/MS_W)
P = Probability

Table 2.10. Pairwise Multiple Comparison Procedure of Timepoints for Animals in Group 1 of the Rat Study

Comparison	t-value	$p < 0.05$
10 min vs. 20 min	2.406	no
10 min vs. 30 min	2.954	no
10 min vs. 40 min	3.452	yes
10 min vs. 60 min	6.421	yes
20 min vs. 30 min	0.548	no
20 min vs. 40 min	1.045	no
20 min vs. 60 min	4.015	yes
30 min vs. 40 min	0.497	no
30 min vs. 60 min	3.467	yes
40 min vs. 60 min	2.969	no

Table 2.11. Paired t-test for R/S ratios Between 10 min and 150 min for Animals in Group 2 of the Rat Study

Comparison	p-value	d.f.
10 min vs. 150 min (Group 2)	0.0161	2

p = probability
d.f. = degrees of freedom

2.2.3 Incubation of Razoxane in Plasma and Rat Liver Homogenate Supernatant Fraction

Razoxane was also incubated in rat plasma and rat liver homogenate supernatant at 37°C and pH 7.4. Aliquots were removed for analysis at periodic intervals.

For razoxane incubated in rat plasma at 37°C and pH 7.4, the dexrazoxane and levrazoxane concentrations are listed in Table 2.12. The concentration for both enantiomers declined continually from the 10 to 60 min timepoints. There were large decreases in dexrazoxane and levrazoxane concentrations at the 150 min timepoint. The *R/S* ratios decreased slightly throughout the study and are listed in Table 2.13.

Razoxane was also incubated in a rat liver supernatant extract containing 2 mg/mL of protein in pH 7.4 Tris buffer at 37°C. The dexrazoxane and levrazoxane concentration are listed in Table 2.14. A large decrease in concentration of both enantiomers was observed at the 30, 60 and 150 min timepoints. A plot of the concentrations of dexrazoxane and levrazoxane for razoxane incubated in plasma and in rat liver supernatant extract against time is illustrated in figure 2.15.

The ratios of the dexrazoxane and levrazoxane concentrations, or *R/S* ratios were listed in Table 2.15. These *R/S* ratios also changed during the study with a value of 1.07 at 10 min and a peak value of 1.95 at 150 min. A plot of the *R/S* ratios for razoxane incubated in plasma and in rat liver supernatant extract against time is shown in figure 2.16. The slopes of the two plots were compared using SigmaStat to conduct an unpaired two-tailed *t*-test. The calculations performed by the program are listed in Table 2.16. The two slopes were found to be statistically different using a *p*-value of 0.05.

Razoxane was also added to rat liver homogenate supernatant containing 115 μM of the DHPase inhibitor 4-chlorobenzenesulfonamide. The mixture was incubated for 150 min at 37°C. The concentration of dexrazoxane and levrazoxane is listed in Table 2.17. The *R/S* ratio was calculated and listed in Table 2.18.

Table 2.12. Incubation of Razoxane in Rat Plasma at 37°C and pH 7.4

Time (min)	Levrazoxane (μg/mL) Trial 1	Levrazoxane (μg/mL) Trial 2	Levrazoxane (μg/mL) Average
10	41.19	41.97	41.58
30	40.17	40.81	40.49
60	37.21	41.36	39.28
150	31.86	32.89	32.38

Time (min)	Dexrazoxane (μg/mL) Trial 1	Dexrazoxane (μg/mL) Trial 2	Dexrazoxane (μg/mL) Average
10	40.14	40.56	40.35
30	40.02	40.15	40.04
60	37.05	41.16	39.10
150	32.10	33.41	32.76

Table 2.13. *R/S* ratios of Razoxane Incubated In Rat Plasma

Time (min)	*R/S* ratios Trial 1	*R/S* ratios Trial 2	Average of *R/S* ratios
10	1.03	1.03	1.03
30	1.00	1.02	1.01
60	1.00	1.00	1.00
150	0.99	0.98	0.99

Table 2.14. Incubation of Razoxane in Rat Liver Homogenate Supernatant (2 mg protein/mL) at 37°C and pH 7.4

Time (min)	Levrazoxane (µg/mL) Trial 1	Levrazoxane (µg/mL) Trial 2	Levrazoxane (µg/mL) Average
10	44.16	45.31	44.74
30	35.04	35.98	35.51
60	30.04	31.99	31.02
150	27.81	27.40	27.61

Time (min)	Dexrazoxane (µg/mL) Trial 1	Dexrazoxane (µg/mL) Trial 2	Dexrazoxane (µg/mL) Average
10	41.28	43.32	42.30
30	29.64	30.10	29.87
60	22.62	22.76	22.69
150	14.00	14.11	14.05

Table 2.15. R/S ratios Incubated in Rat Liver Homogenate Supernatant

Time (min)	*R/S* ratios trial 1	*R/S* ratios trial 2	average *R/S* ratios
10	1.10	1.03	1.07
30	1.21	1.18	1.20
60	1.41	1.36	1.39
150	1.96	1.96	1.96

Table 2.16. *t*-test to Compare Slopes of *R/S* Ratios Between Razoxane Incubated in Rat Plasma and Rat Liver Homogenate Supernatant

Slope Of *R/S* Ratios: Rat Plasma	Slope Of *R/S* Ratios: Rat Liver Homogenate Supernatant	*t* value	*p* value	d.f.
-0.0003 min^{-1}	0.0064 min^{-1}	81.6	0.05	4

*d.f. = degrees of freedom

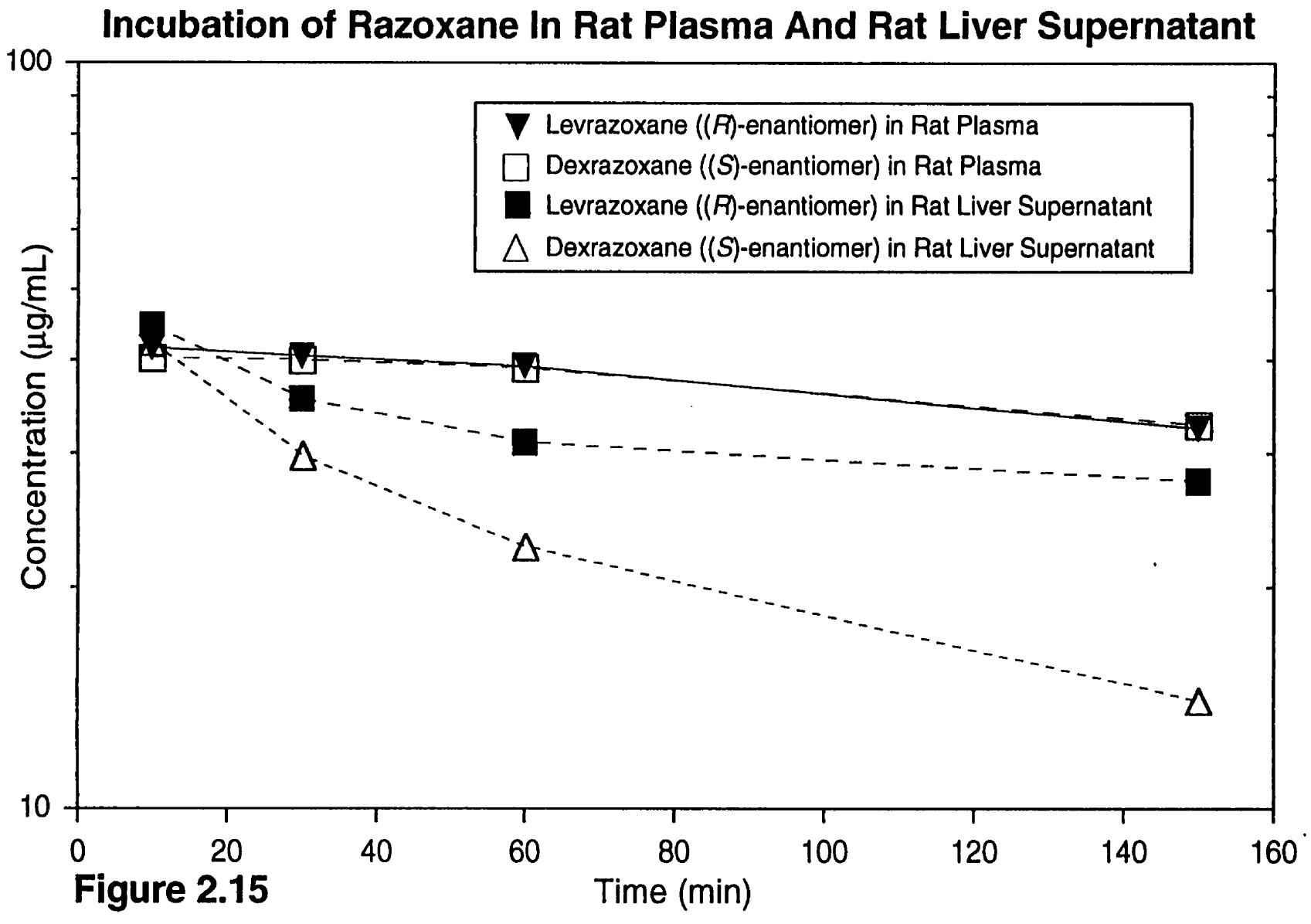

Incubation of Razoxane In Rat Plasma And Rat Liver Supernatant
Levrazoxane ((R)-enantiomer) in Rat Plasma
Dexrazoxane ((S)-enantiomer) in Rat Plasma
Levrazoxane ((R)-enantiomer) in Rat Liver Supernatant
Dexrazoxane ((S)-enantiomer) in Rat Liver Supernatant
Concentration (µg/mL)
100
10
0
20
40
60
80
100
120
140
160
Time (min)

Figure 2.15

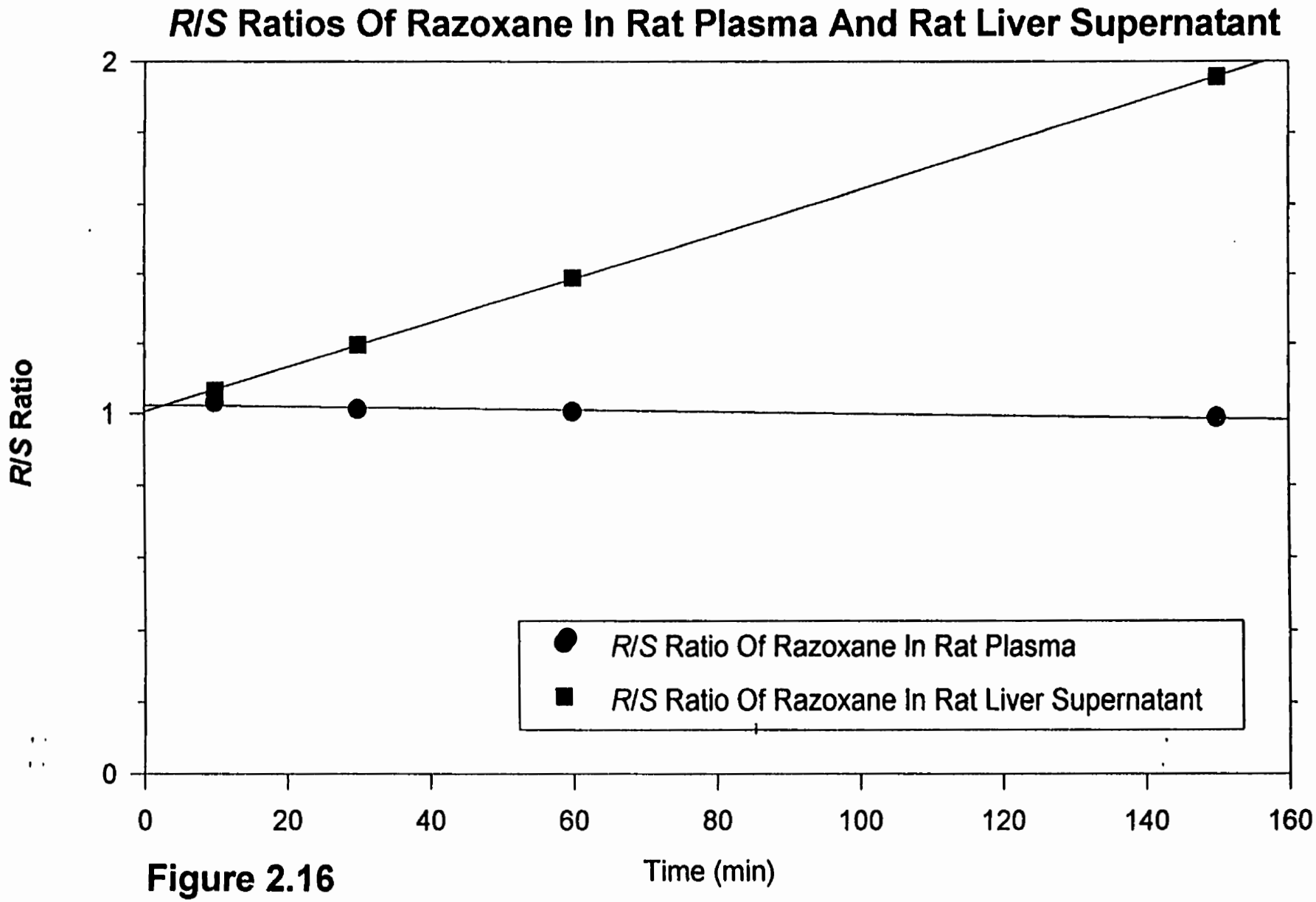

R/S Ratios Of Razoxane In Rat Plasma And Rat Liver Supernatant
R/S Ratio
2
1
0
0
20
40
60
80
100
120
140
160
Time (min)
R/S Ratio Of Razoxane In Rat Plasma
R/S Ratio Of Razoxane In Rat Liver Supernatant

Figure 2.16

Table 2.17. Incubation of Razoxane in Rat Liver Homogenate Supernatant (2 mg protein/mL) at 37°C and pH 7.4 Containing 115 μM 4-chlorobenzenesulfonamide

Time (min)	Levrazoxane (μg/mL) Trial 1	Levrazoxane (μg/mL) Trial 2	Levrazoxane (μg/mL) Average
10	44.16	45.31	44.74
150	30.59	31.61	31.10

Time (min)	Dexrazoxane (μg/mL) Trial 1	Dexrazoxane (μg/mL) Trial 2	Dexrazoxane (μg/mL) Average
10	41.28	43.32	42.30
150	27.82	29.34	28.58

Table 2.18. *R/S* ratios of Razoxane Incubated in Rat Liver Homogenate Supernatant at 37°C and pH 7.4 Containing 115 μM 4-chlorobenzenesulfonamide

Time (min)	*R/S* ratios trial 1	*R/S* ratios trial 2	Average *R/S* ratios
10	1.10	1.03	1.07
150	1.10	1.08	1.09

2.3 DISCUSSION

2.3.1 HPLC Chiral Analysis of Razoxane

The addition of the chiral selector ß-cyclodextrin to the mobile phase did not result in resolution of the enantiomers of razoxane. Various concentrations of ß-cyclodextrin in the mobile phase showed no enantioselectivity. For all concentrations tried, the retention time for the observed peak was much shorter than for razoxane with no ß-cyclodextrin in the mobile phase. Szepesi and Gazdag (1988) noted that changes occurred once the drug enantiomer binds with the cyclodextrin molecule. The polarity of the complex is much higher than that of the drug enantiomer alone, resulting in decreased retention times. This suggests that the ß-cyclodextrin did form a complex with razoxane but wasn't able to discriminate between enantiomers. In a study by Sybilska *et al.* (1986) it was found that there were specific structural requirements for chiral recognition between enantiomers and ß-cyclodextrin. Enantiomers of mephenytoin and various barbiturates were analyzed with ß-cyclodextrin in the mobile phase. Resolution of the enantiomers of mephenytoin, methylphenobarbital and hexobarbital was achieved. These drugs all had the chiral carbon in a ring system. The barbiturates enantiomers with the chiral carbon in a side chain were not resolved, pentobarbital, secobarbital, and thiopental. The chiral carbon in dexrazoxane and levrazoxane is in the chain that links the two rings together and too far away from the site of interaction.

A chiral cellulose tris(3,5-dimethylphenylcarbamate) column was able to separate dexrazoxane from levrazoxane. The hydroxyl groups present in the cellulose were substituted with non-polar 3,5-dimethylphenylcarbamate functional groups. This increases the interaction between the stationary phase and nonpolar compounds.

Standard solutions of dexrazoxane and levrazoxane dissolved in ethanol were analyzed to determine which enantiomer eluted first. Levrazoxane eluted first on the chiral cellulose column and was followed by dexrazoxane. The dexrazoxane sample was found to contain a small amount of levrazoxane, less than 0.5%. No dexrazoxane contamination was found in the levrazoxane sample.

The composition of the mobile phase used for the chiral column was changed in order to alter the resolution between the 2 enantiomers. Increasing the amount of hexane and decreasing the proportion of ethanol present in the mobile phase increased the resolution or R value. A mobile phase of 90/10 (v/v) ethanol/hexane resulted in the enantiomer peaks co-eluting closely together. With a mobile phase of 85/15 (v/v) ethanol/hexane, baseline separation with a resolution value R (R-value) of 1.5 was observed. The other mobile phases with reduced ethanol composition increased the resolution, resulting in larger R values. The distances between the peaks increased greatly. However, while the resolution increased as the hexane concentration increased, the overall analysis time became longer. The peak shape of the enantiomers also degraded, with much tailing. A compromise between resolution and analysis time resulted in using the mobile phase containing 85/15 (v/v) ethanol/hexane for analysis of all the animal study plasma samples and the plasma calibration samples. This mobile phase gave good peak shape while still providing close to baseline separation of the two enantiomers. The enantiomers eluted at retention times away from any potentially interfering peaks present in the plasma.

A small amount of tailing was observed in the peaks of both dexrazoxane and levrazoxane. Addition of diethylamine to the mobile phase can improve peak symmetry and separation (Krstulovic *et al.*, 1988).

Generally a concentration of 0.1% (v/v) diethylamine in the mobile phase is used. Addition of both 0.1 and 0.125% (v/v) diethylamine did not reduce tailing. However, diethylamine dramatically increased the background absorption of the mobile phase. The addition of diethylamine produced a very strong ultraviolet absorption at the detection wavelength of 207 nm. For these reasons, diethylamine was not used in the mobile phase.

2.3.2 Calibration Plot of Dexrazoxane and Levrazoxane

Retention times of levrazoxane and dexrazoxane were 14.7 and 16.5 min respectively (refer to fig. 2.2 and 2.3) for the mobile phase of reagent alcohol/hexanes (85/15 v/v). There were no detectable interfering peaks from the plasma. The drug peak area calibration plots (refer to fig. 2.4) were highly linear over the dexrazoxane and levrazoxane concentration range from 5.0 to 100 μg/mL.

2.3.3 Rat Study

The plasma concentrations of levrazoxane and dexrazoxane are listed in Table 2.4. The average plasma concentrations of levrazoxane and dexrazoxane are listed in Table 2.5. Peak concentration of razoxane occurred at the first timepoint sampled (10 min) with 27.97 μg/mL for levrazoxane and 25.40 μg/mL for dexrazoxane. At the last timepoint of 150 min, the levrazoxane concentration was 3.03 μg/mL. The dexrazoxane concentration was 2.00 μg/mL.

Paired one-tailed *t*-tests were used to analyze the dexrazoxane and levrazoxane plasma concentrations at each timepoint. The results are listed in Table 2.6. One-tailed *t* tests are used to determine a difference in one direction. In this case, the question is whether levrazoxane plasma concentrations are significantly larger than dexrazoxane plasma concentrations. Two-tailed *t*-tests would be used to determine if there is a difference in two directions such

as whether levrazoxane plasma concentrations are significantly larger or smaller than dexrazoxane plasma concentrations (Kuzma, 1992). There was a significant difference between the dexrazoxane and levrazoxane plasma concentrations at 10, 20, 30, 40, 60 and 120 min. At 150 min, the difference between the dexrazoxane and levrazoxane plasma concentrations was not large enough to rule out chance. The standard error for the dexrazoxane and levrazoxane plasma concentrations at 150 min was relatively high compared to the other timepoints. This may explain why the *t*-test could not rule out chance at this timepoint.

The pharmacokinetic parameters were not evaluated for levrazoxane or dexrazoxane. This study was designed to examine if there is stereoselective metabolism between levrazoxane or dexrazoxane. Due to the lack of sensitivity of the HPLC assay, large blood volumes were required at longer timepoints, so the total number of samples was limited. The large number of timepoints required for accurate determination of the pharmacokinetic parameters was not possible due to constraints of the study.

The ratios of the plasma concentrations of levrazoxane and dexrazoxane or the R/S ratios for each animal are listed in Table 2.7. The average R/S ratios are in Table 2.8. The R/S ratio is an indicator of any difference between the plasma concentrations of dexrazoxane and levrazoxane at each timepoint. It is an accurate method to determine differences in metabolism of enantiomers. The R/S ratio increased over time, from 1.12 at 10 min to a maximum value of 1.49 at 150 min. Statistical analysis of the ratios was performed using the program SigmaStat. The R/S ratio values were divided into 2 groups. The first set of animals was sampled at 10, 20, 30, 40 and 60 min. The second set had timepoints at 10, 120 and 150 min.

A one-way repeated measured analysis of variance (ANOVA) was used to analyze group 1 of the animals and the results are listed in Table 2.9. An ANOVA is used to compare the means of more than 2 groups at once (Rosner, 1995).

The difference in the mean *R/S* ratios among the 10, 20, 30, 40 and 60 min timepoints is larger than would be expected by chance. The one-way ANOVA can detect when at least 2 different groups have different means (Rosner, 1995). A multiple comparison procedure was used to isolate the groups that differ from the others and the results are listed in Table 2.10. The multiple comparion procedure also helps to reduce falsely significant differences to be declared. If more than 2 means are being compared, the multiple comparison method is more strict than ordinary *t*-tests (Rosner, 1995). A significant difference between the *R/S* ratios was shown for the 10 and 40, 10 and 60, 20 and 60, and the 30 and 60 min timepoints. The difference in the *R/S* ratios for the 10 and 20, 10 and 30, 20 and 30, 20 and 40, 30 and 40, and the 40 and 60 min timepoints was not large enough to rule out the possibility that it was due to chance.

For the second set of animals, a paired *t*-test also showed a statistically significant difference between the mean *R/S* ratios at 10 and 150 min. The results of the *t*-test are listed in Table 2.11. A paired *t*-test could not be performed for the 10 min and 120 min timepoints due to problems in the sampling procedure. Using a *p*-value of 0.05, the mean values were found to be significantly different than would be expected by chance. The differences in dexrazoxane and levrazoxane metabolism are similar to previous *in vitro* studies.

A series of experiments by Hasinoff *et al.*, (1991) strongly suggested that DHPase was responsible for the enzymatic hydrolysis of dexrazoxane.

Dexrazoxane was incubated in 105,000 *g* soluble supernatant fractions of either porcine liver, kidney or heart homogenates. Little hydrolysis of dexrazoxane was observed for the heart homogenates. The liver and kidney homogenates hydrolysis rates were 1.2, and 1.4 nmol (mg protein)$^{-1}$ min^{-1}, respectively. Addition of 4-chlorobenzenesulfonamide to liver and kidney homogenates resulted in complete inhibition of hydrolysis of dexrazoxane. The compound 4-chlorobenzenesulfonamide has been shown to be a strong inhibitor of dihydropyrimidine amidohydrolase (DHPase) at a concentration of 500 μM (Brooks *et al.*, 1983). This suggests that this enzyme is responsible for dexrazoxane hydrolysis in rat liver and kidney homogenate supernatant.

The V_{max} for incubation of dexrazoxane in various concentrations of purified bovine liver DHPase is similar to the V_{max} for the enzyme's natural substrates dihydrouracil and dihydrothymine (Hasinoff, 1993). It was also found that DHPase acts to open the rings of dexrazoxane and levrazoxane at different rates. DHPase hydrolyses dexrazoxane to its one ring-opened form 4 times faster than for the hydrolysis of levrazoxane. It was found that DHPase does not hydrolyze the one ring-opened forms of dexrazoxane and levrazoxane. Opening of the second ring is probably by base-catalyzed hydrolysis (Hasinoff, 1993).

Another study by Hasinoff (1994b) follows various concentrations of dexrazoxane, levrazoxane and razoxane in a solution of purified bovine liver DHPase at a protein concentration of 18 μg/mL and at 37°C and pH 7.4. The concentration of drugs used is from 0.1 to 0.6 mM, which is substantially higher than plasma concentrations found in rats used for this razoxane metabolism study. DHPase hydrolyzes dexrazoxane 4.9 times faster than levrazoxane. Dexrazoxane is hydrolyzed by DHPase 1.4 times faster than razoxane. The hydrolysis activity of DHPase toward razoxane was

intermediate between dexrazoxane and levrazoxane. This was predicted as razoxane is a racemic drug, composed of equal proportions of dexrazoxane and levrazoxane.

DHPase has been shown to be stereoselective in the ring-opening hydrolysis of different compounds. DHPase can only open the (*R*)-enantiomers of 5-isopropylhydantoin (Dudley and Roberts, 1978), 5-methylhydantoin (Dudley and Roberts, 1978), and 5-phenylhydantoin (Dudley *et al.*, 1974). The substrate for DHPase must meet a fairly specific steric configuration as DHPase can differentiate between molecules which are identical except for their stereochemistry or physical orientation.

The hydrolysis of dexrazoxane and levrazoxane in a isolated rat hepatocyte suspension at pH 7.4 and 37°C was followed (Hasinoff *et al.*, 1994). The parent drugs and the one-ringed open intermediates were analyzed by a non-chiral HPLC method. Each enantiomer was incubated separately in the hepatocyte mixture. Dexrazoxane was metabolized by the isolated hepatocytes 1.8 times faster than for levrazoxane. This ratio corresponds to a similar value found for purified DHPase. Addition of the strong DHPase inhibitor 4-chlorobenzenesulfonic acid resulted in an 82% inhibition of the loss of dexrazoxane. This suggests that DHPase present in hepatocytes catalyzed the ring-opening hydrolysis of dexrazoxane and levrazoxane. The ratios of the rates of formation of the one-ringed intermediates of dexrazoxane and levrazoxane in the hepatocyte suspension were also consistent with DHPase being primarily responsible for metabolism of these drugs.

2.3.4 Incubation of Razoxane in Plasma and Rat Liver Supernatant

In order to determine if other factors are responsible for the *in vivo* differences in razoxane metabolism, two *in vitro* studies were also conducted. Razoxane was incubated in rat plasma at 37°C and pH 7.4. The concentration

timepoints. There was a 20% loss of razoxane at the 150 min timepoint, presumably due to base-catalyzed hydrolysis caused by elevated pH and temperature. No change occurred in the *R*/S ratios of the concentrations of levrazoxane and dexrazoxane throughout the study. This would suggest that the enantiomers of razoxane were stable in rat plasma and did not racemize. Also, no components present in rat plasma appear to be involved in stereoselective metabolism. The chiral carbon in dexrazoxane is in an alkane chain which connects the two rings of razoxane. Racemization of this type of chemical structure is not favoured. Researchers at the University of Muenster found that each of the enantiomers of thalidomide racemized in human and rabbit plasma incubated at 37°C (Knoche and Blaschke, 1994). The *S*-enantiomer appeared to racemize at a faster rate than the *R*-enantiomer. There appears to be no significant species difference in the racemizations rates. It was later shown that serum albumin was responsible for the racemization process of thalidomide in plasma.

In the second *in vitro* study, razoxane was incubated in a rat liver homogenate supernatant containing 2 mg/mL of protein in pH 7.4 Tris buffer at 37°C. A large decrease in concentration of both enantiomers was observed at the 30, 60 and 150 min timepoints. The *R*/*S* ratios of razoxane also changed during the study with a value of 1.07 at 10 min and a peak value of 1.96 at 150 min. With the program SigmaStat, a two-tailed *t*-test was used to compare the slopes of the *R*/*S* ratios for razoxane in rat plasma and the rat liver homogenate supernatant vs. time. The *p*-value is selected by the program user, the program then calculates the *t*-value and the degrees of freedom. The slopes were were found to be statistically different. There was change in the *R*/S ratios of razoxane in rat liver supernatant while the ratios remained relatively constant for razoxane in plasma.

The slope for the plot of *R*/S ratios of razoxane in rat liver supernatant vs. time was linear with a r^2 value of 0.99995. The Michaelis-Menten equation (Garrett and Graham, 1995) is generally used to describe enzyme kinetics where:

$$v = V_{max} * [S]/(K_m + [S])$$

with: v is the initial velocity

[S] is the substrate concentration

K_m is the Michaelis constant

The concentrations of dexrazoxane and levrazoxane in the rat liver homogenate supernatant were relatively low, with [S] << K_m so the equation now reduces to:

$$v = V_{max} * [S]/(K_m)$$

The integrated rate equation, listed below, predicts an exponential decrease in [S] that is different for each enantiomer. The ratio ([*R*]/[*S*]) is not predicted to be linear.

$$k_R = V_{max}{}^R/K_m{}^R \quad [1]$$

$$k_S = V_{max}{}^S/K_m{}^S \quad [2]$$

$$[S] = [S]_o e^{-k_S t} \quad [3]$$

$$[R] = [R]_o e^{-k_R t} \quad [4]$$

$$\frac{[R]}{[S]} = \frac{[R]_o e^{-k_R t}}{[S]_o e^{-k_S t}} \quad [5]$$

$$\frac{[R]_o}{[S]_o} = 1 \quad \text{at } t = 0 \quad [6]$$

$$\frac{[R]}{[S]} = e^{k_S t - k_R t} = e^{(k_S - k_R)t} \qquad [7]$$

$$\frac{[R]}{[S]} = e^{(\frac{V_{max}{}^S}{K_m{}^S} - \frac{V_{max}{}^R}{K_m{}^R})t} \qquad [8]$$

If the term $\frac{V_{max}{}^S}{K_m{}^S}$ is close to $\frac{V_{max}{}^R}{K_m{}^R}$ then the exponential term is smaller than the equation for either [*R*] or [*S*]. In this case equation [8] predicts that the ([*R*]/[*S*]) ratio is more linear than for either [*R*] or [*S*] alone at times where substantial reaction has occured.

Razoxane was also added to rat liver homogenate supernatant containing 115 μM of the strong DHPase inhibitor 4-chlorobenzenesulfonamide. Although some hydrolysis of the enantiomers of razoxane occurred, the *R*/*S* ratio of 1.09 at 150 min was substantially less than in the rat liver homogenate supernatant without the inhibitor. This would suggest that the agent responsible for stereoselective hydrolysis of razoxane is DHPase and is present in the rat liver homogenate supernatant. These findings agree with previous results published in the literature (Hasinoff, 1991; Hasinoff, 1993).

The difference in the final *R*/*S* ratio between the animal study and the rat liver extract incubation study is probably due to the differences in how long it takes for the razoxane enantiomers to reach DHPase in the liver and kidney. In the animal study, once razoxane enters the body, the drugs require time to become distributed in the bloodstream to the liver and heart. Once the drugs are at these organs, they must cross the cell membrane before they can be hydrolyzed by DHPase. In the rat liver homogenate supernatant incubation study, DHPase is immediately available to the razoxane enantiomers.

In the animal study, the plasma concentration of levrazoxane is higher than for dexrazoxane at all timepoints. In the animal study, at 150 min, the plasma concentration of levrazoxane is 50% higher than for dexrazoxane. This suggests that dexrazoxane is metabolized to its active form much faster; so the plasma concentrations would be lower than for levrazoxane at the same timepoint. If dexrazoxane is metabolized to its active form faster than levrazoxane in plasma, then less dexrazoxane would be available to reach the heart and cross the cell membranes into the heart cells to provide cardioprotection. If the hydrolysis intermediates of the drugs are unable to cross cell membranes, the faster metabolism of dexrazoxane in plasma could reduce its cardioprotective abilities as compared to levrazoxane.

The conclusions of the animal study are that the enzyme DHPase is responsible for hydrolysis of razoxane. Also, the plasma concentration of dexrazoxane declined much faster than the plasma concentration of levrazoxane, indicating enzymatic hydrolysis of razoxane *in vivo* is stereoselective.

Chapter 3

Attempted Development of a Fluorescent Derivative for Dexrazoxane and its Hydrolysis Intermediates

3.0 Introduction

A new HPLC method was developed to simultaneously determine the plasma concentrations of dexrazoxane and its hydrolysis intermediate products. Previous HPLC methods for the analysis of dexrazoxane generally used UV detection (Collins *et al.*, 1983, Jakobsen *et al.*, 1994) or electrochemical detection (Lewis *et al.*, 1992). The UV detection methods usually reported low sensitivity due lack of a strong chromophore in dexrazoxane. Electrochemical detection gave good detection limits, but required the use of specialized equipment not commonly found in research laboratories. No HPLC method has been published for the analysis of dexrazoxane hydrolysis intermediate products in plasma samples.

Sisco (1989) evaluated the suitability of 6 derivatives for the derivatization of dexrazoxane. They included the UV active reagents *o*-, *m*- and *p*- nitrobenzyl bromides, phenacyl bromide and the fluorescent reagents 4-bromomethyl-7-methoxycoumarin and 2-bromomethyl-4,5-diphenyloxazole. Dexrazoxane was derivatized by all six reagents, but only the *p*-nitrobenzyl bromide and 2-bromomethyl-4,5-diphenyloxazole produced stable derivatives. The oxazole derivative was approximately an order of magnitude more sensitive than the *p*-nitrobenzyl derivative.

The 2-bromomethyl-4,5-diphenyloxazole derivative is not commercially available. Aldous *et al.* (1960), developed a one-step reaction in which 2-methyl-4,5-diphenyloxazole was brominated by *N*-bromosuccinimide in the presence of a catalyst (see figure 3.1). The 2-methyl-4,5-diphenyloxazole is readily available from commercial sources (Aldrich Chemical Co, Milwaukee, WI).

Sisco developed a method suitable to derivatize 2-bromomethyl-4,5-diphenyloxazole with dexrazoxane extracted from plasma samples. Solid

phase extraction cartridges were used to separate dexrazoxane from plasma samples and the drug was eluted off the column with acetonitrile. The acetonitrile was evaporated to dryness, and a 1.0 mL aliquot containing 500 µg each of the oxazole and 18-crown-6-ether dissolved in acetonitrile was added to the dexrazoxane residue. Approximately 3 mg of anhydrous potassium carbonate was added to the mixture and stirred constantly at room temperature for 3 hours. The 18-crown-6-ether and potassium carbonate were added as catalysts to increase the reactivity and decrease the reaction time. A fluorescent bis-*N*-oxazole-dexrazoxane derivative formed with 2 oxazoles bonding to each dexrazoxane molecule. An overall view of the derivatization reaction is illustrated in figure 3.2.

The settings for the fluorescent detector were an excitation wavelength of 287 nm and an emission wavelength of 365 nm. Sisco (1989) reported a quantification limit of 10 ng/mL of dexrazoxane based on a signal to noise ration of 3:1. A plot of the peak area of the fluorescent dexrazoxane derivative was linear for the dexrazoxane concentration range of 10 ng/mL to 50 µg/mL.

In view of the success of derivatizing dexrazoxane with 2-bromomethyl-4,5-diphenyloxazole, an attempt was made to derivatize the one-ring opened hydrolysis intermediates of dexrazoxane. One oxazole molecule should be able to react with the imide nitrogen on the intact ring in the hydrolysis intermediates. The oxazole derivative was produced in our laboratory using a modified version of the method developed by Aldous *et al.* (1960). The 2-bromomethyl-4,5-diphenyloxazole was characterized by elemental analysis and nuclear magnetic resonance.

Pure dexrazoxane standards were dissolved in acetonitrile and reacted with 2-bromomethyl-4,5-diphenyloxazole under various conditions. After a linear calibration curve was prepared for the fluorescent oxazole-dexrazoxane

derivative, an attempt was made to derivatize the hydrolysis intermediates of dexrazoxane. However, due to the many side reactions occurring in the derivatization reaction mixture, many peaks were present in the HPLC chromatograms of the blank samples and hydrolysis intermediates. Modification of both the derivatization reaction conditions and the HPLC conditions were unable to resolve the fluorescent derivatives of the hydrolysis intermediates. After much experimentation, this approach of using 2-bromomethyl-4,5-diphenyloxazole to derivatize the hydrolysis intermediates of dexrazoxane was abandoned. This chapter outlines the attempts made to derivatize dexrazoxane and its hydrolysis intermediates with the fluorescent derivative 2-bromomethyl-4,5-diphenyloxazole.

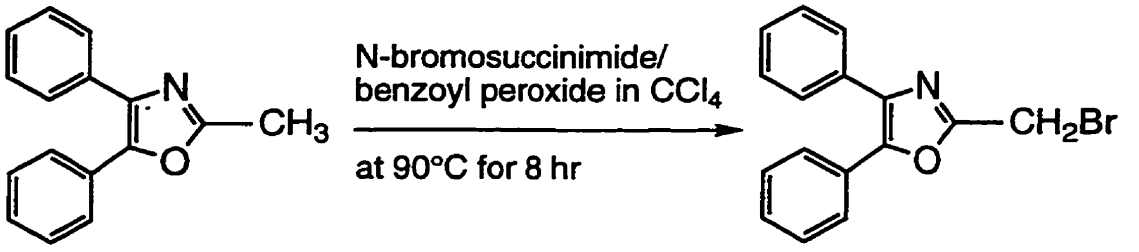


Figure 3.1 Preparation of 2-bromomethyl-4,5-diphenyloxazole by bromination of 4,5-diphenyloxazole with N-bromosuccinimide

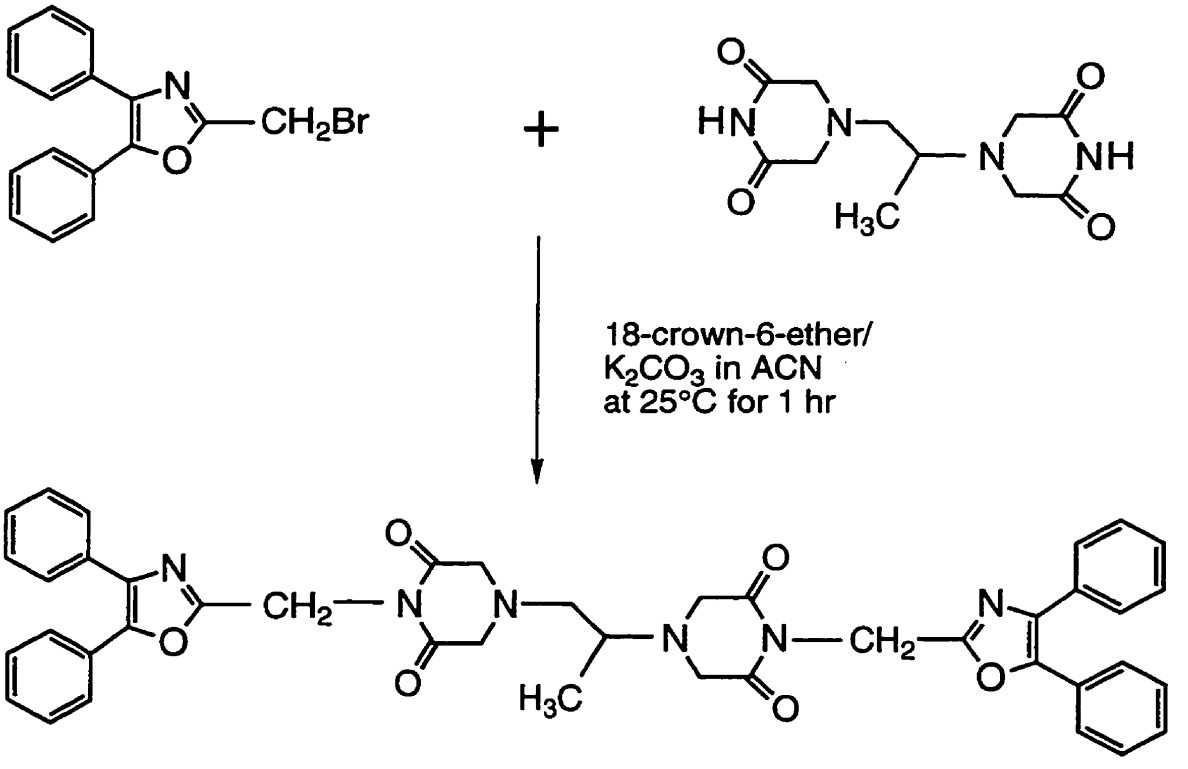


Figure 3.2 Derivatization of dexrazoxane with 2-bromomethyl-4,5-diphenyl oxazole

3.1 EXPERIMENTAL

3.1.1 Materials

3.1.1.1 Chemicals

Dexrazoxane (lot 91D23FY) was a gift from Pharmacia & Upjohn (Columbus, OH). Glacial acetic acid, reagent grade carbon tetrachloride, 4-8 mesh molecular sieves, concentrated phosphoric acid, potassium carbonate, potassium hydroxide, potassium dihydrogen phosphate, sodium acetate trihydrate, sodium hydroxide and washed sea sand were obtained from Fisher Scientific (Nepean, Canada). HPLC grade acetonitrile and methanol, volumetric grade hydrochloric acid and sodium hydroxide solutions were obtained from Mallinckrodt (Mississauga, Canada). Benzoyl peroxide, *N*-bromosuccinimide and 18-crown-6-ether were from Sigma Chemical Co. (St. Louis, MO). AnalaR Grade ethylenediaminetetraacetic acid disodium salt was from BDH Chemicals Ltd. (Poole, England). The 2-methyl-4,5-diphenyloxazole was obtained from Aldrich Chemical Co. (Milwaukee, WI).

3.1.1.2 Analysis of 2-Bromomethyl-4,5,-Diphenyloxazole

A 2-bromomethyl-4,5,-diphenyloxazole sample was sent to Guelph Chemical Laboratories Ltd. (Guelph, ON) for elemental analysis. A sample was also sent to the Department of Chemistry, University of Manitoba for nuclear magnetic resonance (NMR) analysis.

3.1.1.3 Instrumentation

3.1.1.3.1 Chromatographic

The HPLC system consisted of a Varian 9010 pump, Varian Star integration software, and a Rheodyne injector. The system was fitted with a Brownlee Labs NewGuard column containing an 1.5 cm X 3.2 mm I.D. reversed-phase C-18 guard cartridge (Applied Biosystems, Santa Clara, CA, USA). An μBondapak 4.6 mm I.D. X 250 mm reversed-phase C-18 column

with a particle size of 10 µm (Waters Chromatography, Mississauga, ON) was used for analysis.

For the HPLC quantitation of the sodium hydroxide hydrolysis products of dexrazoxane, a Varian 9050 programmable wavelength detector was used. A program was used with the detection wavelength at 205 nm for the first 10 minutes and then switched to 215 nm for the rest of the analysis. The sample loop volume was 20 µL.

For the analysis of the fluorescent derivatives of dexrazoxane, a Shimadzu RF551 fluorescence HPLC monitor was used. The excitation wavelength was 287 nm and the emission wavelength was 365 nm. The response time was 1.5 s, the range was set to X1 and the sensitivity was set to "low". The sample loop volume was 20 µL. For most analysis, peak areas were calculated automatically using the Varian Star integration software.

3.1.2 Methods

3.1.2.1 Preparation of the Fluorescent Derivative 2-Bromomethyl-4,5-Diphenyloxazole

The compound 2-bromomethyl-4,5-diphenyloxazole was prepared using a modification of a method by Aldous *et al.* (1960). In a 100 mL round bottom flask, 2.5 g of 4,5-diphenyl-2-methyloxazole, 0.211 g of *N*-bromosuccinimide and 0.05 g of benzoyl peroxide were mixed in 50 mL carbon tetrachloride. A drying tube was attached and the flask refluxed in an oil bath at 90°C for 8 hr.

The reaction mixture was allowed to cool briefly and then suction filtered to remove unreacted *N*-bromosuccinimide. The residue was washed with 2 mL of carbon tetrachloride. The filtrate was purified using flash chromatography (Still *et al.*, 1978).

The column used for flash chromatography was prepared by filling a 50

mm by 100 cm burette with 60 cm of 230-400 mesh silica gel. A 5 mm layer of washed sea sand was placed on top. The column was conditioned by filling the column with petroleum ether, sealing the burette with a glass stopper and applying gentle air pressure. The flow rate of the petroleum ether was approximately 100 mL/min.

The air pressure was turned off, the stopper removed and the reaction mixture was transferred to the column by glass pasteur pipette. The flask was rinsed with 2 mL of carbon tetrachloride and transferred to the column. The column was then filled with petroleum ether to 5 mm above the level of the sea sand layer. The column was then resealed, the air pressure turned on and the ether eluted off at a rate of 5 cm per min until it was 5 mm above the level of the sea sand layer. The air pressure was turned off, the stopper removed and the column was filled with 4.5% (v/v) ethyl acetate in petroleum ether to 5 mm above the level of the sea sand layer. The air pressure was turned on and 20 mL fractions were collected in 50 mL glass culture tubes. Twenty fractions were collected and every second fraction spotted onto a 5 cm x 10 cm silica gel (250 microns) thin layer chromatography (TLC) glass plate (Analtech, Inc. , Newark, DE). An aliquot from the reaction mixture was also spotted on the plate. The plate was then put into a 200 mL glass jar with a 12 cm high piece of filter paper around the inside. The jar contained a 1 cm layer of 4.5% (v/v) ethyl acetate in petroleum ether. The plate was removed once the solvent traveled up the plate and reached the top. The plate was removed and allowed to dry in a fume hood. The plate was developed in a 250 mL glass screw topped jar with a 1 cm layer of iodine crystals in the bottom. After a 5 min exposure, the plate was removed. Fractions that did not have spots that corresponded to the reaction mixture but had a new spot were collected. Fractions 7, 8, and 9 were combined and

then evaporated to a thick yellow oil in a rotary evaporator. The product was recrystallized in 10% (v/v) diethyl ether in petroleum ether. A sample was sent to Guelph Chemical Laboratories Ltd. for elemental analysis. A nuclear magnetic resonance (NMR) run was performed by the Department of Chemistry, University of Manitoba.

3.1.2.2 Preparation of Sodium Hydroxide Hydrolysis Intermediate Products of Dexrazoxane

Using a method developed by Hasinoff (1994b), 5 mg of dexrazoxane was added to a 10 mL glass culture tube and dissolved in 1.0 mL of double distilled water. An 80 µL aliquot of 1.0 M NaOH solution was added, the tube capped and the mixture incubated in a 25°C water bath for 40 min. The reaction was quenched by the addition of 80 µL of 1.0 M HCl, which preserved the stability of the intermediates. The sample was sealed with a disposable plastic cap and stored at -80°C until required for use.

3.1.2.3 Preparation of Potassium Phosphate Hydrolysis Intermediate Products of Dexrazoxane

Using a method developed by Hasinoff (1994b), 5 mg of dexrazoxane were added to a 10 mL glass culture tube and dissolved in 1.0 mL of 50 mM KH_2PO_4, pH 7.4. The KH_2PO_4 solution was titrated with 6 M KOH solution to pH 7.4. The mixture incubated in a 37°C water bath for 18 hours. The reaction was quenched by the addition of 36 µL of 1.0 M HCl, which preserved the stability of the intermediates. The sample was sealed with a disposable plastic cap and stored at -80°C until required for use.

3.1.2.4 HPLC Quantitation of the Hydrolysis Intermediates of Dexrazoxane

A modification of a method developed by Hasinoff (1994a) was used to quantify the levels of dexrazoxane, the intermediates **B**, **C** (fig. 1.2) and the active drug ADR-925 in the NaOH and KH_2PO_4 hydrolysis intermediates of

dexrazoxane. Samples from each stock hydrolysis solution were diluted 1:10 with 10 mM HCl before analysis.

The initial mobile phase was 100% 500 µM Na_2EDTA solution (pH 4.5). The pH of the Na_2EDTA solution was not adjusted. A linear gradient was used from 0 to 10 min to increase the amount of methanol from 0 to 8% (v/v). This mobile phase of Na_2EDTA/methanol (92/8 v/v) was maintained to the end of the run at 18 min. The column was then re-equilibrated at the initial mobile phase for 8 min.

A computer program was developed that converted the peak areas of dexrazoxane and its hydrolysis intermediates into concentrations. The calibration factors for dexrazoxane and ADR-925 were obtained from pure samples of these compounds. The calibration factors for **B** and **C** were obtained from a ^{1}H-NMR determination of the concentrations of a partially hydrolyzed dexrazoxane sample also simultaneously analyzed by HPLC (Hasinoff, 1994a). Aliquots of 30 µL were injected into the HPLC.

3.1.2.5 Preparation of the Fluorescent Derivatives of Dexrazoxane

3.1.2.5.1 Preparation of Dexrazoxane Solutions

A 1.0 mg/mL dexrazoxane stock solution was prepared by dissolving 1.0 mg of dexrazoxane and made up to 1.0 mL with 10 mM HCl in a volumetric container. The stock solution was transferred to a 1.5 mL microcentrifuge tube and stored at -80°C when not in use.

Dexrazoxane calibration standards were prepared ranging in concentration from 50, 100 and 500 ng/mL and 1, 5, and 10 µg/mL. Various aliquots of the stock solution were added to 5 mL volumetric flasks and made up with acetonitrile. The calibration standard solutions were transferred to 5 mL glass culture tubes, capped with plastic lids and stored at -80°C when not in use.

3.1.2.5.2 Preparation of Phosphate and Sodium Hydroxide Hydrolysis Intermediate Products of Dexrazoxane Solutions

Solutions of 1/5, 1/10, 1/50 and 1/100 dilutions were made of both the KH_2PO_4 and NaOH hydrolysis intermediate products. The 1/5 dilution of the NaOH hydrolysis intermediate products had 200 µg/mL each of intermediates **B** and **C** and dexrazoxane. The 1/10, 1/50 and 1/100 dilutions contained 100, 20 and 10 µg/mL respectively of intermediates **B** and **C** and dexrazoxane. The 1/5 dilution of the KH_2PO_4 hydrolysis sample contained 300 µg/mL of intermediate **B**, 100 µg/mL of intermediate **C** and 260 µg/mL of dexrazoxane. The 1/10 dilution contained 150 µg/mL of **B**, 50 µg/mL of **C** and 130 µg/mL of dexrazoxane. The 1/50 dilution contained 30 µg/mL of **B**, 10 µg/mL of **C** and 26 µg/mL of dexrazoxane. The 1/100 dilution contained 15 µg/mL of **B**, 5 µg/mL of **C** and 13 µg/mL of dexrazoxane.

A 400 µL aliquot of the intermediate was made up with acetonitrile in a 2.0 mL volumetric container for the 1/5 dilution sample. A 200 µL aliquot of the intermediate was made up with acetonitrile in a 2.0 mL volumetric container for the 1/10 dilution sample. The 1/50 dilution sample was prepared by taking a 100 µL aliquot of the intermediate and made up to 5.0 mL with acetonitrile. The 1/100 dilution sample was prepared by taking a 50 µL aliquot of the intermediate and made up to 5.0 mL with acetonitrile.

3.1.2.5.3 Preparation of Dry Acetonitrile

Excess water was removed from acetonitrile by stirring overnight over molecular sieves. About 20 g of molecular sieves were added to 250 mL of acetonitrile. The molecular sieves were dried at 80°C overnight and allowed to cool in a desiccator before being added to the acetonitrile. The acetonitrile and molecular sieves were allowed to stand undisturbed for 24 hr.

3.1.2.5.4 Preparation of 2-Bromomethyl-4,5-Diphenyloxazole Solution

Solutions of various concentrations of 2-bromomethyl-4,5-diphenyloxazole were prepared by dissolving 2-bromomethyl-4,5-diphenyloxazole in dried acetonitrile. Concentrations of 0.5, 1.0, 2.0 and 4.0 mg/mL were prepared. The solutions were kept tightly stoppered and stored at -80°C when not in use.

3.1.2.5.5 Preparation of 18-Crown-6-Ether Solution

Concentrations of 0.5, 1.0, 2.0 and 4.0 mg/mL 18-crown-6-ether solution were prepared in dried acetonitrile. The solutions were kept tightly stoppered and stored at -80°C when not in use.

3.1.2.5.6 Preparation of Potassium Carbonate

Potassium carbonate was ground in a mortar and pestle, dried overnight at 80°C and allowed to cool in a desiccator. The potassium carbonate was kept in a tightly stopped glass container and stored in a desiccator when not in use.

3.1.2.5.7 Derivatization of Dexrazoxane

Derivatization of dexrazoxane was based on a modified method by Sisco (1989). Aliquots of 1.0 mL dexrazoxane solution were transferred to 1.5 mL microcentrifuge tube and evaporated to dryness under a stream of argon in a 40°C water bath. Aliquots of 0.5 mL of 2-bromomethyl-4,5-diphenyloxazole and 0.5 mL of 18-crown-6-ether solution were added to the dried dexrazoxane residue. About 3 mg of potassium carbonate and a 1.5 X 8 mm stir bar were added to the mixture. The mixture was stirred for 60 min at room temperature. Blank samples were also prepared. No dexrazoxane was added to the microcentrifuge tubes, but the reagents were mixed together and stirred for 60 min at room temperature.

To find appropriate mobile phase conditions, samples containing 5

µg/mL of dexrazoxane were derivatized with 2.0 mg/mL solutions of 2-bromomethyl-4,5-diphenyloxazole and 18-crown-6-ether solution The total concentration of each reagent in the sample was 1.0 mg/mL.

For evaluation of the derivatization process, four sets of derivatization conditions were tried with samples containing 5 µg/mL of dexrazoxane. For set 1, the concentration of 2-bromomethyl-4,5-diphenyloxazole and 18-crown-6-ether in the samples was each 250 µg/mL. For set 2, the concentration of 2-bromomethyl-4,5-diphenyloxazole and 18-crown-6-ether in the samples was each 500 µg/mL. For set 3, the concentration of 2-bromomethyl-4,5-diphenyloxazole and 18-crown-6-ether in the samples was each 0.750 µg/mL. For set 4, the concentration of 2-bromomethyl-4,5-diphenyloxazole and 18-crown-6-ether was each 1.0 mg/mL. For preparation of the dexrazoxane calibration plot, the concentration of 2-bromomethyl-4,5-diphenyloxazole and 18-crown-6-ether was each 500 µg/mL.

The reaction was quenched by placing the samples in ice water for 5 min. Between analysis, the derivatized samples were stored at 4°C. For long term storage, the samples were stored at -80°C.

3.1.2.5.8 Attempted Derivatization of Dexrazoxane Hydrolysis Intermediate Products

The attempted derivatization of dexrazoxane hydrolysis intermediates was based on a modification of a method by Sisco (1989). Aliquots of 1.0 mL dexrazoxane hydrolysis intermediates in acetonitrile were transferred to 1.5 mL microcentrifuge tubes and evaporated to dryness under a stream of argon in a 40°C water bath.

Aliquots of 0.5 mL of 2-bromomethyl-4,5-diphenyloxazole and 0.5 mL of 18-crown-6-ether solution were added to the dried dexrazoxane residue. About 3 mg of potassium carbonate and a 1.5 X 8 mm stir bar were added to

the mixture. The mixture was stirred at room temperature.

Three sets of derivatization conditions were tried. For set 1, the concentration of 2-bromomethyl-4,5-diphenyloxazole and 18-crown-6-ether solution was each 2.0 mg/mL. The stirring time was 60 min. For set 2, the concentrations of the derivatizing reagents were kept at 2.0 mg/mL but the stirring time was increased to 150 min. For set 3, the concentrations of the derivatizing reagents were changed to 4.0 mg /mL. The stirring time remained at 150 min. The reaction was quenched by placing the samples in ice water for 5 min. Between analysis, the derivatized samples were stored at 4°C. For long term storage, the samples were stored at -80°C.

3.1.2.6 HPLC Method for the Fluorescent Derivatives of Dexrazoxane

3.1.2.6.1 Preparation of Sodium Acetate Buffer

Sodium acetate buffer was made by dissolving 3.4 g of sodium acetate trihydrate in 1 L of water. The pH was adjusted to 5.0 with glacial acetic acid.

3.1.2.6.2 HPLC Conditions for the Analysis of the Fluorescent Derivative of Dexrazoxane

For gradient 1, the flow rate was 2.5 mL/min. The mobile phase was acetate buffer/acetonitrile (39/61 v/v) for the first 21 min. From 21 to 25 min, a gradient was used to change the mobile phase to 100% acetonitrile. From 25 to 30 min the conditions were returned to acetate buffer/acetonitrile (39/61 v/v).

For gradient 2, the conditions for the first 22 min were acetate buffer/acetonitrile (43/57 v/v). From 22 to 25 min, the mobile phase was changed to 100% acetonitrile. From 25 to 28 min, the conditions were returned to acetate buffer/acetonitrile (43/57 v/v). The flow rate was 1.0 mL/min.

3.1.2.6.3 HPLC Conditions for the Attempted Analysis of the Fluorescent Derivatives of Dexrazoxane Hydrolysis Intermediates

For gradient 1, the conditions for the first 22 min were acetate buffer/acetonitrile (43/57 v/v). From 22 to 25 min, a gradient was used to change the mobile phase to 100% acetonitrile. From 25 to 28 min, the conditions were returned to acetate buffer/acetonitrile (43/57 v/v). The flow rate was 1.0 mL/min.

For gradient 2, the mobile phase was acetate buffer/acetonitrile (47/53 v/v) for the first 37 min. From 37 to 40 min, a gradient was used to change the mobile phase to 100% acetonitrile. From 40 to 43 min, the conditions were returned to acetate buffer/acetonitrile (47/53 v/v). The flow rate was 1.0 mL/min.

For gradient 3, the mobile phase was acetate buffer/acetonitrile (65/35 v/v) for the first 62 min From 62 to 65 min, a gradient was used to change the mobile phase to acetate buffer/acetonitrile (49/51 v/v). The mobile phase was kept at this composition from 65 to 112 min. Another gradient was used to change the mobile phase to 100% acetonitrile from 112 to 115 min. The conditions were held at 100% acetonitrile for 1 min from 115 to 116 min. From 116 to 119 min, the mobile phase was returned to acetate buffer/acetonitrile (65/35 v/v). The flow rate was 1.0 mL/min.

In gradient 4, the conditions for the first 139 min were acetate buffer/acetonitrile (72/28 v/v). From 139 to 140 min, a gradient was used to change the mobile phase to 100% acetonitrile. The conditions were held at 100% acetonitrile for 10 min from 140 to 150 min. From 150 to 152 min the conditions were returned to acetate buffer/acetonitrile (72/28 v/v). The flow rate was 1.0 mL/min.

3.2 RESULTS

3.2.1 HPLC Quantitation of the Sodium Hydroxide and Potassium Phosphate Hydrolysis Intermediate Products of Dexrazoxane

The NaOH hydrolysis intermediate products of dexrazoxane contained equimolar concentrations of intermediates **B** and **C** and dexrazoxane. About 1 mg/mL of each compound was in the NaOH hydrolysis mixture.

The phosphate buffer (pH 7.4) hydrolysis intermediate products of dexrazoxane contained equimolar concentrations of intermediate **B** and dexrazoxane. There is approximately 3.45 times more **B** than **C**. About 1.3 mg/mL of dexrazoxane, 1.4 mg/mL of compound **B** and 0.4 mg/mL of compound **C** was in the KH_2PO_4 hydrolysis mixture.

3.2.2 Derivatization With 2-Bromomethyl-4,5-Diphenyloxazole

3.2.2.1 Analysis of 2-Bromomethyl-4,5-Diphenyloxazole

Elemental analysis was performed on the fluorescent derivative 2-bromomethyl-4,5-diphenyloxazole. The results were carbon (C) 61.18%, hydrogen (H) 3.70%, nitrogen (N) 4.35%, oxygen (O) 5.27% and bromine (Br) 24.25%. The theoretical values for 2-bromomethyl-4,5-diphenyloxazole are C 61.17%, H 3.85%, N 4.46%, O 5.09% and Br 25.43%. A melting point of the compound was found to be 62°C. Nuclear magnetic resonance (NMR) spectra of the starting material 2-methyl-4,5-diphenyloxazole and 2-bromomethyl-4,5-diphenyloxazole are illustrated in figures 3.3 and 3.4. The ^1H NMR data for 2-methyl-4,5-diphenyloxazole was: δ 2.5 ppm (3H, singlet, CH_3 at the 2 position of the oxazole ring) δ 7.3 and 7.6 ppm (10H, split multiplets, phenyl rings). For 2-bromomethyl-4,5-diphenyloxazole, the ^1H NMR data was the following: δ 4.5 ppm (2H, singlet, CH_2 at the 2 position of the oxazole ring) δ 7.3 and 7.6 ppm (10H, split multiplets, phenyl rings).

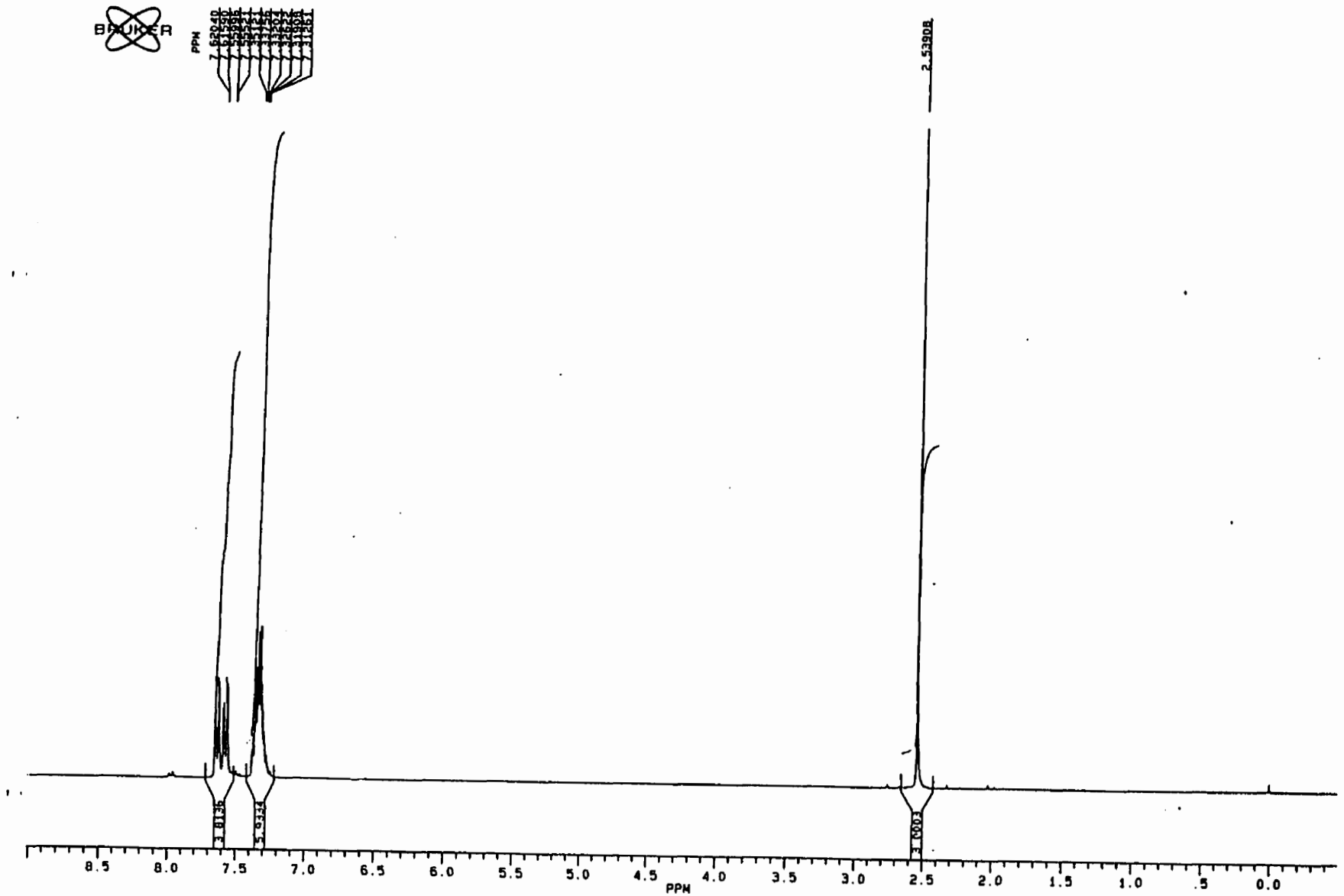


Figure 3.3. ^{1}H-NMR spectrum of 2-methyl-4,5-diphenyloxazole

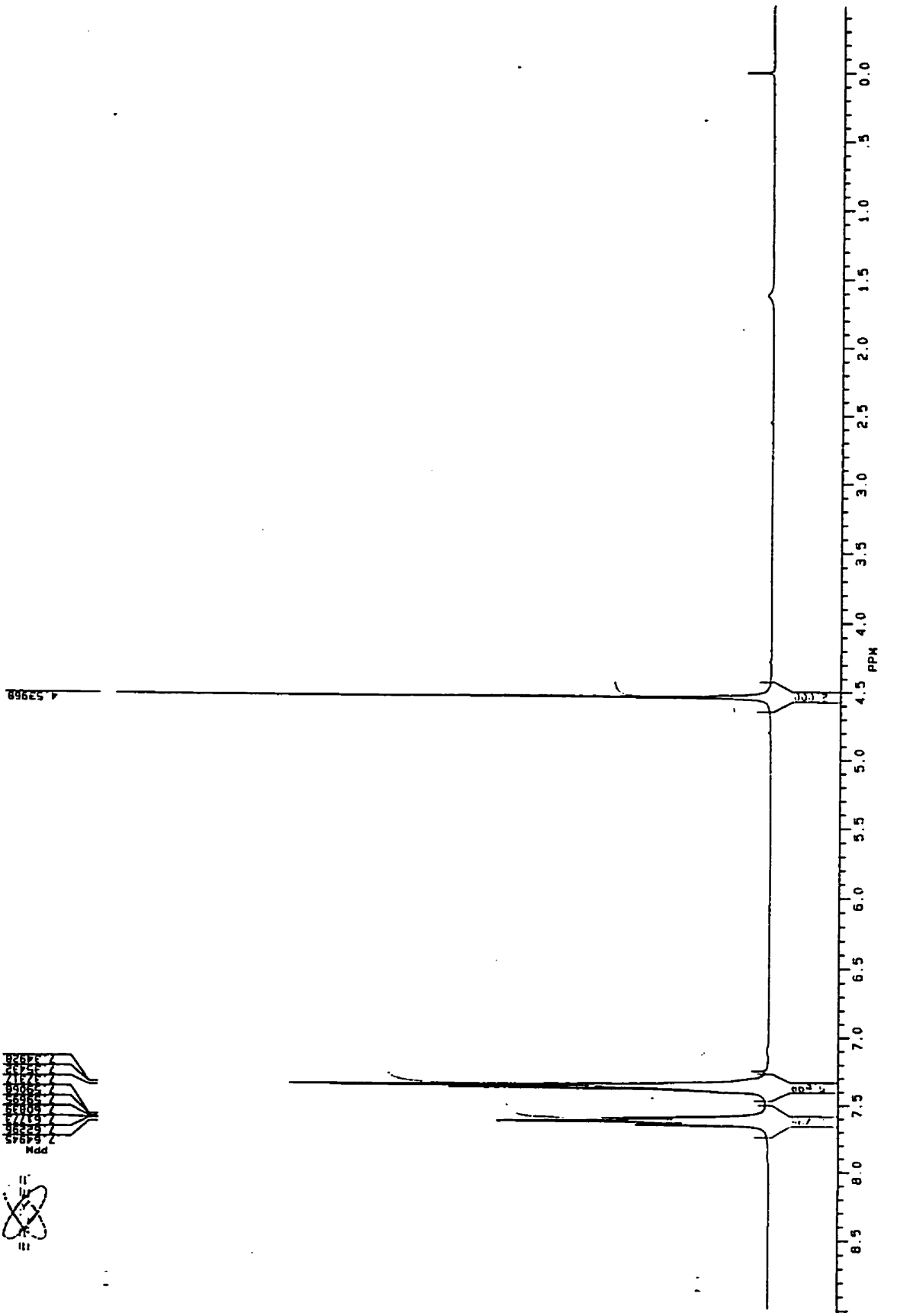


Figure 3.4. ^{1}H-NMR spectrum of 2-bromomethyl-4,5-diphenyloxazole

3.2.2.2 Selection of HPLC Conditions for Analysis of the Fluorescent Derivative of Dexrazoxane

For the selection of an appropriate mobile phase, samples containing 5 µg/mL of dexrazoxane were derivatized with 2.0 mg/mL 2-bromomethyl-4,5-diphenyloxazole and crown-6-ether solutions. Final concentration of derivatizing reagents in the sample was each 1.0 mg/mL.

Samples were analyzed by 2 different sets of HPLC conditions listed in section 3.1.2.6.2. Using HPLC gradient 1, many peaks were present in the blank and the dexrazoxane sample. These peaks eluted together and the dexrazoxane peak was unable to be separated. For HPLC gradient 2, the flow rate was reduced from 2.5 to 1.0 mL/min and the proportion of acetonitrile reduced by 4%. The dexrazoxane peak was identified and had a retention time of 21 min and was well resolved from any endogenous peaks. These HPLC conditions were used to analyze both test samples of the derivatization reaction and samples for the dexrazoxane fluorescent derivative calibration plot.

3.2.2.3 Optimization of Derivatization Conditions for Dexrazoxane Samples

Samples containing 5 µg/mL of dexrazoxane were derivatized with four different concentrations of 2-bromomethyl-4,5-diphenyloxazole and crown-6-ether solutions. The concentration of 2-bromomethyl-4,5-diphenyloxazole and crown-6-ether in the samples were 250, 500, 750 and 1000 µg/mL for samples 1 to 4 respectively. A blank sample was also prepared with 1000 µg/mL each of 2-bromomethyl-4,5-diphenyloxazole and crown-6-ether.

Dexrazoxane was derivatized at all concentrations of 2-bromomethyl-4,5-diphenyloxazole and crown-6-ether. The peak areas of the dexrazoxane fluorescent derivative were similar at all concentrations of reagents used. As the concentration of derivatizing reagents decreased, the size of the endogenous peaks also decreased. For preparation of the dexrazoxane

calibration curve, samples contained 500 µg/mL each of 2-bromomethyl-4,5-diphenyloxazole and crown-6-ether.

3.2.2.4 Dexrazoxane Fluorescent Derivatives Calibration Plot

Dexrazoxane samples with concentrations of 50, 100, 500 ng/mL and 1, 5, and 10 µg/mL were derivatized. The total concentration of 2-bromomethyl-4,5-diphenyloxazole and crown-6-ether in the sample was 500 µg/mL each. The calibration plot for the dexrazoxane derivatives was prepared by plotting the derivative peak areas against their concentrations. The data for the calibration plot are listed in Table 3.1 and the calibration plot is illustrated in figure 3.5. Good linearity was observed over the concentration range used. The detection limit was approximately 0.03 µg/mL with a signal:noise ratio of 3:1. The slope and y-intercepts for the calibration plot are listed in Table 3.2.

Table 3.1 Peak Areas of the Fluorescent Derivatives of Dexrazoxane vs. Concentration

Dexrazoxane Concentration (µg/mL)	Peak Area (arbitrary units)
0.05	106,493
0.10	183,361
0.50	1,441,267
1.0	3,059,354
5.0	16,238,634
10.0	31,685,924

Table 3.2 Data of the Calibration Plot for the Fluorescent Derivatives of Dexrazoxane

Fluorescent Derivatives Of Dexrazoxane
Slope ± SEM = 3,192,303 ± 22, 641 $(\mu g/mL)^{-1}$
Y-intercept ± SEM = -72,804 ± 103, 863
$r^2 = 0.9998$

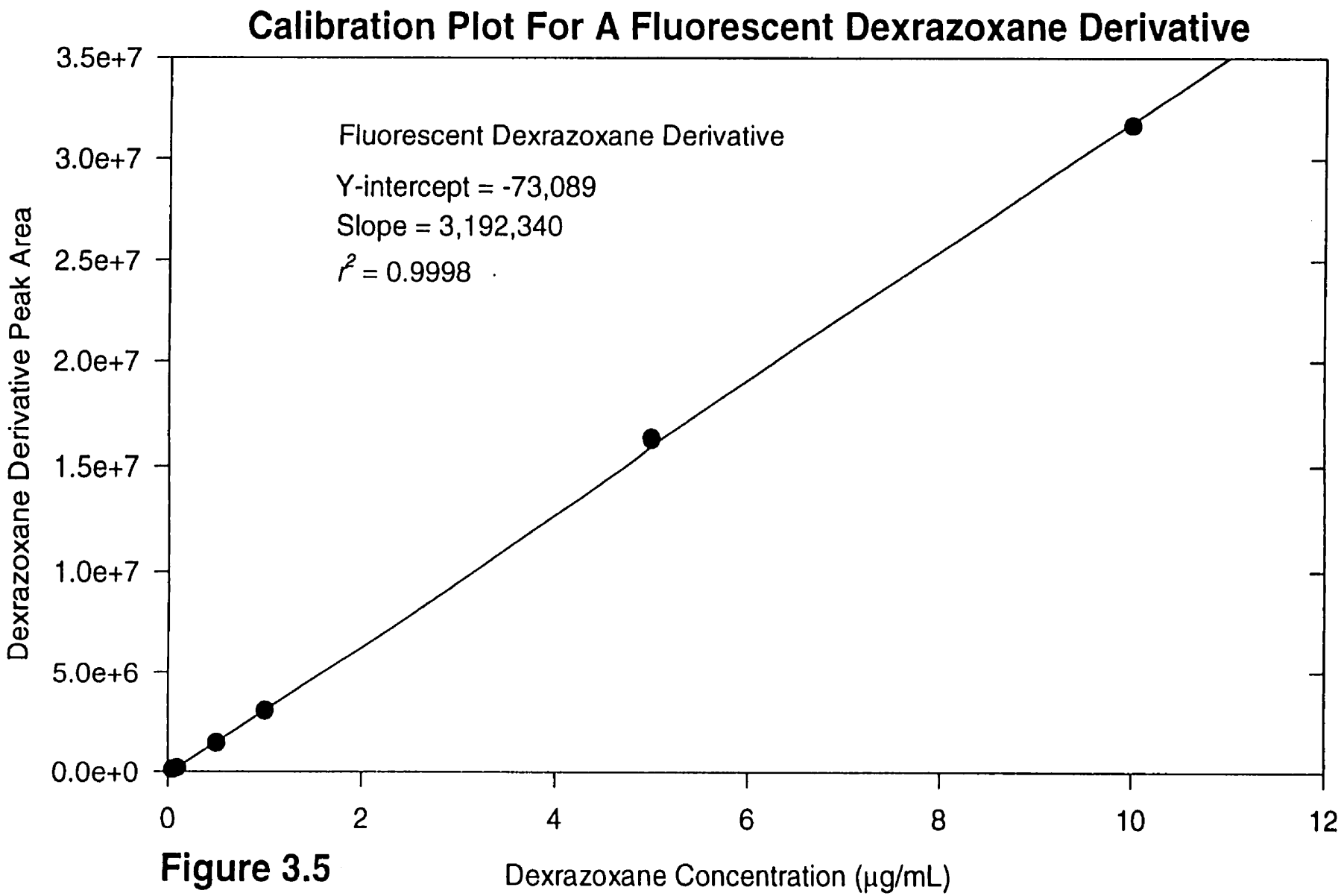

Calibration Plot For A Fluorescent Dexrazoxane Derivative
Fluorescent Dexrazoxane Derivative
Y-intercept = -73,089
Slope = 3,192,340
r^2 = 0.9998
Dexrazoxane Derivative Peak Area
3.5e+7
3.0e+7
2.5e+7
2.0e+7
1.5e+7
1.0e+7
5.0e+6
0.0e+0
0
2
4
6
8
10
12
Dexrazoxane Concentration (µg/mL)

Figure 3.5

3.2.2.5 Attempted Derivatization and HPLC Analysis of Dexrazoxane Hydrolysis Intermediates

Attempts were made to derivatize samples containing 1/50 and 1/100 dilutions of the NaOH and KH_2PO_4 hydrolysis intermediates. The 1/50 dilution of the NaOH hydrolysis intermediate products had 20 µg/mL each of intermediates **B** and **C** and dexrazoxane. The 1/100 dilution contained 10 µg/mL of intermediates **B** and **C** and dexrazoxane. The 1/50 dilution of the KH_2PO_4 hydrolysis sample contained 30 µg/mL of **B**, 10 µg/mL of **C** and 26 µg/mL of dexrazoxane. The 1/100 dilution contained 15 µg/mL of **B**, 5 µg/mL of **C** and 13 µg/mL of dexrazoxane. A 5 µg/mL dexrazoxane sample and a blank were also prepared. Samples contained 1.0 mg/mL each of 2-bromomethyl-4,5-diphenyloxazole and crown-6-ether, twice the concentration used for the dexrazoxane calibration plot samples. The samples were derivatized for 60 min before the reaction was quenched by cooling the samples in ice water. Three different sets of HPLC conditions were tried and were listed in section 3.1.2.6.3. Chromatograms for all samples contained many endogenous peaks which eluted close together and the intermediate peaks were unable to be identified. The third set of HPLC conditions used for analysis resulted in a retention time of 96 min for the fluorescent dexrazoxane derivative, but the chromatogram was still filled with endogenous peaks. Increasing the time for the derivatization reaction to 150 min resulted in some peaks increasing in size but the intermediates were still unable to be identified.

Next, 1/5 and 1/10 dilutions of both NaOH and KH_2PO_4 hydrolysis intermediates or 10 times more concentrated than the previous samples, were derivatized along with a blank and a 70 µg/mL dexrazoxane sample. The amount of 2-bromomethyl-4,5-diphenyloxazole and crown-6-ether in the

sample was doubled to 2.0 mg/mL and the derivatization time was 150 min. The third HPLC gradient was used to analyze these samples. The dexrazoxane peak was easily identified by its large size. Several other large peaks were present in the intermediate samples but not in the blank or dexrazoxane sample. The chromatogram for a blank derivatized sample was illustrated in figure 3.6.

A fourth gradient listed in section 3.1.2.6.3, was tried in order to separate the peaks out even further. Under these conditions, the dexrazoxane derivative peak eluted at 116 min. Comparison of the suspected intermediate peaks between samples found no correlation between peak size, concentration or type of buffer used to hydrolyze dexrazoxane. There were still a very large number of peaks in the blank which made identification of the derivatized products of the hydrolysis intermediates very difficult. This approach was abandoned.

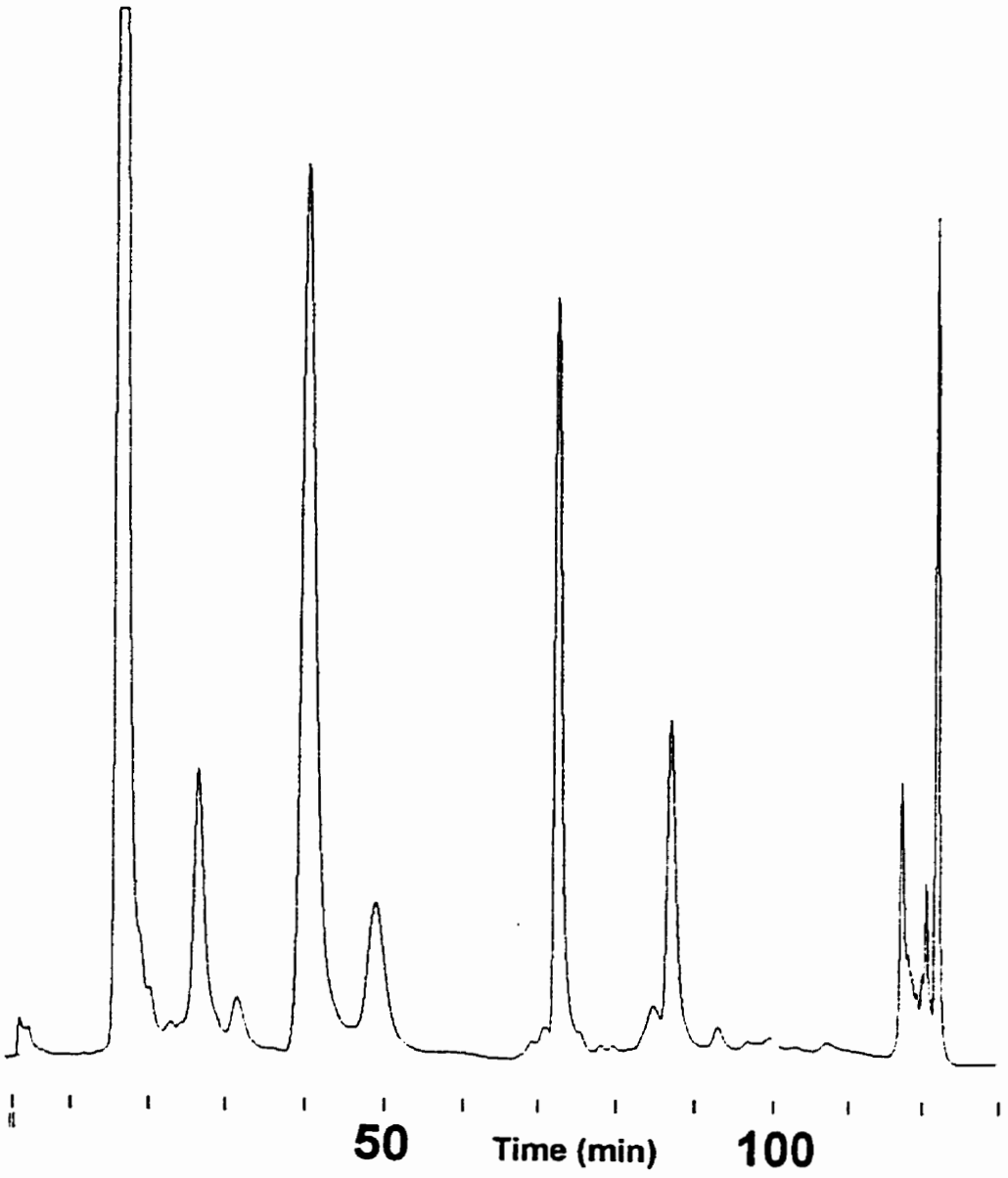


Figure 3.6 Chromatogram for a blank sample containing 500 µg/mL of 2-bromomethyl-4,5-diphenyloxazole, 500 µg/mL of crown-16-ether, 3mg/mL potassium carbonate. Reaction time was 150 min at 25°C. The HPLC conditions are: 0 to 62 min, 0.25 M sodium acetate buffer (pH 5.0)/acetonitrile (65/35 v/v); 62 to 65 min, 0.25 M sodium acetate buffer (pH 5.0)/acetonitrile (49/51 v/v); 65 min to 112 min, 0.25 M sodium acetate buffer (pH 5.0)/acetonitrile (49/51 v/v); 112 to 115 min, 100% acetonitrile; 115 to 116, 100% acetonitrile; 116 to 119 min, 0.25 M sodium acetate buffer (pH 5.0)/acetonitrile (65/35 v/v). Flow rate was 1.0 mL/min and a 3.9 mm I.D. X 300 mm µBondapak C-18 column was used. Fluorescent detection was used with an excitation wavelength of 287 nm and an emission wavelength of 365 nm.

3.3 DISCUSSION

3.3.1 HPLC Quantitation of the Sodium Hydroxide and Potassium Phosphate Hydrolysis Intermediate Products of Dexrazoxane

The dexrazoxane intermediates were produced by NaOH hydrolysis at 25°C or in pH 7.4 phosphate buffer at 37°C. Intermediates **B** and **C** were produced in nearly equimolar concentrations in the NaOH solution. In the pH 7.4 phosphate buffer, 3.45 times more of intermediate **B** was produced than for intermediate **C**. These results agree with data from Hasinoff (1993, 1994a, 1994b).

3.3.2 Derivatization With 2-Bromomethyl-4,5-Diphenyloxazole

3.3.2.1 Analysis of 2-Bromomethyl-4,5-Diphenyloxazole

The results of the elemental analysis of 2-bromomethyl-4,5-diphenyloxazole agree closely with the theoretical values. The experimental values were within 0.01% of theoretical values for carbon, 0.15% for hydrogen, 0.11% for nitrogen, 0.18% for oxygen and 0.18% for bromine. The NMR spectra for the starting material 2-methyl-4,5-diphenyloxazole had a single peak with 3 hydrogens at δ = 2.5 ppm. For 2-bromomethyl-4,5-diphenyloxazole, a single peak with 2 hydrogens at δ = 4.5 ppm was observed. The loss of one hydrogen and a shift downfield of 2 ppm for the methyl group in 2-bromomethyl-4,5-diphenyloxazole is consistent with bromination of the methyl group. Electron withdrawing atoms such as bromine cause large downfield shifts. The other peaks in the spectra were similar for both 2-methyl-4,5-diphenyloxazole and 2-bromomethyl-4,5-diphenyloxazole. The two split multiplets at δ = 7.3 and 7.6 ppm with an total integration of 10 hydrogens are consistent with hydrogens on the phenyl rings.

It should be mentioned that the experimentally found melting point of 62°C was substantially different from the melting point of 104-106°C found by

Aldous *et al.*, (1960). The discrepancy may be due to the different methods used for purification of the oxazole. Aldous used vacuum distillation after refluxing to separate the brominated oxazole from the reaction mixture. Due to lack of vacuum distillation apparatus available to us, flash chromatography was used instead. These different methods of purification may have resulted in two different crystal structures of the purified products, leading to different melting points. However, all other methods of characterization used showed good agreement between experimentally found data and theoretically expected values.

3.3.2.2 Selection of HPLC Conditions for Analysis of the Fluorescent Derivatives of Dexrazoxane

The first HPLC mobile phase conditions tried for the analysis of the fluorescent derivative of dexrazoxane were similar to those used by Sisco (1989). However, the peaks eluted together closely and the dexrazoxane peak could not be identified. This may be due to the different columns used by our study and the one reported by Sisco (1989). The column conditions Sisco used were two hand packed C-18 columns of 15 cm in length connected in series. The chromatographic conditions in this study used a single 30 cm C-18 column. Reducing the flow rate to 1.0 mL/min and decreasing the amount of acetonitrile in the mobile phase resulted in the dexrazoxane derivative peak being well resolved from endogenous peaks.

Varying the concentration of 2-bromomethyl-4,5-diphenyloxazole and crown-6-ether from 250 to 1000 μg/mL still resulted in derivatization of dexrazoxane. The peak areas for the dexrazoxane derivative at all concentrations of reagents were the same. At lower concentrations, the endogenous peaks formed as a result of reactions between the oxazole, crown-6-ether and potassium carbonate were much smaller in size. The

concentration of 2-bromomethyl-4,5-diphenyloxazole and crown-6-ether used for the calibration plot samples was changed to 500 µg/mL to reduce the size of the endogenous peaks.

A linear calibration plot was obtained for the dexrazoxane phenyloxazole derivative over the range from 50 ng/mL to 10 µg/mL. Once a linear plot had been obtained, derivatization was attempted on the dexrazoxane hydrolysis intermediates.

3.3.2.4 Derivatization and Analysis of Dexrazoxane Hydrolysis Intermediates

Three different HPLC conditions, listed in section 3.1.2.6.3, were used, each with decreasing proportions of acetonitrile. For all conditions, chromatograms with a large number of peaks were produced, including the blank sample containing only the derivatizing reagents. The third mobile phase was then used

Four different HPLC conditions were used, each with decreasing proportion of acetonitrile. For all conditions, chromatograms with a large number of peaks were produced and it was impossible to determine the intermediate peaks. This approach was abandoned.

Chapter 4

Development of a Method to Isolate Dexrazoxane and its Hydrolysis Intermediates From Rat Plasma

4.0 Introduction to Method Development for the Isolation and Quantitation of Dexrazoxane and its Hydrolysis Intermediates From Rat Plasma.

Several solid phase extraction cartridges were evaluated for their ability to recover dexrazoxane and its intermediates from buffer. Of the cartridges tried, only Sep-pak anion exchange cartridges gave good recoveries of about 95% of the intermediates from buffer. These cartridges were then used to separate dexrazoxane and its intermediates from rat plasma. Maximum recoveries of only 50% were achieved in plasma, so this approach was abandoned.

Some protein precipitation methods were tried in an effort to separate dexrazoxane and its intermediates from rat plasma. Good recoveries of over 80% were obtained for dexrazoxane and the intermediates **B** and **C** from rat plasma with a simple acetonitrile protein precipitation procedure. The processed plasma samples were then analyzed using a reversed-phase C-18 HPLC method and post-column derivatization with NaOH.

4.1 EXPERIMENTAL

4.1.1 Materials

4.1.1.1 Chemicals

Dexrazoxane (lot 91D23FY) was a gift from Pharmacia & Upjohn (Columbus, OH). Concentrated phosphoric acid, potassium hydroxide, potassium phosphate, and sodium hydroxide were obtained from Fisher Scientific (Nepean, Canada). HPLC grade acetonitrile and methanol, volumetric grade hydrochloric acid and sodium hydroxide solutions were obtained from Mallinckrodt (Mississauga, Canada). 1-heptanesulfonic acid sodium salt, Sigma Ultra grade Tris base and Tris hydrochloride were from Sigma Chemical Co. (St. Louis, MO). AnalaR Grade ethylenediaminetetraacetic acid disodium salt was from BDH Chemicals Ltd. (Poole, England).

4.1.1.2 Instrumentation

4.1.1.2.1 Chromatographic

The HPLC system consisted of a Varian 9010 pump, Varian Star integration software, and a Rheodyne injector. The system was fitted with a Brownlee Labs NewGuard column containing an 1.5 cm X 3.2 mm I.D. reversed-phase C-18 guard cartridge (Applied Biosystems, Santa Clara, CA, USA). An μBondapak 4.6 mm I.D. X 250 mm reversed-phase C-18 column with a particle size of 10 μm (Waters Chromatography, Mississauga, ON) was used for analysis. For the analysis of the hydrolysis intermediates of dexrazoxane, separation was also tried with a Nova-pak 4.6 mm I.D. X 250 mm reversed-phase C-18 column with a particle size of 4 μm (Waters Chromatography, Mississauga, ON)

For the HPLC quantitation of the sodium hydroxide hydrolysis products of dexrazoxane, a Varian 9050 programmable wavelength detector was used. A program was used with the detection wavelength at 205 nm for

the first 10 minutes and then switched to 215 nm for the rest of the analysis. The sample loop volume was 20 µL.

For the post column derivatization of the sodium hydroxide hydrolysis products of dexrazoxane, a Varian 9050 programmable wavelength UV detector was used. The wavelength used was 227 nm during method development and 235 nm for analysis of plasma samples from the animal study and the calibration plot. The sample loop volume was 50 µL.

For most analyses, peak areas were calculated automatically using the Varian Star integration software. For the analysis of the animal study and the calibration plot, peak areas were calculated manually using the Varian Star integration software. Manual integration was used to give more consistent results between chromatograms. The start of the intermediate **B** peak was defined as starting 0.60 min before the retention time of 6.0 min for the maximum amplitude of the peak. When intermediates **B** and **C** eluted closely together, the end of peak **B** and the start of peak **C** was defined as the lowest point in the valley. The end time for peak **C** was defined as ending 0.60 min after the retention time of 7.0 min for the maximum amplitude of the peak. The start of the dexrazoxane peak was defined as being 16.4 min and the stop of the peak was set to 17.6 min.

4.1.1.2.2 Post-Column Derivatization With Sodium Hydroxide

An Eldex A-30-S-Peek pump (Eldex Laboratories, Inc., San Carlos, CA) was used to deliver 30 mM NaOH solution at a flow rate of 0.25 mL/min through a pulsation dampener (Varian Scientific) to a 3-way mixing tee (Upchurch Scientific, Oak Harbor, WA). The outlet tubing from the HPLC column was also connected to the mixing tee. The outflow of the tee was then attached to a reaction bed. The bed consisted of ~0.5 m of 0.02 mm I.D. flexible HPLC tubing tightly knitted into a rectangle approximately 3 cm X 4 cm. The

outflow from the reaction web went directly to the HPLC UV-detector. Refer to figure 4.1 for a diagram of the post-column derivatization apparatus.

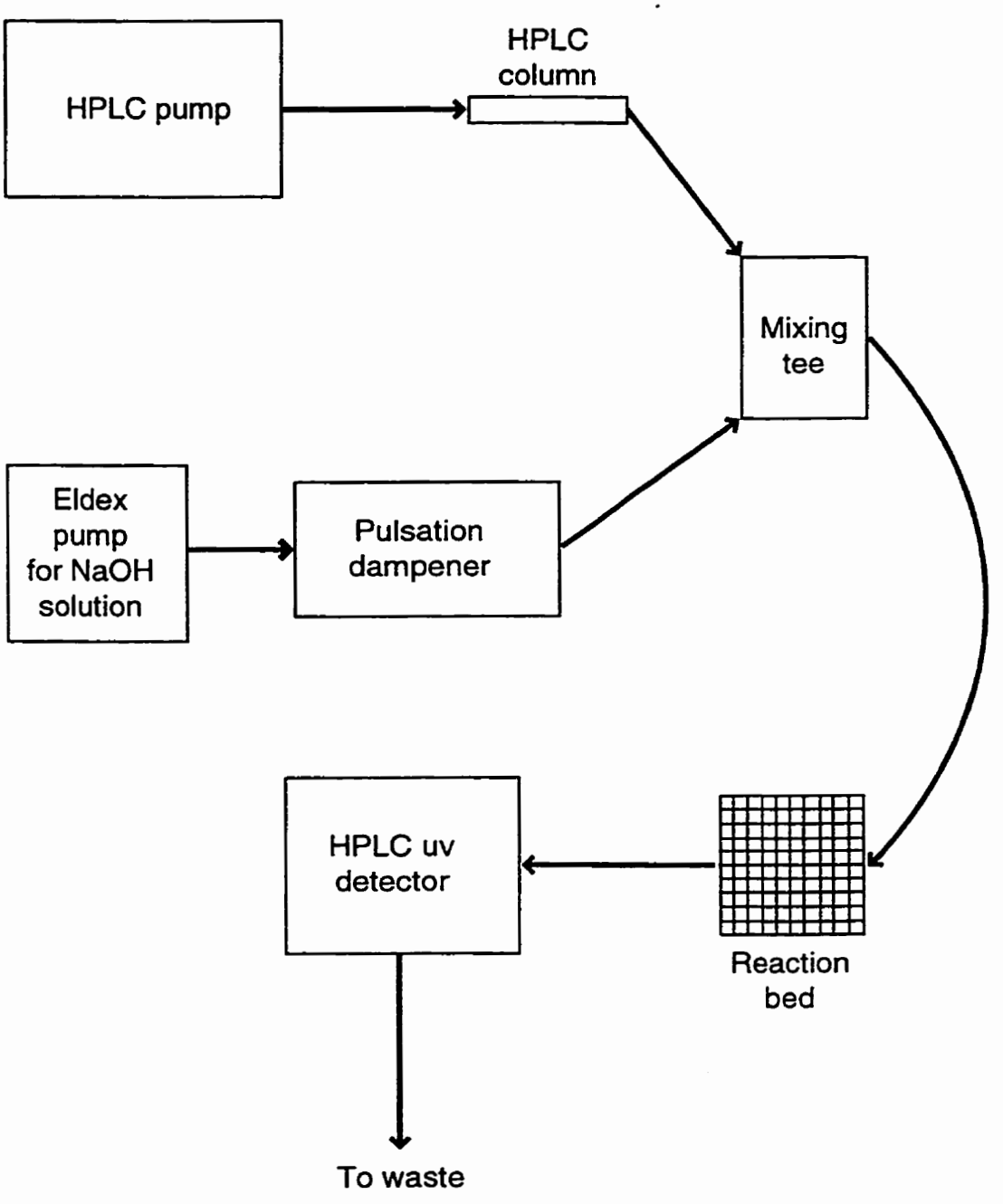


Figure 4.1 Diagram of post-column derivatization of dexrazoxane and its hydrolysis intermediates with NaOH.

4.1.2 Methods

4.1.2.1 Preparation of Sodium Hydroxide Hydrolysis Intermediate Products of Dexrazoxane

Using a method developed by Hasinoff (1994b), 5 mg of dexrazoxane were added to a 10 mL glass culture tube and dissolved in 1.0 mL of double distilled water. An 80 µL aliquot of 1.0 M NaOH solution was added, the tube capped and the mixture incubated in a 25°C water bath for 40 min. The reaction was quenched by the addition of 80 µL of 1.0 M HCl, which preserved the stability of the intermediates. The sample was sealed with a disposable plastic cap and stored at -80°C until required for use.

4.1.2.2 Preparation of Potassium Phosphate Hydrolysis Intermediate Products of Dexrazoxane

Using a method developed by Hasinoff (1994b), 5 mg of dexrazoxane were added to a 10 mL glass culture tube and dissolved in 1.0 mL of 50 mM KH_2PO_4, pH 7.4. The KH_2PO_4 solution was titrated with 6 M KOH solution to pH 7.4. The mixture incubated in a 37°C water bath for 18 hours. The reaction was quenched by the addition of 36 µL of 1.0 M HCl, which preserved the stability of the intermediates. The sample was sealed with a disposable plastic cap and stored at -80°C until required for use.

4.1.2.3 HPLC Quantatition of the Hydrolysis Intermediates of Dexrazoxane

A modification of a method developed by Hasinoff (1994a) was used to quantify the levels of dexrazoxane, the intermediates **B**, **C** (refer to fig. 1.2) and the active drug ADR-925 in the NaOH and KH_2PO_4 hydrolysis intermediates of dexrazoxane. Samples from each stock hydrolysis solution were diluted 1:10 with 10 mM HCl before analysis.

The initial mobile phase was 100% 500 µM Na_2EDTA solution (pH 4.5). The pH of the Na_2EDTA solution was not adjusted. A linear gradient was used from 0 to 10 min to increase the amount of methanol from 0 to 8%. This

mobile phase of Na_2EDTA/methanol (92/8 v/v) was maintained to the end of the run at 18 min. The column was then re-equilibrated at the initial mobile phase for 8 min.

A computer program was developed that converted the peak areas of dexrazoxane and its hydrolysis intermediates into concentrations. The calibration factors for dexrazoxane and ADR-925 were obtained from pure samples of these compounds. The calibration factors for **B** and **C** were obtained from a ^{1}H-NMR determination of the concentrations of a partially hydrolyzed dexrazoxane sample also simultaneously analyzed by HPLC (Hasinoff, 1994a). Aliquots of 30 µL were injected into the HPLC.

4.1.2.4 Preparation of Phosphate and Sodium Hydroxide Hydrolysis Intermediate Products of Dexrazoxane Standard Solutions

Solutions of 1/5, 1/10, 1/50 and 1/100 dilutions were made of both the KH_2PO_4 and NaOH hydrolysis intermediate products. The 1/5 dilution of the NaOH hydrolysis intermediate products had 200 µg/mL each of intermediates **B** and **C** and dexrazoxane. The 1/10, 1/50 and 1/100 dilutions contained 100, 20 and 10 µg/mL respectively of intermediates **B** and **C** and dexrazoxane. The 1/5 dilution of the KH_2PO_4 hydrolysis sample contained 300 µg/mL of intermediate **B**, 100 µg/mL of intermediate **C** and 260 µg/mL of dexrazoxane. The 1/10 dilution contained 150 µg/mL of **B**, 50 µg/mL of **C** and 130 µg/mL of dexrazoxane. The 1/50 dilution contained 30 µg/mL of B, 10 µg/mL of **C** and 26 µg/mL of dexrazoxane. The 1/100 dilution contained 15 µg/mL of **B**, 5 µg/mL of **C** and 13 µg/mL of dexrazoxane.

A 400 µL aliquot of the intermediates was made up with acetonitrile in a 2.0 mL volumetric container for the 1/5 dilution sample. A 200 µL aliquot of the intermediates was made up with acetonitrile in a 2.0 mL volumetric container for the 1/10 dilution sample. The 1/50 dilution sample was

prepared by taking a 100 µL aliquot of the intermediates and made up to 5.0 mL with acetonitrile. The 1/100 dilution sample was prepared by taking a 50 µL aliquot of the intermediates and made up to 5.0 mL with acetonitrile.

4.1.2.5 Evaluation of Solid Phase Extraction Methods for the Recovery of Dexrazoxane Hydrolysis Intermediates in Buffer

Solid phase extraction methods were evaluated for their ability to recover dexrazoxane and its hydrolysis intermediates. Reversed-phase extraction cartridges from two manufacturers were used to recover dexrazoxane and its hydrolysis intermediates from buffer. Anion exchange extraction cartridges were also used to recover dexrazoxane and its hydrolysis intermediates from buffer and rat plasma.

4.1.2.5.1 Empore (3M) High Performance Disks

C-2, C-8, C-18 and SBX-XC (poly(styrenedivinylbenzene) copolymer) Empore High Performance disks (3M New Products Department, St. Paul, MN, USA) were used according to the manufacturer's instructions. The Empore disks were 3 mm in diameter and their syringe barrels had a capacity of 3 mL of sample. They were conditioned with 0.5 mL acetonitrile and then 1 mL of 0.2 M potassium phosphate buffer, pH 7.0 under a vacuum of 15 m m Hg. A vacuum manifold from Waters Chromatography (Milford, MA, USA) was used for processing of the extraction disks.

For each sample, 20 µL of the NaOH hydrolysis intermediates stock solution was added to 2 mL of 0.2 M potassium phosphate buffer, pH 7.0 in a 10 mL glass culture tube. The samples were transferred to one each of the C-2, C-8, C-18 and SBX-XC Empore disks. The samples were extracted through the disks using a vacuum of 15 mm of Hg. The disks were washed with 2 portions of 1 mL of double distilled water under a vacuum of 15 mm of Hg.

The disks were then eluted with 2 portions of 0.5 mL of acetonitrile,

collected in 10 mL glass culture tubes and the extracts evaporated to dryness under a stream of argon in a 40°C water bath. The samples were stored at -80°C until ready for analysis. Just before analysis, the samples were thawed at room temperature, reconstituted in 1.0 mL of 10 mM HCl and analyzed by HPLC.

4.1.2.5.2 Sep-Pak C-8 and C-18 Solid Phase Extraction Cartridges

C-8 and C-18 Sep-Pak Plus solid phase extraction cartridges (Waters Chromatography, Milford, MA, USA) were conditioned by eluting through 5 mL of acetonitrile followed by 5 mL of double distilled water under vacuum (5 mm Hg). For each sample, 20 µL of the NaOH hydrolysis intermediates stock solution were added to 2 mL of 0.2 M potassium phosphate buffer (pH 7.0) in a 10 mL glass culture tube. The buffer solution was run through the cartridges under vacuum (5 mm Hg). The cartridges were then washed with 5 mL of double-distilled water and dried under vacuum (15 mm Hg) for 5 min. The cartridges were then eluted with 2 portions of 2 mL acetonitrile. The acetonitrile extracts were collected in a 10 mL disposable glass culture tube and evaporated to dryness under a stream of argon gas in a 40°C water bath. The samples were stored at -80°C until ready for analysis. Just before analysis, the samples were thawed at room temperature, reconstituted in 1.0 mL of 10 mM HCl and analyzed by HPLC.

4.1.2.5.3 Sep-Pak QMA Anion Exchange Solid Phase Extraction Cartridges

Sep-Pak Accell Plus QMA anion exchange solid phase extraction cartridges (Waters Chromatography, Milford, MA, USA) were used at two different pHs, one at pH 6.0 and the other at pH 7.0 Both Tris buffers were prepared to their respective pHs at 20°C. The buffers were also used at 20°C. The cartridges were conditioned by eluting through 4 mL of the appropriate Tris buffer under vacuum (5 mm Hg). For each sample, 20 µL of the NaOH

hydrolysis intermediates stock solution were added to 3 mL of the appropriate 1 mM Tris buffer in a 10 mL glass culture tube. The final concentration in the reconstituted samples was 10 µg/mL each of intermediates **B** and **C** and dexrazoxane. The Tris buffer solutions were run through the cartridges under vacuum (5 mm Hg). The cartridges were then washed with 5 mL of double-distilled water and dried under vacuum (15 mm Hg) for 5 min. The cartridges were then eluted with 2 portions of 3 mL 100 mM HCl. The HCl extracts were collected in a 10 mL disposable glass culture tube and evaporated to dryness under a stream of argon gas in a 40°C water bath. The samples were stored at -80°C until ready for analysis. Just before analysis, the samples were thawed at room temperature, reconstituted in 1.0 mL of 10 mM HCl and analyzed by HPLC.

4.1.2.6 Recovery of Dexrazoxane Hydrolysis Intermediates From Plasma

4.1.2.6.1 Sep-Pak QMA Anion Exchange Solid Phase Extraction Cartridges

Plasma samples were treated in one of four ways before being eluted through the QMA extraction cartridges. In all methods, 20 µL of the NaOH hydrolysis intermediates stock solution were added to a 1.5 mL microcentrifuge tube containing 200 µL of blank rat plasma. The final concentration in the reconstituted samples was 10 µg/mL each of intermediates **B** and **C** and dexrazoxane.

For method 1, an aliquot of 30 µL of 100 mM Na_2EDTA solution was added to the plasma. The concentration of Na_2EDTA in the samples was 3 mM and released the hydrolysis intermediates of dexrazoxane that may have complexed with Ca^{2+} or Mg^{2+} ions present in the plasma. The plasma proteins were precipitated with 70 µL of 6 M HCl. Samples were vortexed at medium speed, centrifuged at 14,000 *g* for 1 min and the supernatant was removed to another microcentrifuge tube. The pellet was rinsed two times

with 100 µL of 500 mM HCl and the washings transferred to the supernatant that had been removed. The pellet was then discarded. The supernatant was transferred to a 10 mL glass culture tube containing 4 mL of 1 mM Tris buffer (pH 7.0). The pH was adjusted to pH 7.0 with 85 µL of 5 M NaOH solution.

In method 2, 20 µL of 100 mM HCl were added to the plasma sample. A 300 µL aliquot of acetonitrile was added to precipitate proteins present in the sample. Samples were vortexed at medium speed for 15 s, centrifuged at 14,000 *g* for 1 min and the supernatant was removed to another microcentrifuge tube. The pellet was rinsed two times with 100 µL acetonitrile and the washings transferred to the supernatant that had been removed. The pellet was then discarded. The acetonitrile supernatant was evaporated to dryness by a stream of argon gas in a 40°C water bath. The residue was reconstituted in 1 mM Tris buffer (pH 7.0) and transferred to a 10 mL glass culture tube. The microcentrifuge tube was rinsed twice with 100 µL of 1 mM Tris buffer (pH 7.0) and transferred to the culture tube containing the reconstituted plasma extract. An aliquot of 3 mL of 1 mM Tris buffer (pH 7.0) was added to the culture tube and vortex mixed for 20 s.

For method 3, the procedure used in method 2 was changed slightly. An aliquot of 10 µL of 42.5% (wt/vol) phosphoric acid was added with the hydrolysis intermediates to the plasma sample. The phosphoric acid was added to prevent further hydrolysis of dexrazoxane and its hydrolysis intermediates.

For method 4, the samples were treated the same as in method 2, except an aliquot of 30 µL of 100 mM Na_2EDTA solution was added to the plasma at the same time as the HCl. The Na_2EDTA released the hydrolysis intermediates of dexrazoxane that may have complexed with Ca^{2+} or Mg^{2+} ions present in the plasma.

The QMA cartridges were conditioned by eluting through 4 mL of 1 mM Tris buffer (pH 7.0) under vacuum (5 mm Hg). The Tris buffer solutions containing the plasma extracts were run through the cartridges under vacuum (5 mm Hg). The cartridges were then washed with 5 mL of double-distilled water and dried under vacuum (15 mm Hg) for 5 min The cartridges were then eluted with 2 portions of 3 mL 100 mM HCl. The HCl extracts were collected in a 10 mL disposable glass culture tube and evaporated to dryness under a stream of argon gas in a 40°C water bath. The samples were stored at -80°C until ready for analysis. Just before analysis, the samples were thawed at room temperature, reconstituted in 1.0 mL of 10 mM HCl and analyzed by HPLC.

4.1.2.7 Evaluation of Methods for the Separation of Dexrazoxane Hydrolysis Intermediates From Plasma

Three methods were evaluated in their ability to separate dexrazoxane and its hydrolysis intermediate from rat plasma. For all methods, 20 µL of the NaOH hydrolysis intermediates stock solution were added to a 1.5 mL microcentrifuge tube containing 200 µL of blank rat plasma.

In method 1, 30 µL of 100 mM Na_2EDTA solution were added to the plasma. The Na_2EDTA released any of the hydrolysis intermediates of dexrazoxane that may have complexed with Ca^{2+} or Mg^{2+} ions present in the plasma. The plasma proteins were precipitated with 70 µL of 6 M HCl. Samples were vortexed at medium speed, centrifuged at 14,000 *g* for 1 min and the supernatant was removed to another microcentrifuge tube. The pellet was rinsed two times with 100 µL of 500 mM HCl and the washings transferred to the supernatant that had been removed. The pellet was then discarded. The supernatant was evaporated to dryness by a stream of argon gas in a 40°C water bath. The samples were stored at -80°C until ready for analysis.

Just before analysis, the samples were thawed at room temperature, reconstituted in 200 µL of 10 mM HCl and analyzed by HPLC.

For method 2, the procedure used in method 1 was changed slightly. An aliquot of 10 µL of 42.5% (wt/vol) phosphoric acid was added with the hydrolysis intermediates to the plasma sample. The phosphoric acid was added to prevent further hydrolysis of dexrazoxane and its hydrolysis intermediates.

In method 3, 20 µL of 100 mM HCl were added to the plasma sample. A 300 µL aliquot of acetonitrile was added to precipitate proteins present in the sample. Samples were vortexed at medium speed for 20 s, centrifuged at 14,000 *g* for 1 min and the supernatant was removed to another microcentrifuge tube. The pellet was rinsed two times with 100 µL acetonitrile and the washings transferred to the supernatant that had been removed. The pellet was then discarded. The acetonitrile supernatant was evaporated to dryness by a stream of argon gas in 40°C water bath. The samples were stored at -80°C until ready for analysis. Just before analysis, the samples were thawed at room temperature, reconstituted in 200 µL of 10 mM HCl and analyzed by HPLC.

For method 4, the samples were treated the same as in method 3, except an aliquot of 30 µL of 100 mM Na_2EDTA solution was added to the plasma at the same time as the HCl. The Na_2EDTA released any of the hydrolysis intermediates of dexrazoxane that may have bonded with Ca^{2+} or Mg^{2+} ions present in the plasma.

4.1.2.8 HPLC Method for the Analysis of Dexrazoxane Hydrolysis Intermediates

4.1.2.8.1 Preparation of Na_2EDTA Solution (pH 4.5)

A 500 µM Na_2EDTA solution (pH 4.5), was made by dissolving 0.744 g of ethylenediaminetetraacetic acid disodium salt in 4 L of double distilled

water. The pH was not adjusted.

4.1.2.8.2 Preparation of Na_2EDTA Solution (pH 3.5)

A 500 µM Na_2EDTA (pH 3.5), solution was made by dissolving 0.744 g of ethylenediaminetetraacetic acid disodium salt in 4 L of double distilled water. The pH was adjusted to 3.5 with 6 M hydrochloric acid.

4.1.2.8.3 Preparation of 1-Heptanesulfonic Acid

A 20 mM 1-heptanesulfonic acid solution was made by dissolving 4.40 g of 1-heptanesulfonic acid sodium salt in 1 L of double distilled water.

4.1.2.8.4 HPLC Conditions for the Analysis of Dexrazoxane Hydrolysis Intermediates

For gradient 1, the initial mobile phase was 100% 500 µM Na_2EDTA solution (pH 4.5). A linear gradient was used from 0 to 10 min to increase the amount of methanol from 0 to 8%. This mobile phase of Na_2EDTA/methanol (92/8, v/v) was maintained to the end of the run at 18 min. The column was then re-equilibrated at the initial mobile phase for 8 min.

For gradient 2, the initial mobile phase was 100% 500 µM Na_2EDTA solution (pH 3.5). A linear gradient was used from 0 to 10 min to increase the amount of methanol from 0 to 8%. This mobile phase of Na_2EDTA/methanol (92/8 v/v) was maintained to the end of the run at 18 min. The column was then re-equilibrated at the initial mobile phase for 8 min.

In gradient 3, the initial mobile phase was 500 µM Na_2EDTA (pH 3.5)/heptanesulfonic acid (90/10 v/v). A linear gradient was used from 0 to 10 min to increase the amount of methanol from 0 to 8%. This mobile phase of Na_2EDTA/heptanesulfonic acid/methanol (82/10/8 v/v/v) was maintained to the end of the run at 18 min. The column was then re-equilibrated with the

initial mobile phase for 8 min. Gradient 3 was also used with a Nova-pak 4.6 mm I.D. X 250 mm reversed-phase C-18 column with a particle size of 4 µm (Waters Chromatography, Mississauga, ON)

4.1.2.8.5 HPLC Conditions for the Analysis of Dexrazoxane and its Hydrolysis Intermediates in Rat Plasma

The initial mobile phase was 500 µM Na_2EDTA (pH 3.5)/heptanesulfonic acid (90/10 v/v). A linear gradient was used from 0 to 10 min to increase the amount of methanol from 0 to 8%. This mobile phase of Na_2EDTA/heptanesulfonic acid/methanol (82/10/8 v/v/v) was maintained to the end of the run at 18 min. The column was then re-equilibrated with the initial mobile phase for 8 min. The outflow from the C-18 column was derivatized with NaOH solution as outlined in section 4.1.1.2.2.

4.1.2.9 Preparation of Dexrazoxane Hydrolysis Intermediate Standard Solutions

Two dexrazoxane NaOH hydrolysis intermediate standard solutions with concentrations of 5, and 50 µg/mL each of dexrazoxane and intermediates **B** and **C** were prepared. The 50 µg/mL standard was made by taking a 50 µl aliquot of the 500 µg/mL standard solution and making up to 500 µL with 10 mM HCl in a 1.5 mL microcentrifuge tube. The 5 µg/mL standard was prepared by taking a 50 µl aliquot of the 50 µg/mL standard solution and made up to 500 µL with 10 mM HCl in a 1.5 mL microcentrifuge tube.

4.1.2.10 Preparation of the Calibration Curve in Rat Plasma

Aliquots of dexrazoxane hydrolysis intermediates calibration standard solutions were added to 500 µL volumes of acidified (20 µL of 500 mM hydrochloric acid per mL of plasma) blank plasma. The final dexrazoxane hydrolysis intermediates concentration in plasma after extraction and reconstitution was from 1 µg/mL to 50 µg/mL. For the 1 and 2 µg/mL plasma

calibration samples, 25 and 50 µL respectively of the 5 µg/mL hydrolysis intermediates standard solution were added. For the 5, 10 and 20 µg/mL plasma calibration samples, 12.5, 25 and 50 µL respectively of the 50 µg/mL hydrolysis intermediates standard solution were added. For the 50 µg/mL plasma calibration samples, 12.5 µL of the 500 µg/mL hydrolysis intermediates standard solution were added. A blank plasma sample was also extracted using plasma free of dexrazoxane and treated with 20 µL of 500 m M hydrochloric acid per mL of plasma.

4.1.2.11 Separation Of Dexrazoxane Hydrolysis Intermediates From Plasma

The plasma samples were thawed at room temperature before use. In a 1.5 mL microcentrifuge tube, 37 µL of 200 mM Na_2EDTA solution were added to 500 µL of acidified plasma (20 µL of 500 mM HCl per mL of plasma). The Na_2EDTA released any of the hydrolysis intermediates of dexrazoxane that may have complexed with Ca^{2+} or Mg^{2+} ions present in the plasma. A 750 µL aliquot of acetonitrile was added to precipitate any proteins present in the sample. Samples were vortexed at medium speed, centrifuged at 14,000 *g* for 1 min and the supernatant removed to another microcentrifuge tube. The pellet was rinsed two times with 100 µL acetonitrile and the washings transferred to the supernatant that had been removed. The pellet was then discarded. The acetonitrile supernatant was evaporated to dryness by a stream of argon gas in a 40°C water bath. The tubes were closed and then stored at -80°C. Samples were allowed to warm to room temperature and then reconstituted in 125 µL of 10 mM HCl just prior to analysis. The plasma samples were concentrated 4 times for analysis. Two aliquots of 60 µL each of the plasma samples were injected into the HPLC.

4.1.2.12 Recoveries of Dexrazoxane and its Hydrolysis Intermediates From Rat Plasma

The 5 and 50 µg/mL dexrazoxane hydrolysis intermediate standard solutions described in section 4.1.2.9 were analyzed using post-column derivatization (see section 4.1.2.8.5). Recovery of each intermediate and dexrazoxane from plasma was calculated by dividing the peak areas of the 5 and 50 µg/mL plasma calibration plot samples by the peak areas of the corresponding standard.

4.2 RESULTS

4.2.1 HPLC Quantitation of Dexrazoxane and the Sodium Hydroxide and Potassium Phosphate Hydrolysis Intermediate Products of Dexrazoxane

The NaOH hydrolysis intermediate products of dexrazoxane contained equimolar concentrations of intermediates **B** and **C** and dexrazoxane. About 1 mg/mL of each compound was in the NaOH hydrolysis mixture.

The phosphate buffer (pH 7.4) hydrolysis intermediate products of dexrazoxane contained equimolar concentrations of intermediate **B** and dexrazoxane. There is approximately 3.45 times more **B** than **C**. About 1.3 mg/mL of dexrazoxane, 1.4 mg/mL of compound **B** and 0.4 mg/mL of compound **C** was in the KH_2PO_4 hydrolysis mixture.

4.2.2 Evaluation of Attempted Solid Phase Extraction Methods for the Recovery of Dexrazoxane Hydrolysis Intermediates in Buffer

Several solid phase extraction cartridges were evaluated for recovery of dexrazoxane hydrolysis intermediates in buffer. Samples containing 10 µg/mL of the intermediates **B** and **C** and dexrazoxane were extracted with C-2, C-8, C-18 and SBX-XC (poly(styrenedivinylbenzene) copolymer) Empore extraction disks. HPLC analysis of reconstituted extracts eluted with acetonitrile from all the disks found no intermediates **B** and **C**. Recoveries of over 95% for dexrazoxane were found for the C-18 disk. No dexrazoxane was

recovered from the other disks. Analysis of the buffer solutions which passed through the disks found all the intermediates and dexrazoxane. All the disks were unable to retain the intermediates. Only the C-18 disk retained dexrazoxane until it was eluted off with acetonitrile.

Sep-Pak Plus C-8 and C-18 cartridges were used in an attempt to extract samples containing 10 µg/mL each of the intermediates **B** and **C** and dexrazoxane in pH 7.0 phosphate buffer. Reconstituted extracts eluted with acetonitrile from the cartridges found no intermediates. Good recoveries of over 95% were observed for dexrazoxane from both the C-8 and C-18 cartridges. The intermediates were not retained on the cartridges and passed through with the phosphate buffer used to load the samples.

Sep-Pak Plus QMA anion exchange cartridges were used to recover dexrazoxane and the intermediates at two different pH levels. Samples containing 10 µg/mL each of the intermediates **B** and **C** and dexrazoxane in 1 mM Tris buffer either at pH 6.0 and pH 7.0 were run through the cartridges. At pH 7.0, virtually all the intermediates **B** and **C** were in the reconstituted extracts eluted from the cartridges. However, no dexrazoxane was found in these extracts. Analysis of the buffer used to load the intermediates and dexrazoxane onto the cartridges was found to contain all of the dexrazoxane. At pH 6.0, the intermediates **B** and **C** and dexrazoxane were not found in reconstituted extracts eluted from the cartridges. All of the intermediates and dexrazoxane were recovered in the Tris buffer which was used to load the samples onto the cartridges.

4.2.3 Recovery of Dexrazoxane Hydrolysis Intermediates From Plasma

4.2.3.1 Sep-Pak QMA Anion Exchange Solid Phase Extraction Cartridges

Plasma samples containing 10 µg/mL each of dexrazoxane hydrolysis intermediates and dexrazoxane were processed in one of four ways. Once the

samples were processed, they were made up in Tris buffer (pH 7.0) before elution through Sep-Pak QMA anion exchange cartridges.

Sample 1 was treated with HCl to precipitate plasma proteins and Na_2EDTA to chelate Ca^{2+} or Mg^{2+} ions bound to dexrazoxane or its hydrolysis intermediates. No intermediates or dexrazoxane were recovered from the extract eluted from the anion exchange cartridge.

The plasma sample treated with acetonitrile to precipitate the plasma proteins had about 50% recovery of intermediates **B** and **C**. Dexrazoxane was not found in the extracts eluted from the cartridges. Neither of the hydrolysis intermediates nor dexrazoxane was recovered with the anion exchange cartridge for sample 3 containing phosphoric acid. Sample 4 was treated in the same manner as sample 2, but 3 mM Na_2EDTA was added to the sample to chelate divalent cations bound to dexrazoxane or its hydrolysis intermediates. No intermediates or dexrazoxane were recovered from the extract eluted with 100 mM HCl from the anion exchange cartridge.

4.2.3.2 Methods for Recovery of Dexrazoxane and its Hydrolysis Intermediates From Plasma

Only 50% of the hydrolysis intermediates **B** and **C** and dexrazoxane were recovered from the sample with hydrochloric acid protein precipitation and NaOH neutralization. The same level of recovery was also found for the sample precipitated with acetonitrile. Good recovery of the hydrolysis intermediates **B** and **C** and dexrazoxane was observed for the sample with acetonitrile protein precipitation and 3 mM Na_2EDTA. Good recovery was also found with the sample treated with acetonitrile and phosphoric acid added to prevent further hydrolysis of dexrazoxane.

4.2.4 HPLC Conditions for the Analysis of Dexrazoxane Hydrolysis Intermediates

Gradient 1 gave baseline separation of ADR-925, intermediates **B** and **C**

and dexrazoxane when used with new μBondapak C-18 columns. As the columns were used, the degree of resolution decreased over time and the intermediates eluted very closely together. Changing the pH of the Na_2EDTA solution from pH 4.5 to pH 3.5 increased the separation of the intermediates and ADR-925.

The use of 1-heptanesulfonic acid with pH 3.5 Na_2EDTA solution in the mobile phase gave even better resolution between ADR-925, and the intermediates **B** and **C**. The peak shape was also improved with no signs of tailing. For all later analysis, 1-heptanesulfonic acid was present in the mobile phase. The use of a Nova-pak 4.6 mm I.D. X 250 mm reversed-phase C-18 column with the same chromatographic conditions resulted in less separation between the intermediates **B** and **C**. The ADR-925 peak also eluted very close to the solvent front. The use of this column was discontinued and the μBondapak column originally used was also used for all later analysis.

4.2.5 HPLC Conditions for the Analysis of Dexrazoxane Hydrolysis Intermediates in Rat Plasma Samples

Plasma samples were prepared for analysis using method 3 in section 4.1.2.7. However, the samples were concentrated four-fold compared to the plasma concentration to increase the level of quantitation required for the animal study. Both blank and 10 μg/mL plasma samples were analyzed with gradient 3 as described in section 4.1.2.8.4. and at a detection wavelength of 205 nm. The concentrated blank plasma samples were found to contain an endogenous peak which eluted very closely to the intermediate **B** peak.

Plasma samples containing dexrazoxane and its intermediates were then analyzed using gradient 3 (see section 4.1.2.8.4), followed by post-column derivatization with 30 mM NaOH solution in a reaction bed. A pump delivered NaOH solution at a flow rate of 0.25 mL/min to a 3 way mixing tee

and mixed with the elutants coming off the HPLC column. Outflow from the tee went to a reaction bed consisting of tightly knitted flexible HPLC tubing The many twists and turns in the web created turbulent flow to ensure complete mixing between the NaOH solution and the compounds eluting off the column. The detection wavelength used was 227 nm. The endogenous plasma which co-eluted with the intermediate **B** peak was still present but there were less background interferences from other plasma peaks. Other wavelengths at 232, 235 and 240 nm were also tried. The interferences from the endogenous plasma peaks decreased substantially as the wavelength increased. The interfering plasma peak with intermediate **B** decreased only slightly at all the wavelengths tried. The size of the intermediate peaks and dexrazoxane decreased approximately 5% at 232 nm, 10% at 235 nm and 25% at 240 nm. The wavelength of 235 nm was selected for use as the optimum compromise between sensitivity and freedom from interferences from endogenous plasma components.

4.2.6 Calibration Plot for Dexrazoxane and its Hydrolysis Intermediates in Rat Plasma

To test the assay, calibration plots were prepared in treated plasma samples with a range of 5 to 50 µg/mL for intermediate **B**, 1 to 50 µg/mL for intermediate **C**, and 1 to 50 µg/mL for dexrazoxane. Data for intermediate **B** is listed in Table 4.1, intermediate **C**, is listed in Table 4.2 and dexrazoxane is listed in Table 4.3. The calibration plot for intermediates **B** and **C**, and dexrazoxane is illustrated in figure 4.2.

Table 4.1 Peak Area vs. Plasma Concentrations for Intermediate **B**

Concentration (μg/mL)	Average Peak Area (Arbitrary Units)	SEM (Arbitrary Units)
5	111,319	7.943
10	339,284	3,585
20	806,144	192
50	2,243,825	21,911

Table 4.2 Peak Area vs. Plasma Concentrations for Intermediate **C**

Concentration (μg/mL)	Average Peak Area (Arbitrary Units)	SEM (Arbitrary Units)
1	79,176	14,687
2	122,031	5,853
5	265,545	15,925
10	564,084	15,254
20	1,031,834	4171
50	2,526,960	14,365

Table 4.3 Peak Area vs. Plasma Concentrations for Dexrazoxane

Concentration (μg/mL)	Average Peak Area (Arbitrary Units)	SEM (Arbitrary Units)
1	59,908	1,267
2	121,920	820
5	342,869	439
10	860,323	1,358
20	1,558,115	39,305
50	4,298,172	20,793

Statistical analysis was performed on the calibration plots and listed in Table 4.4 for intermediates **B** and **C** and dexrazoxane.

Table 4.4 Linear Regression for Calibration Plot of Dexrazoxane and its Intermediates in Rat Plasma

	Intermediate **B**	Intermediate **C**	Dexrazoxane
Y-intercept ± SEM	-133,985 ± 7,237	31,644 ± 10,195	-61,676 ± 36,673
slope ± SEM $(\mu g/mL)^{-1}$	47,488 ± 263	49,997 ± 454	86,459 ± 1632
r^2-value	0.9999	0.9998	0.9986

The recovery of dexrazoxane and intermediates **B** and **C** from rat plasma samples were calculated at two different concentrations. The recoveries at 5 and 50 μg/mL are listed in Table 4.5.

Table 4.5 Recoveries of Intermediates **B**, **C** and Dexrazoxane From Rat Plasma Samples

	Intermediate **B** 5 μg/mL	Intermediate **C** 5 μg/mL	Dexrazoxane 5 μg/mL
Ave. Peak Area in Plasma ± SEM	111,319 ± 7,943	265,545 ± 15,925	342,869 ± 439
Ave. Peak Area of Standard ± SEM	278,395 ± 21,911	290,138 ± 13,819	463,330 ± 12,423
% Recovery	40.0	91.5	74.0

	Intermediate **B** 50 μg/mL	Intermediate **C** 50 μg/mL	Dexrazoxane 50 μg/mL
Ave. Peak Area in Plasma ± SEM	2,243,825 ± 21,911	2,526,960 ± 14,365	4,298,172 ± 20,793
Ave. Peak Area of Standard± SEM	2,723,697 ± 29,367	2,910,459 ± 26,895	4,432,460 ± 29,458
% Recovery	82.4	86.8	97.0

Calibration Plot For Dexrazoxane And Intermediates B and C In Rat Plasma

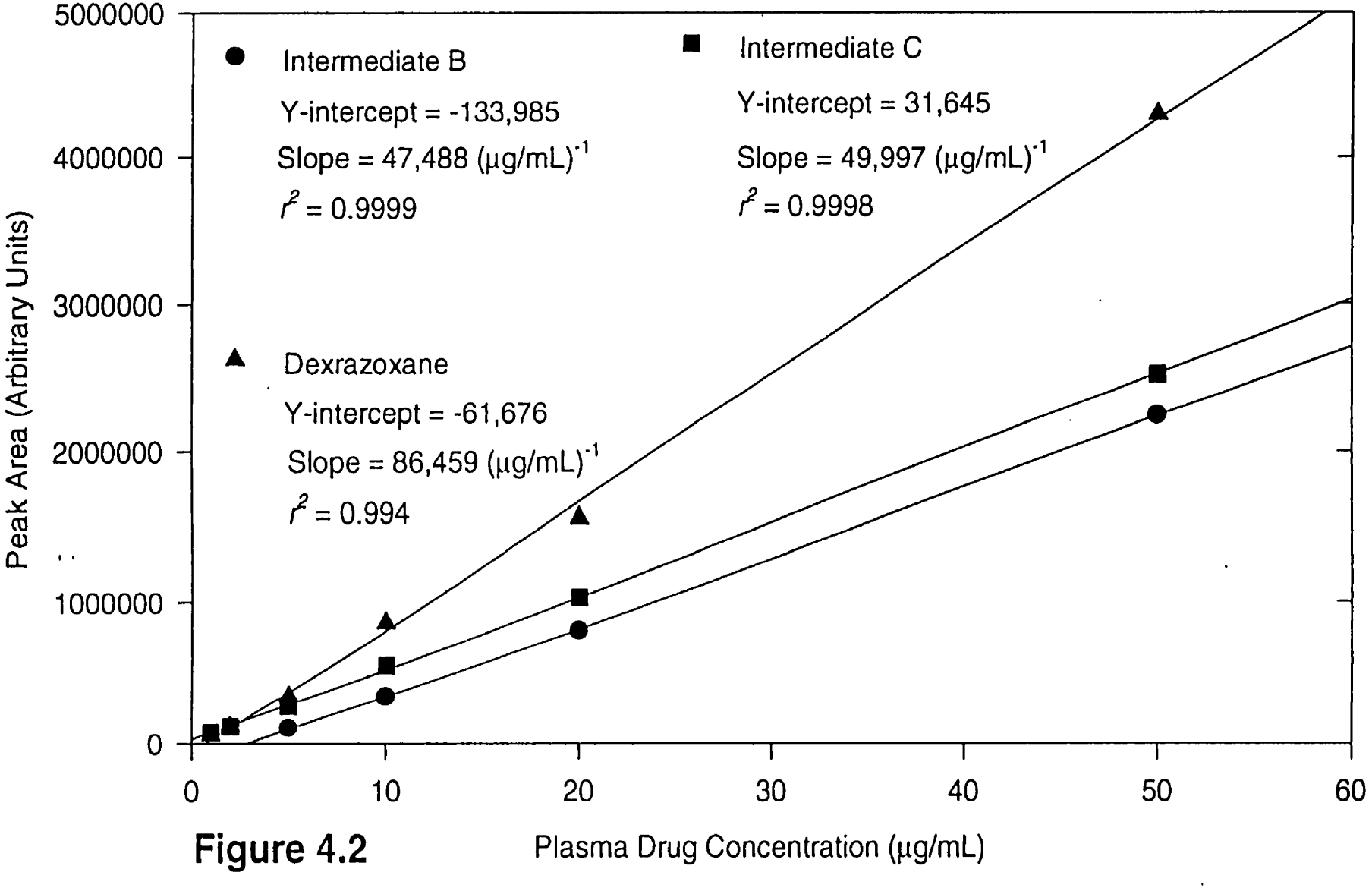


Figure 4.2

4.3 DISCUSSION

4.3.1 HPLC Quantitation of the Sodium Hydroxide and Potassium Phosphate Hydrolysis Intermediate Products of Dexrazoxane

The dexrazoxane intermediates were produced by NaOH hydrolysis at 25°C or in pH 7.4 phosphate buffer at 37°C. Intermediates **B** and **C** were produced in nearly equimolar concentrations in the NaOH solution. In the pH 7.4 phosphate buffer, 3.45 times more of intermediate **B** was produced than for intermediate **C**. These results agree with data from Hasinoff (1993, 1994a).

4.3.2 Evaluation of Solid Phase Extraction Methods for the Recovery of Dexrazoxane Hydrolysis Intermediates in Buffer

All reversed-phase solid phase extraction cartridges tried were unable to retain the dexrazoxane hydrolysis intermediates. Analysis of the buffers which passed through the cartridges found all of the intermediates. The dexrazoxane hydrolysis intermediates were probably too polar to be retained by the reversed-phase material. Recoveries of over 95% for dexrazoxane was found for the Empore C-8 and C-18 disks and the Sep-Pak C-8 and C-18 cartridges. Several methods have been published for the solid phase extraction of dexrazoxane from plasma using either C-8 or C-18 cartridges (Sisco, 1989; Lewis *et al.*, 1992; Jakobsen *et al.*, 1994)

The Sep-Pak QMA anion exchange cartridges were able to completely recover intermediates **B** and **C** and ADR-925 at pH 7.0. Changing the pH of the Tris buffer to 6.0 resulted in recoveries of less than 10% for intermediates **B** and **C** and ADR-925. The results suggested that the Sep-Pak QMA cartridges are sensitive to the pH of the buffer used to load the samples. The QMA cartridges did not retain dexrazoxane, which is not surprising as the drug is not anionic under the conditions used. The QMA cartridges were then used

in an attempt to extract the hydrolysis intermediates of dexrazoxane from rat plasma.

4.3.3 Recovery of Dexrazoxane Hydrolysis Intermediates From Plasma

4.3.3.1 Sep-Pak QMA Anion Exchange Solid Phase Extraction Cartridges

The dexrazoxane hydrolysis intermediates **B** and **C** in the plasma sample that used 6 M HCl to precipitate proteins were not recovered with the QMA anion exchange cartridge. The high levels of chloride in the sample may have interfered with the anion exchange process.

The plasma sample treated with acetonitrile gave 50% recovery of the intermediates **B** and **C** with the QMA cartridges. The addition of Na_2EDTA resulted in no recovery of either of the intermediates. The addition of phosphoric acid also resulted in no recovery of the intermediates. The lack of recovery of the intermediates is probably due to anionic EDTA and phosphate ion displacing the dexrazoxane intermediates on the anionic cartridges. Due to the low drug recoveries, the QMA anion exchange cartridges were not used for further experiments.

4.3.3.2 Methods for Recovery of Dexrazoxane and its Hydrolysis Intermediates From Plasma

The partial recovery of the hydrolysis intermediates **B** and **C** from the plasma sample treated with either HCl or acetonitrile protein precipitation may be due to Ca^{2+} or Mg^{2+} ions binding with the intermediates. The addition of 3 mM Na_2EDTA gave almost complete recovery of the intermediates. The intermediates are good chelating agents (Buss and Hasinoff, 1993). The addition of phosphoric acid also gave good recovery of the intermediates. Good recoveries of the intermediates may also be due to the phosphoric acid preventing the binding of the intermediates to metal ions present in the plasma.

The four methods tried for the separation of dexrazoxane and its

hydrolysis intermediates from plasma were evaluated in their ability to recover the drugs. The method of acetonitrile protein precipitation and addition of Na_2EDTA gave the best recoveries of dexrazoxane and its hydrolysis intermediates from plasma and was chosen for use in the treatment of plasma samples in the rat study.

4.3.4 HPLC Conditions for the Analysis of Dexrazoxane Hydrolysis Intermediates

The HPLC conditions used gave good separation of the intermediates **B** and C when used with a μBondapak C-18 column. ADR-925 eluted quite close to the solvent peak. Changing the pH of the Na_2EDTA solution from pH 4.5 to 3.5 improved the separation between the intermediates and ADR-925. This is probably due to a carboxylic acid functional group in the intermediates and two carboxylic acid groups in the ADR-925 structure. The addition of the ion pair reagent 1-heptanesulfonic acid to pH 3.5 Na_2EDTA improved the peak shapes of the drugs and the separation of ADR-925 from the solvent front. The separation between the intermediates also improved. Again, interactions between 1-heptanesulfonic acid and carboxylic acid functional groups in the intermediates and the ADR-925 structure were probably responsible for the increase in separation. For all later analyses, 1-heptanesulfonic acid was present in the mobile phase. The use of a Nova-pak C-18 column resulted in less separation between the two intermediates. It was originally thought the smaller particle size of the Nova-pak column would result in better separation between the intermediates. For all future analyses, the μBondapak column was used.

4.3.5 HPLC Conditions for the Analysis of Dexrazoxane Hydrolysis Intermediates in Plasma Samples

Analysis of concentrated plasma samples with the previously discussed HPLC mobile phase conditions and a detection wavelength of 205 nm found a

large endogenous plasma peak which co-eluted with intermediate **B**. Hasinoff (1990) had found that a maximum absorption peak for dexrazoxane shifts from 205 nm in neutral pH buffers to a maximum peak at 227 nm under more basic conditions. The mechanism is the weakly acidic imide group which ionizes to its anionic form in basic pH. It was also found that the absorption value at basic pH increases approximately 2.25 times over the value found at pH 7.4. Changing the detection wavelength from 205 nm to other higher values reduced the interferences from plasma components.

A method was developed for the post column reaction of dexrazoxane and its hydrolysis intermediates with 30 mM NaOH solution. A pump was used to deliver NaOH at a flow rate of 0.25 mL/min where the solution was mixed with the elutants coming off the HPLC column in a 3 way mixing tee. To ensure complete mixing between the NaOH and the compounds eluting from the column, outflow from the tee went to a reaction bed consisting of tightly knitted flexible HPLC tubing. The many twists and turns in the web created turbulent flow so mixing between the solutions occurred.

Analysis of concentrated plasma blanks and samples containing the dexrazoxane intermediates at wavelengths of 227, 232, 235 and 240 n m detected less plasma interferences at the higher wavelengths. The peaks for the intermediates and dexrazoxane also decreased. The wavelength of 235 n m was picked as a compromise between reduced plasma interferences and reduced sensitivity for the intermediates.

4.3.6 Calibration Plot of the Dexrazoxane Hydrolysis Intermediates B and C, and Dexrazoxane

The retention times for intermediate **B**, **C** and dexrazoxane were 6, 7 and 17 min respectively for the HPLC conditions used to analyze these samples. An endogenous plasma peak co-eluted with the intermediate **B** peak

but a highly linear calibration plot from 5 to 50 μg/mL was still obtained (r^2 value of 0.9999). Highly linear calibration plots were obtained for intermediate **C** and dexrazoxane from 1 to 50 μg/mL (r^2 values of 0.9998 and 0.9986 respectively).

4.3.7 Recoveries of Dexrazoxane and its Intermediates B and C From Rat Plasma Samples

The recoveries of dexrazoxane and its intermediates **B** and **C** from rat plasma were calculated for 2 concentrations. At a concentration of 5 μg/mL, the recovery of intermediate **B** was 40.0% or approximately half of the recovery values for intermediate **C** or dexrazoxane. Intermediate **B** has a similar molecular structure and identical molecular weight to intermediate **C**, suggesting that the recoveries from plasma should also be similar. This low recovery value is probably due to the co-elution of an endogenous plasma peak which affected integration of the intermediate **B** peak. The peak area of intermediate **B** was manually integrated as described in Section 4.1.1.2.1. When the intermediate **B** peak is automatically integrated by the Varian Star software, area from the endogenous plasma peak is also included, resulting in a total peak area much larger than the peak area for the intermediate **B** standard at the same concentration. At the high concentration of 50 μg/mL, the endogenous plasma peak does not interfere to the same degree, as suggested by a recovery of 82% for intermediate **B**. Since the calibration plot samples were prepared in rat plasma and processed in the same manner as for the rat metabolism study, this should compensate for low recoveries at the low end of the concentration scale. The calibration plot for intermediate **B** was linear with an r^2 value of 0.9999.

Chapter 5

Metabolism of Dexrazoxane to its Hydrolysis Intermediates in the Rat

5.0 Introduction to the *In Vivo* Metabolism of Dexrazoxane to Its Hydrolysis Intermediates

The experiments in this chapter examine the metabolism of dexrazoxane to its hydrolysis intermediates in the rat. An *in vivo* study was conducted with the i.v. administration of dexrazoxane to male Sprague-Dawley rats. Dexrazoxane and its hydrolysis intermediates were separated from rat plasma with a simple acetonitrile protein precipitation step. Plasma samples were then analyzed using a reversed-phase C-18 HPLC method and post-column derivatization with NaOH solution.

The plasma concentrations of dexrazoxane and its intermediates **B** and **C** (fig. 1.2) were studied up to 180 min. The plasma concentration ratios **B/C** were calculated over time. These ratios were compared with the product ratio **B/C** for dexrazoxane reacted with the enzyme DHPase in a previous study (Hasinoff, 1993). DHPase is believed to be responsible for the enzyme catalyzed hydrolysis of dexrazoxane to the intermediates **B** and **C** (Hasinoff *et al.*, 1991; Hasinoff, 1993).

5.1 EXPERIMENTAL

5.1.1 Materials

5.1.1.1 Chemicals

Dexrazoxane (lot 91D23FY and lot 91N03A) was a gift from Pharmacia & Upjohn (Columbus, OH). Concentrated phosphoric acid, potassium hydroxide, potassium dihydrogen phosphate and sodium hydroxide were obtained from Fisher Scientific (Nepean, Canada). HPLC grade acetonitrile and methanol, volumetric grade hydrochloric acid and sodium hydroxide solutions were obtained from Mallinckrodt (Mississauga, Canada). Saline solution (0.9% wt/vol sodium chloride USP), was obtained from Astra Pharmaceuticals, Inc. (Mississauga, Canada). Heparin, and 1-heptanesulfonic acid sodium salt were from Sigma Chemical Co. (St. Louis, MO). AnalaR grade ethylenediaminetetraacetic acid disodium salt was from BDH Chemicals Ltd. (Poole, England). Ketamine, pentobarbital sodium salt and xylazine were obtained from Central Animal Care Services, University of Manitoba. Lidocaine HCl 2% (wt/vol) solution with 0.05% (wt/vol) epinephrine was obtained from MTC Pharmaceuticals (Cambridge, ON).

5.1.1.2 Animals

Six male Sprague-Dawley rats were obtained from the Central Animal Care Services, University of Manitoba. Rats with weights between 320 and 560 grams were used in the i.v. administration study. The rats were allowed food and water *ad libitum* prior to the study. The animals were transported from the animal care facility to the Faculty of Pharmacy immediately before the start of the study. The animals were not acclimatized before the study was started.

5.1.1.3 Instrumentation

5.1.1.3.1 Chromatographic

The HPLC system consisted of a Varian 9010 pump, Varian Star integration software, and a Rheodyne injector. The system was fitted with a

Brownlee Labs NewGuard column containing an 1.5 cm X 3.2 mm I.D. reversed-phase C-18 guard cartridge (Applied Biosystems, Santa Clara, CA, USA). A μBondapak 4.6 mm I.D. X 250 mm reversed-phase C-18 column with a particle size of 10 μm (Waters Chromatography, Mississauga, ON) was used for analysis. For the detection of the post column derivatives of the sodium hydroxide hydrolysis products of dexrazoxane, a Varian 9050 programmable wavelength UV detector was used. The wavelength used was 235 nm for analysis of plasma samples from the animal study and the calibration plot. The sample loop volume was 50 μL.

For most analyses, peak areas were calculated automatically using the Varian Star integration software. For the analysis of the animal study and the calibration plot, peak areas were calculated manually using the Varian Star integration software. Manual integration was used to give more consistent results among chromatograms. The start of the intermediate **B** peak was defined as 0.60 min before the retention time of 6.0 min for the maximum amplitude of the peak. When intermediates **B** and **C** eluted closely together, the end of peak **B** and the start of peak **C** was defined as the lowest point in the valley. The end time for peak **C** was defined as 0.60 min after the retention time of 7.0 min for the maximum amplitude of the peak. The start of the dexrazoxane peak was defined as 16.4 min and the end of the peak was set to 17.6 min.

5.1.1.3.2 Post-Column Derivatization With Sodium Hydroxide

An Eldex A-30-S-Peek pump (Eldex Laboratories, Inc., San Carlos, CA) was used to deliver 30 mM NaOH solution at a flow rate of 0.25 mL/min through a pulsation dampener (Varian Scientific) to a 3-way mixing tee (Upchurch Scientific, Oak Harbor, WA). The analytes eluting from the HPLC column were also connected to the mixing tee. The outflow of the tee was then attached to a reaction bed. The bed consisted of ~0.5 m of 0.02 mm I.D. flexible

HPLC tubing tightly knitted into a rectangle approximately 3 cm X 4 cm. The outflow from the reaction web went directly to the HPLC UV-detector. (Refer to figure 4.1).

5.1.1.4 Statistical Analysis

Statistical analysis of the animal and incubation data was performed using SigmaStat for Windows (Jandel Scientific, 1994). Paired one-tailed *t*-tests were used to analyze the data.

5.1.2 Methods

5.1.2.1 Preparation of Solutions

The NaOH hydrolysis intermediate products of dexrazoxane were prepared as described in section 4.1.2.1 and analyzed by the method in section 4.1.2.3. Na_2EDTA (pH 4.5) solution, Na_2EDTA (pH 3.5) solution and 1-heptanesulfonic acid were prepared according to sections 4.1.2.8.1, 4.1.2.8.2 and 4.1.2.8.3, respectively.

5.1.2.2 HPLC Analysis of Dexrazoxane i.v. Solution

The dexrazoxane used in the rat metabolism study was analyzed for the amount of the hydrolysis intermediates present as impurities. Two lots of dexrazoxane were analyzed, lot 91D23FY and lot 91N03A. A 1000 µg/mL solution of each lot of dexrazoxane was prepared in 0.9% (wt/vol) saline and analyzed as described previously in section 4.1.2.3.

5.1.2.3 HPLC Method for the Analysis of Dexrazoxane Hydrolysis Intermediates in Rat Plasma

The gradient used for HPLC analysis is described in section 4.1.2.8.5 The outflow from the C-18 column was derivatized with NaOH solution as described in section 5.1.1.4.2.

5.1.2.4 Rat Study

5.1.2.4.1 Surgical Procedure: Femoral Vein Cannulation

The rat was anesthetized with a combination of ketamine (90 mg/kg) and

xylazine (10 mg/kg) administered i.p. The degree of anesthesia was checked by gently stretching out the front limb and checking if the rat retracted the limb. The blink response was also examined by gently touching the eye with the tip of a blunt probe. Surgery was performed only after the rat's response to both stimuli was negative. Through out the study, the rat was kept warm by a 60 watt lamp 20 cm from the rat. Breathing and response to stimuli were monitored throughout the study.

A 4 cm transverse incision was made 2 cm below the rib cage to the right leg to penetrate the skin. The skin and fat were gently separated from the muscle surface. Saline (0.9% wt/vol NaCl) was used to moisten the surface. The femoral artery and vein were located and gently separated from each other with the tip of a 6 cm hemostat. Two pieces of 3-O surgical silk were loosely tied around the vein 1 cm apart. A 0.5 x 3 cm piece of cardboard was placed underneath the isolated femoral vein between the surgical silk. Several drops of lidocaine solution were applied to the vein and surrounding area. Gentle tension was applied on the front piece of silk and a 5/8" 24 gauge Jelco winged catheter (Critikon Inc., Tampa FL) was slowly inserted into the vein until it stopped. The internal wire guide was removed, and blood flow was observed. To prevent clotting, 100 µL of saline containing heparin (20 units/mL) was injected into the cannula before it was closed.

5.1.2.4.2 Dosing Protocol: Intravenous Administration of Dexrazoxane

The dosage for each rat was 40 mg/kg in a 10 mg/mL solution of dexrazoxane dissolved in 0.9% (wt/vol) saline solution. For 4 rats, the dexrazoxane used was lot 91D23FY. For 2 other rats, lot 91N03A was used. The dexrazoxane solution was administered via an i.v. infusion through the femoral vein cannula. For a 400 g rat, the volume of drug infused was 1.6 mL and occurred over a period of 30 s. After the infusion, 100 µL of saline containing

heparin (20 units/mL) were injected into the cannula before it was closed.

5.1.2.4.3 Blood Collection

Before each blood collection, several drops of lidocaine were applied to the femoral vein and surrounding area. A 1 mL sample of blood was taken through the femoral vein cannula prior to the administration of dexrazoxane. This sample was used as a blank to determine if endogenous compounds eluted at the same time as dexrazoxane and its hydrolysis intermediates. One mL aliquots of blood were removed at 5, 15, 30, 60, 120 and 180 min post infusion. At the end of this study, the rats did not appear to be in hypovolemic shock. All rats were still alive at the end of the study. Immediately after blood collection, fluid volume was replaced by infusing 1 mL of 0.9% (wt/vol) saline. A 100 µL aliquot of saline containing heparin (20 units/mL) was injected into the cannula before it was closed to prevent clotting.

The blood samples (1 mL aliquots) were added to 1.5 mL polyethylene microcentrifuge tubes containing 15 µL of heparin in saline (1000 units per mL of saline solution). The blood was immediately centrifuged at 11,750 *g* for 2 min. The plasma was removed and an aliquot of 20 µL of 500 mM HCl per mL of plasma was added to prevent further hydrolysis of dexrazoxane. The treated plasma samples were then stored at -80°C. After the last blood collection, the animals were euthanized with pentobarbital sodium salt at a dosage of 22 mg/kg, administered by i.v. into the femoral cannula.

5.1.2.4.4 Preparation of Dexrazoxane Hydrolysis Intermediate Standard Solutions

Three dexrazoxane NaOH hydrolysis intermediate standard solutions with concentrations of 5, 50 and 500 µg/mL each of dexrazoxane and intermediates **B** and **C** were prepared. For the 500 µg/mL standard, a 250 µL aliquot of the dexrazoxane hydrolysis intermediate stock solution was added to a

1.5 mL microcentrifuge tube and made up to 500 µL with 10 mM HCl. The 50 µg/mL standard was made by taking a 50 µl aliquot of the 500 µg/mL standard solution and making up to 500 µL with 10 mM HCl in a 1.5 mL microcentrifuge tube. The 5 µg/mL standard was prepared by taking a 50 µl aliquot of the 50 µg/mL standard solution and made up to 500 µL with 10 mM HCl in a 1.5 mL microcentrifuge tube.

5.1.2.4.5 Preparation of the Calibration Curve

Aliquots of dexrazoxane hydrolysis intermediates calibration standard solutions were added to 500 µL volumes of acidified (20 µL of 500 mM hydrochloric acid per mL of plasma) blank plasma. The final dexrazoxane hydrolysis intermediates concentration in plasma after extraction and reconstitution was from 1 µg/mL to 500 µg/mL. For the 1 and 2 µg/mL plasma calibration samples, 25 and 50 µL respectively of the 5 µg/mL hydrolysis intermediates standard solution were added. For the 5, 10 and 20 µg/mL plasma calibration samples, 12.5, 25 and 50 µL respectively of the 50 µg/mL hydrolysis intermediates standard solution were added. For the 50, 100 and 200 µg/mL plasma calibration samples, 12.5, 25 and 50 µL respectively of the 500 µg/mL hydrolysis intermediates standard solution were added. For the 500 µg/mL plasma calibration sample, 62.5 µL of the hydrolysis intermediates stock solution were added. A blank plasma sample was also extracted using plasma free of dexrazoxane and treated with 20 µL of 500 mM HCl per mL of plasma.

5.1.2.4.6 Separation of Dexrazoxane Hydrolysis Intermediates From Plasma

The plasma samples were thawed at room temperature before use. In a 1.5 mL microcentrifuge tube, 37 µL of 200 mM Na_2EDTA solution were added to 500 µL of acidified plasma (20 µL of 500 mM HCl per mL of plasma). The Na_2EDTA released any of the hydrolysis intermediates of dexrazoxane that may have complexed with Ca^{2+} and Mg^{2+} ions present in the plasma. A 750 µL aliquot of

acetonitrile was added to precipitate any proteins present in the sample. Samples were vortexed at medium speed, centrifuged at 14,000 *g* for 1 min and the supernatant removed to another microcentrifuge tube. The pellet was rinsed two times with 100 µL acetonitrile and the washings transferred to the supernatant that had been removed. The pellet was then discarded. The acetonitrile supernatant was evaporated to dryness in a 40°C water bath by a stream of argon gas. The tubes were closed and then stored at -80°C. Samples were allowed to warm to room temperature and then reconstituted in 125 µL of 10 mM HCl just prior to analysis. The plasma samples were concentrated 4 times for analysis. Two 60 µL aliquots of each rat plasma sample were analyzed by HPLC.

5.2 RESULTS

5.2.1 HPLC Quantitation of Dexrazoxane and the Sodium Hydroxide and Potassium Phosphate Hydrolysis Intermediate Products of Dexrazoxane

The NaOH hydrolysis intermediate products of dexrazoxane contained equimolar concentrations of intermediates **B** and **C** and dexrazoxane. About 1 mg/mL of each compound was in the NaOH hydrolysis mixture.

The phosphate buffer (pH 7.4) hydrolysis intermediate products of dexrazoxane contained equimolar concentrations of intermediate **B** and dexrazoxane. There was approximately 3.45 times more **B** than **C**. About 1.3 mg/mL of dexrazoxane, 1.4 mg/mL of compound **B** and 0.4 mg/mL of compound **C** were in the KH_2PO_4 hydrolysis mixture.

5.2.2 HPLC Analysis of Dexrazoxane i.v. Solution

The dexrazoxane i.v. solution used in the rat metabolism study was analyzed for the amount of the hydrolysis intermediates present as contaminants. Two lots of dexrazoxane were analyzed, lot 91D23FY used in the first study of 4 rats and lot 91N03A used in the second study of 2 rats. A 1000 µg/mL solution of each lot of dexrazoxane was prepared in 0.9% (wt/vol) saline

and was analyzed. Dexrazoxane lot 91D23FY used in the study of 4 rats contained 0.577% (wt/wt) intermediate **B** and 0.378% (wt/wt) intermediate **C** for a 10 mg/mL solution of dexrazoxane. Dexrazoxane lot 91N03A used in the second study of 2 rats contained 0.056% (wt/wt) intermediate **B** and 0.0206% (wt/wt) intermediate **C** in a 10 mg/mL solution of dexrazoxane dissolved in 0.9% (wt/vol) saline.

5.2.3 HPLC Quantitation of Dexrazoxane and its Hydrolysis Intermediate Products in Rat Plasma Samples

A simple method was used to treat plasma samples before analysis by HPLC and is described in section 5.1.2.4.6. Recoveries of dexrazoxane and the intermediates **B** and **C** were good and listed in Table 4.5.

The HPLC method used for analysis of dexrazoxane and its hydrolysis intermediates is described in section 5.1.2.3. The outflow from the C-18 HPLC column was then reacted by post column derivatization with 30 mM NaOH. The NaOH solution was delivered at a flow rate of 0.25 mL/min and reacted with the elutants in a reaction bed (Refer to section 5.1.1.3.3. A diagram of the post-column derivatization apparatus is illustrated in figure 4.1). The detection wavelength used was 235 nm.

A typical chromatogram for a blank plasma sample and plasma samples for rat 1 at 5, 60 and 180 min are shown in figures 5.1 to 5.4. Endogenous plasma peaks eluted with ADR-925, so no determination of ADR-925 was possible. An endogenous plasma peak eluted at 5.4 min, just before intermediate **B**, which eluted at 6.0 min. At lower concentrations of intermediate **B** there was some interference from this plasma peak. The intermediate **C** peak was well resolved from both the intermediate **B** peak and endogenous plasma components and eluted at 6.9 min. Dexrazoxane eluted at 17 min and was also resolved from endogenous plasma components.

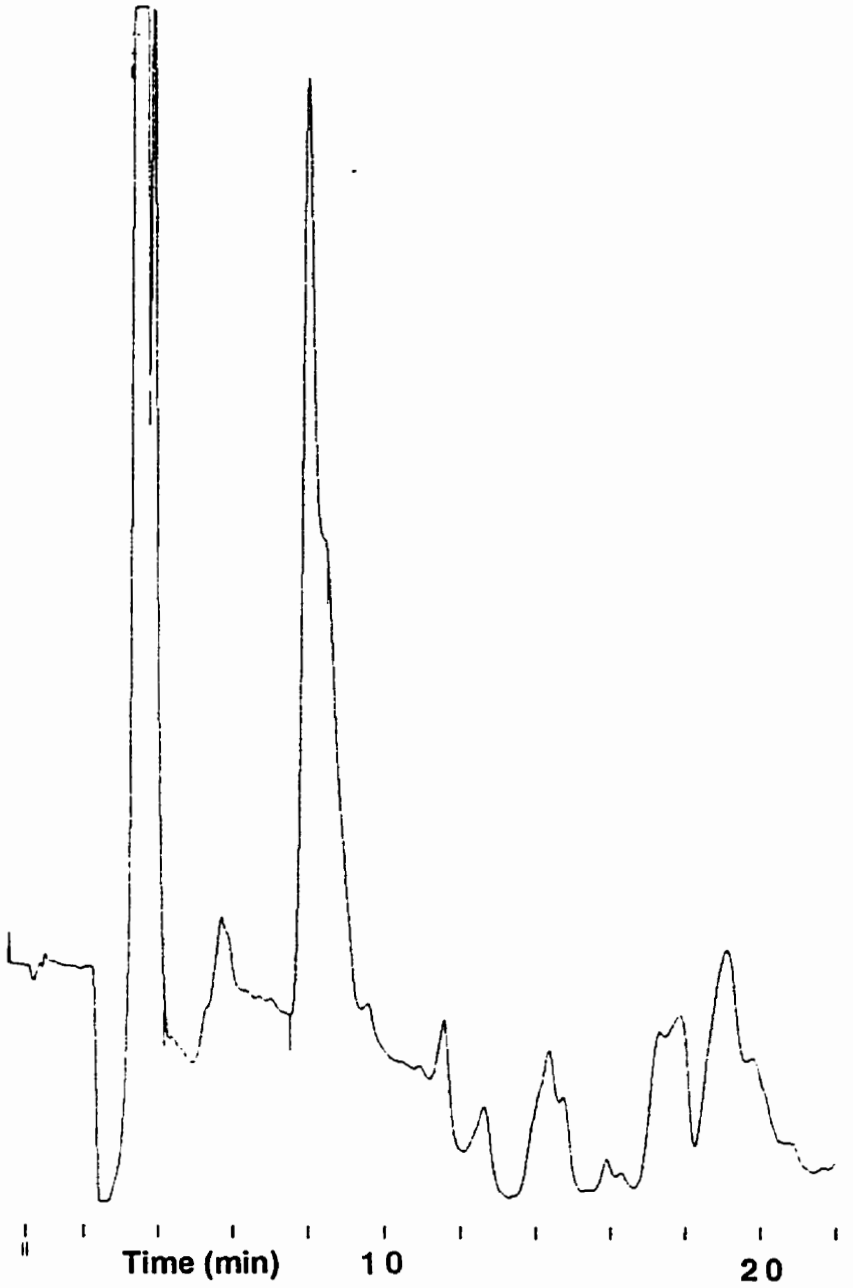


Figure 5.1 Chromatogram for a plasma sample from Rat 1, 5 min before i.v. administration of dexrazoxane at a dose of 40 mg/kg. The sample was concentrated 4 times and reconstituted in 125 µL of 10 mM HCl. The gradient conditions used were: 0 min, 500 µM EDTA (pH 3.5)/20 mM 1-heptanesulfonic acid (90/10); 10 min, EDTA (pH 3.5)/1-heptane sulfonic acid/methanol (82/10/8 v/v/v). The mobile phase conditions at 10 min were held for another 14 min until the end of the HPLC run. HPLC flow rate was 1.0 mL/min and the column used was a 3.9 X 300 mm C-18 µBondapak column. Post column reaction with 30 mM NaOH at a flow rate of 0.25 mL/min was used for the analytes eluted off the HPLC column. Detection wavelength was 235 nm.

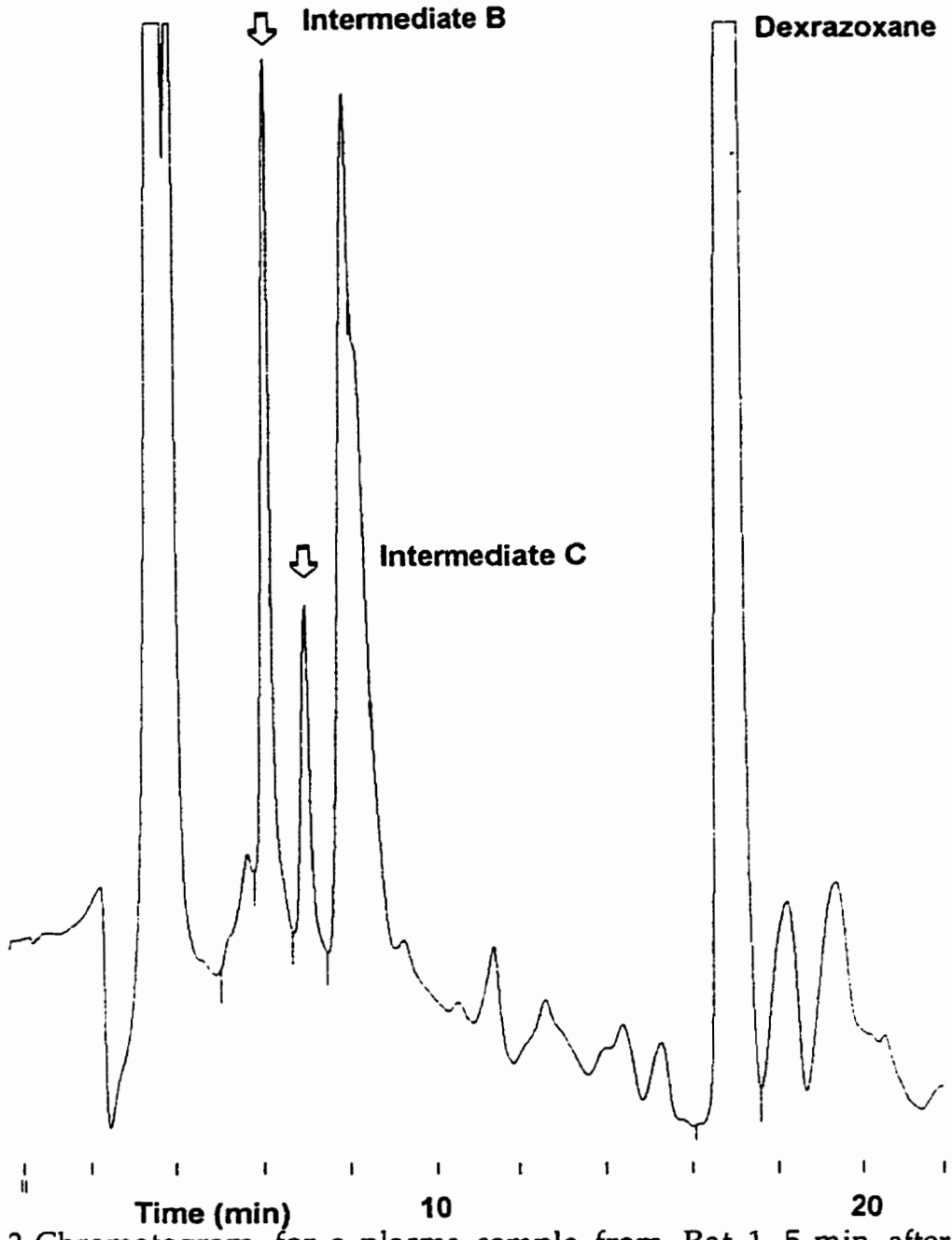


Figure 5.2 Chromatogram for a plasma sample from Rat 1, 5 min after i.v. administration of dexrazoxane at a dose of 40 mg/kg. The sample was concentrated 4 times and reconstituted in 125 µL of 10 mM HCl. The gradient conditions used were: 0 min, 500 µM EDTA (pH 3.5)/20 mM 1-heptanesulfonic acid (90/10); 10 min, EDTA (pH 3.5)/1-heptane sulfonic acid/methanol (82/10/8 v/v/v). The mobile phase conditions at 10 min were held for another 14 min until the end of the HPLC run. HPLC flow rate was 1.0 mL/min and the column used was a 3.9 X 300 mm C-18 µBondapak column. Post column reaction with 30 mM NaOH at a flow rate of 0.25 mL/min was used for the analytes eluted off the HPLC column. Detection wavelength was 235 nm.

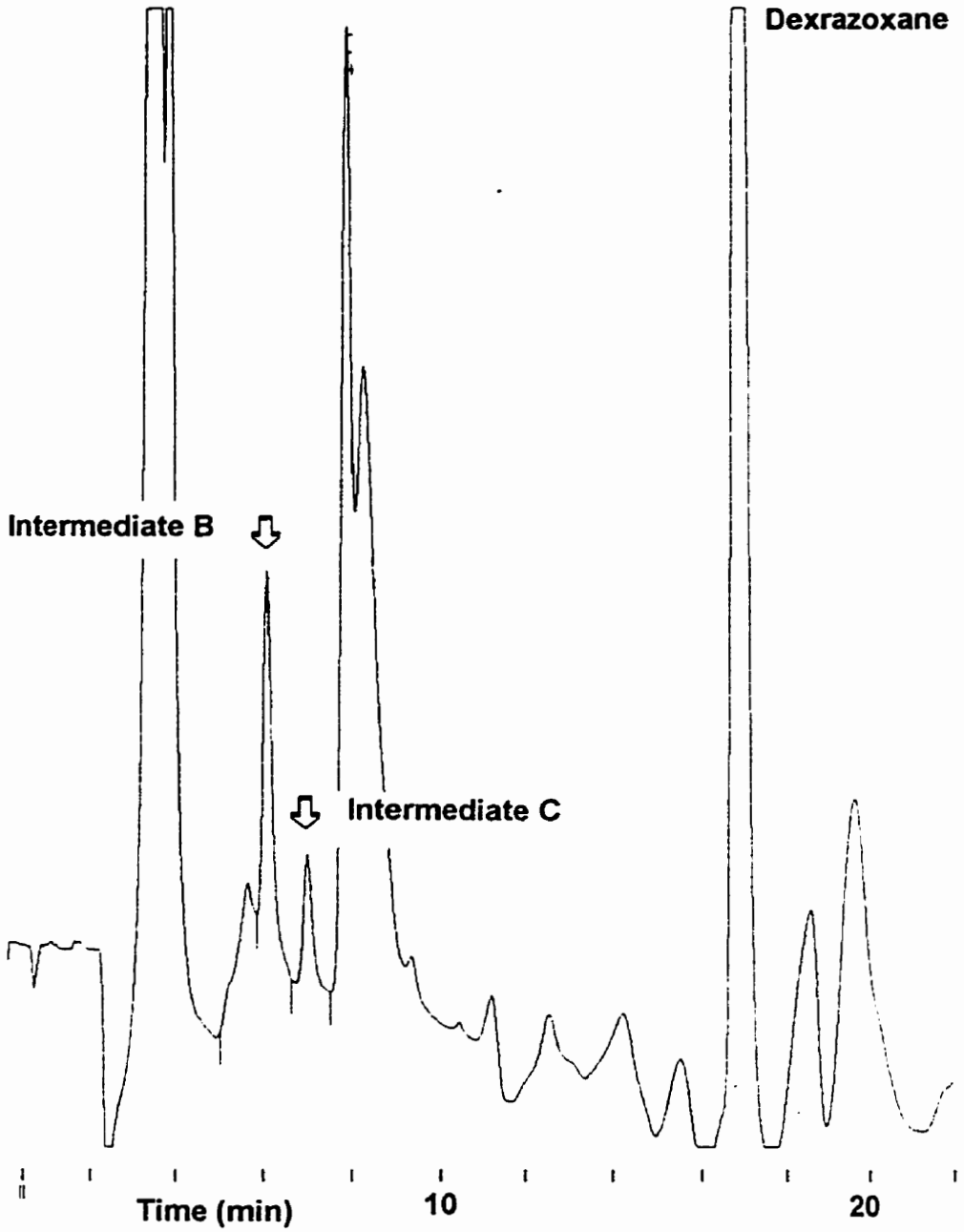


Figure 5.3 Chromatogram for a plasma sample from Rat 1, 15 min after i.v. administration of dexrazoxane at a dose of 40 mg/kg. The sample was concentrated 4 times and reconstituted in 125 µL of 10 mM HCl. The gradient conditions used were: 0 min, 500 µM EDTA (pH 3.5)/20 mM 1-heptanesulfonic acid (90/10); 10 min, EDTA (pH 3.5)/1-heptane sulfonic acid/methanol (82/10/8 v/v/v). The mobile phase conditions at 10 min were held for another 14 min until the end of the HPLC run. HPLC flow rate was 1.0 mL/min and the column used was a 3.9 X 300 mm C-18 µBondapak column. Post column reaction with 30 mM NaOH at a flow rate of 0.25 mL/min was used for the analytes eluted off the HPLC column. Detection wavelength was 235 nm.

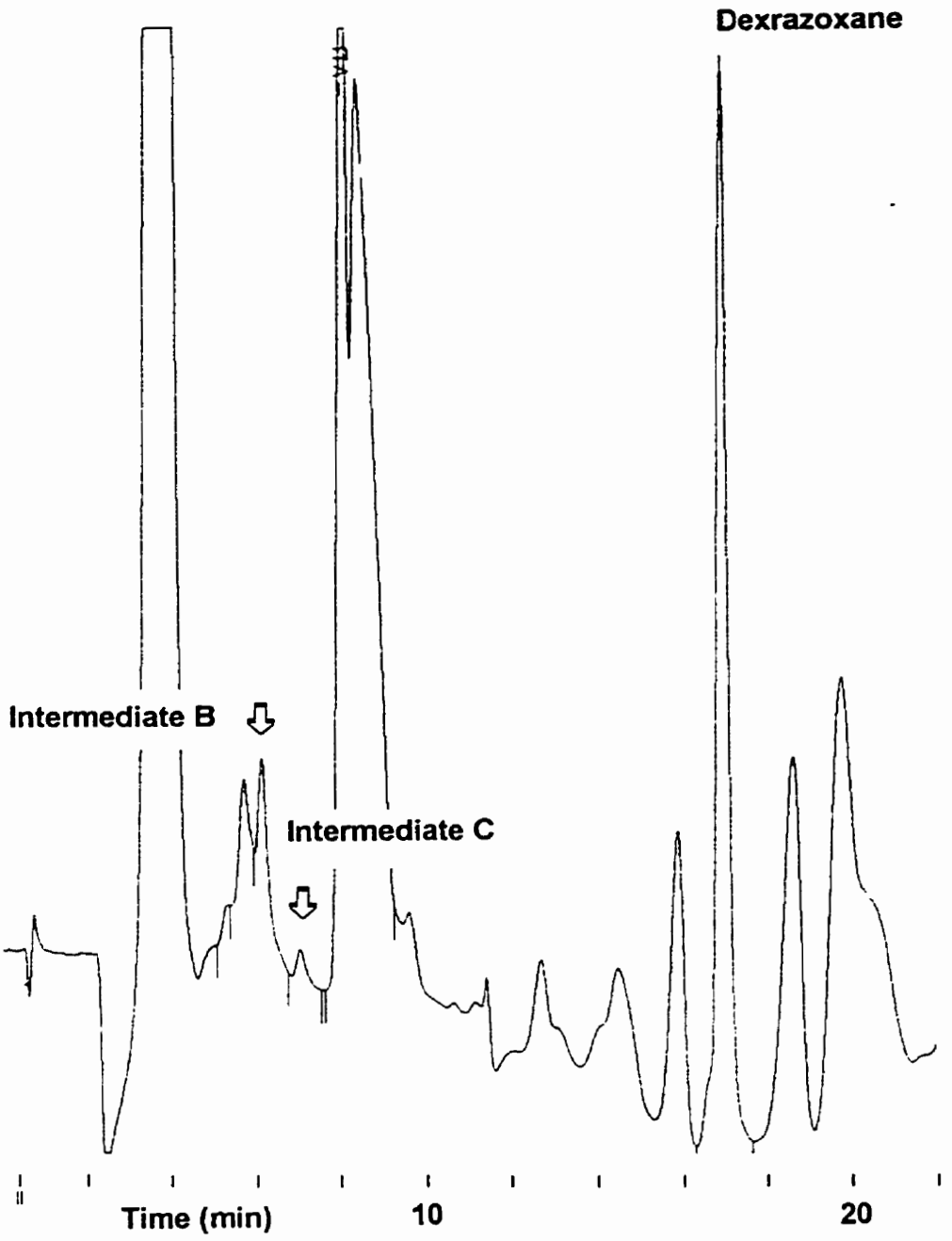


Figure 5.4 Chromatogram for a plasma sample from Rat 1, 180 min after i.v. administration of dexrazoxane at a dose of 40 mg/kg. The sample was concentrated 4 times and reconstituted in 125 µL of 10 mM HCl. The gradient conditions used were: 0 min, 500 µM EDTA (pH 3.5)/20 mM 1-heptanesulfonic acid (90/10); 10 min, EDTA (pH 3.5)/1-heptane sulfonic acid/methanol (82/10/8 v/v/v). The mobile phase conditions at 10 min were held for another 14 min until the end of the HPLC run. HPLC flow rate was 1.0 mL/min and the column used was a 3.9 X 300 mm C-18 µBondapak column. Post column reaction with 30 mM NaOH at a flow rate of 0.25 mL/min was used for the analytes eluted off the HPLC column. Detection wavelength was 235 nm.

Calibration plots were prepared by adding dexrazoxane to blank plasma samples with a range of 5 to 50 μg/mL for intermediate **B**, 1 to 50 μg/mL for intermediate **C**, and two ranges for dexrazoxane. A low concentration range calibration plot was prepared from 1 to 50 μg/mL and a high concentration range from 20 to 500 μg/mL. Details on the calibration plot for the intermediates and the low concentration range for dexrazoxane can be found in section 4.2.6. Data for intermediate **B** are listed in Table 4.1, intermediate **C**, in Table 4.2 and dexrazoxane in Table 4.3 for the low concentration range and Table 5.1 for the high concentration range. The low range calibration plot for intermediates **B** and **C**, and dexrazoxane is illustrated in figure 4.2. The high range concentration plot for dexrazoxane can be seen in figure 5.5.

Table 5.1 Peak Area vs. Plasma Concentrations For Dexrazoxane (High Concentration)

Concentration (μg/mL)	Average Peak Area (Arbitrary Units)	SEM (Arbitrary Units)
20	1,558,115	39,305
50	4,295,172	20,793
100	8,919,945	65,421
250	19,693,570	226,290
500	34,109,255	734,665

Table 5.2 Linear Regression For Calibration Plot of Dexrazoxane (High Concentration)

Y-intercept ± SEM	1,342,542 ± 856,051
slope ± SEM $(\mu g/mL)^{-1}$	67,243 ± 3356
r^2-value	0.9963

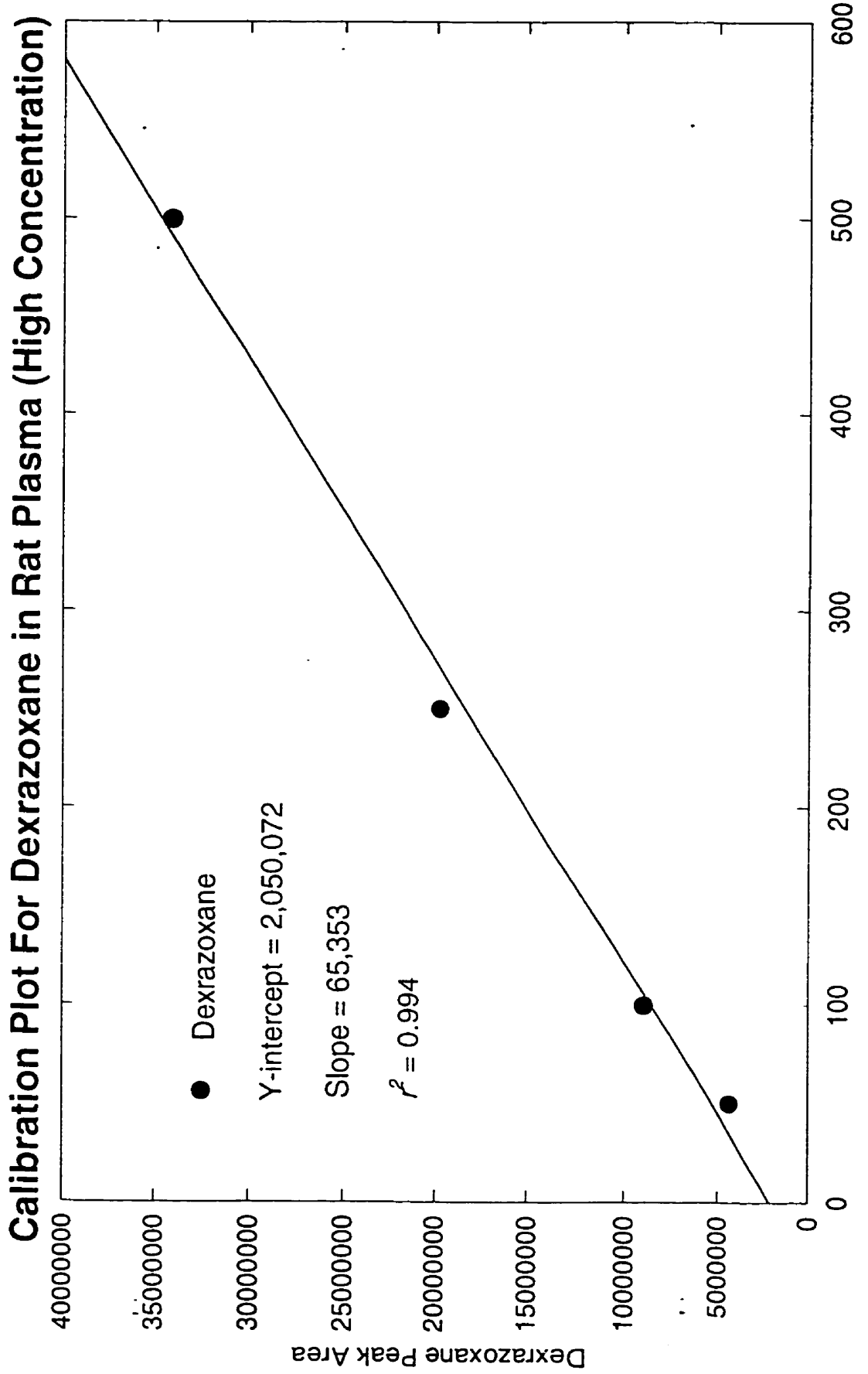

Calibration Plot For Dexrazoxane in Rat Plasma (High Concentration)
Dexrazoxane
Y-intercept = 2,050,072
Slope = 65,353
r^2 = 0.994
Dexrazoxane Peak Area
Dexrazoxane Plasma Concentration (µg/mL)
40000000
35000000
30000000
25000000
20000000
15000000
10000000
5000000
0
0
100
200
300
400
500
600

Figure 5.5

5.2.4 Rat Study

Plasma samples were taken from 4 rats at 5, 15, 30, 60, 120 and 180 min. Table 5.3 lists the plasma concentrations of intermediates **B** and **C** and dexrazoxane for each animal. These concentrations were plotted against time and are illustrated in figures 5.6 through 5.9.

Table 5.3 Plasma Concentrations of Intermediates **B**, **C** And Dexrazoxane After i.v. Dose of 40 mg/kg of Dexrazoxane in 0.9% (wt/vol) Saline (Dexrazoxane lot 91D23FY)

Rat 1

Time (min)	Intermediate **B** (μg/mL)	Intermediate **C** (μg/mL)	Dexrazoxane (μg/mL)
5	7.80	2.57	86.90
15	4.81	1.58	33.59
30	3.48	0.96	22.75
60	2.18	0.29	13.87
120	1.90	0.25	8.73
180	1.40	bloq	6.24

*bloq: below limit of quantitation

Rat 2

Time (min)	Intermediate **B** (μg/mL)	Intermediate **C** (μg/mL)	Dexrazoxane (μg/mL)
5	17.64	8.56	98.97
15	6.56	2.22	68.80
30	6.43	1.44	50.27
60	7.59	1.12	37.30
120	9.91	1.59	29.87
180	11.62	1.91	25.12

Rat 3

Time (min)	Intermediate B (µg/mL)	Intermediate C (µg/mL)	Dexrazoxane (µg/mL)
5	5.20	1.38	75.79
15	3.22	0.81	46.73
30	2.89	0.67	27.77
60	2.32	0.36	21.44
120	1.45	bloq	6.73
180	1.03	bloq	3.75

*bloq: below limit of quantitation

Rat 4

Time (min)	Intermediate B (µg/mL)	Intermediate C (µg/mL)	Dexrazoxane (µg/mL)
5	5.13	1.69	76.54
15	3.78	1.33	43.78
30	2.38	0.83	26.65
60	1.82	0.42	13.53
120	2.26	0.70	10.50
180	1.93	bloq	9.68

*bloq: below limit of quantitation

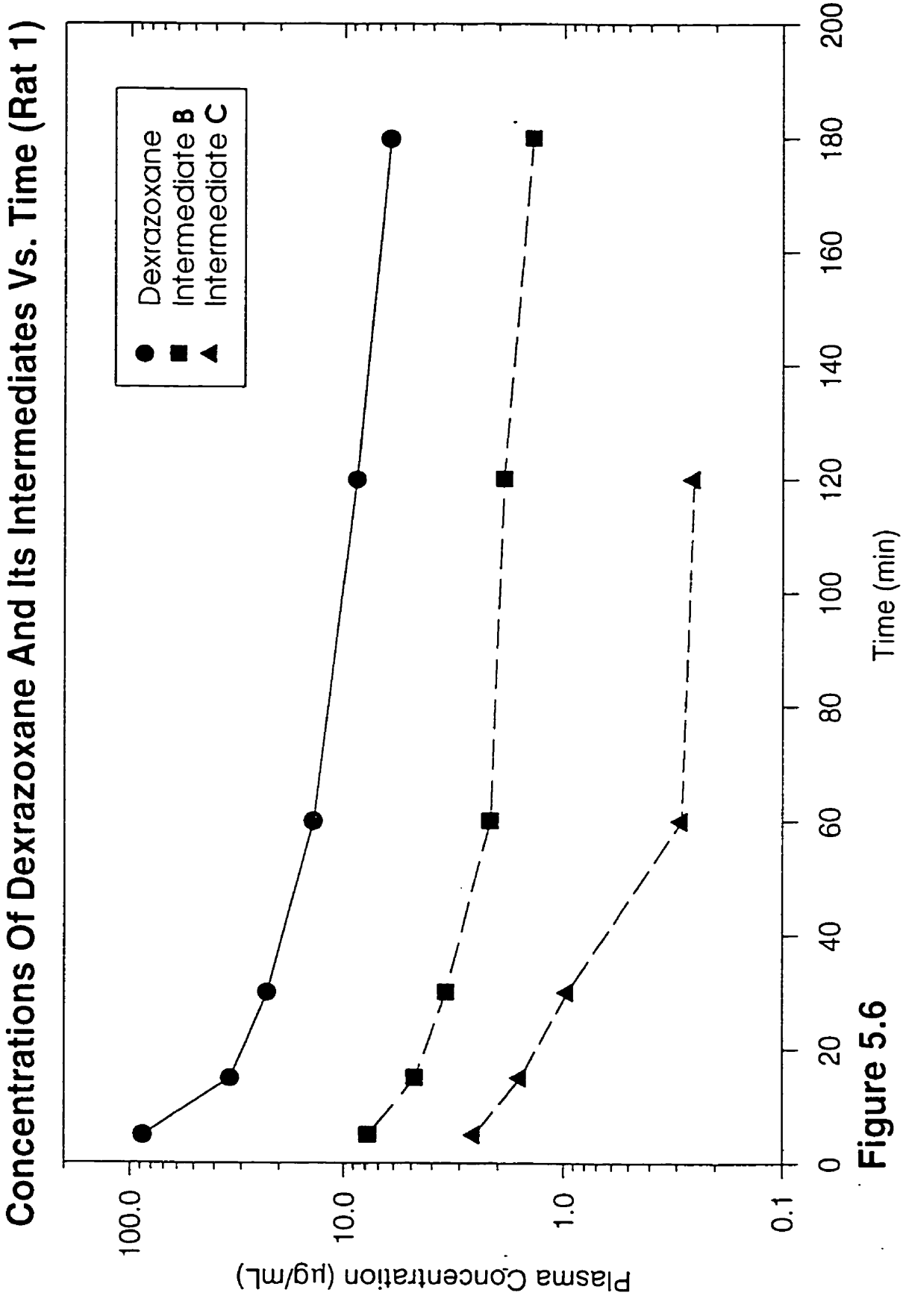

Concentrations Of Dexrazoxane And Its Intermediates Vs. Time (Rat 1)
Dexrazoxane
Intermediate B
Intermediate C
Plasma Concentration (µg/mL)
100.0
10.0
1.0
0.1
0
20
40
60
80
100
120
140
160
180
200
Time (min)

Figure 5.6

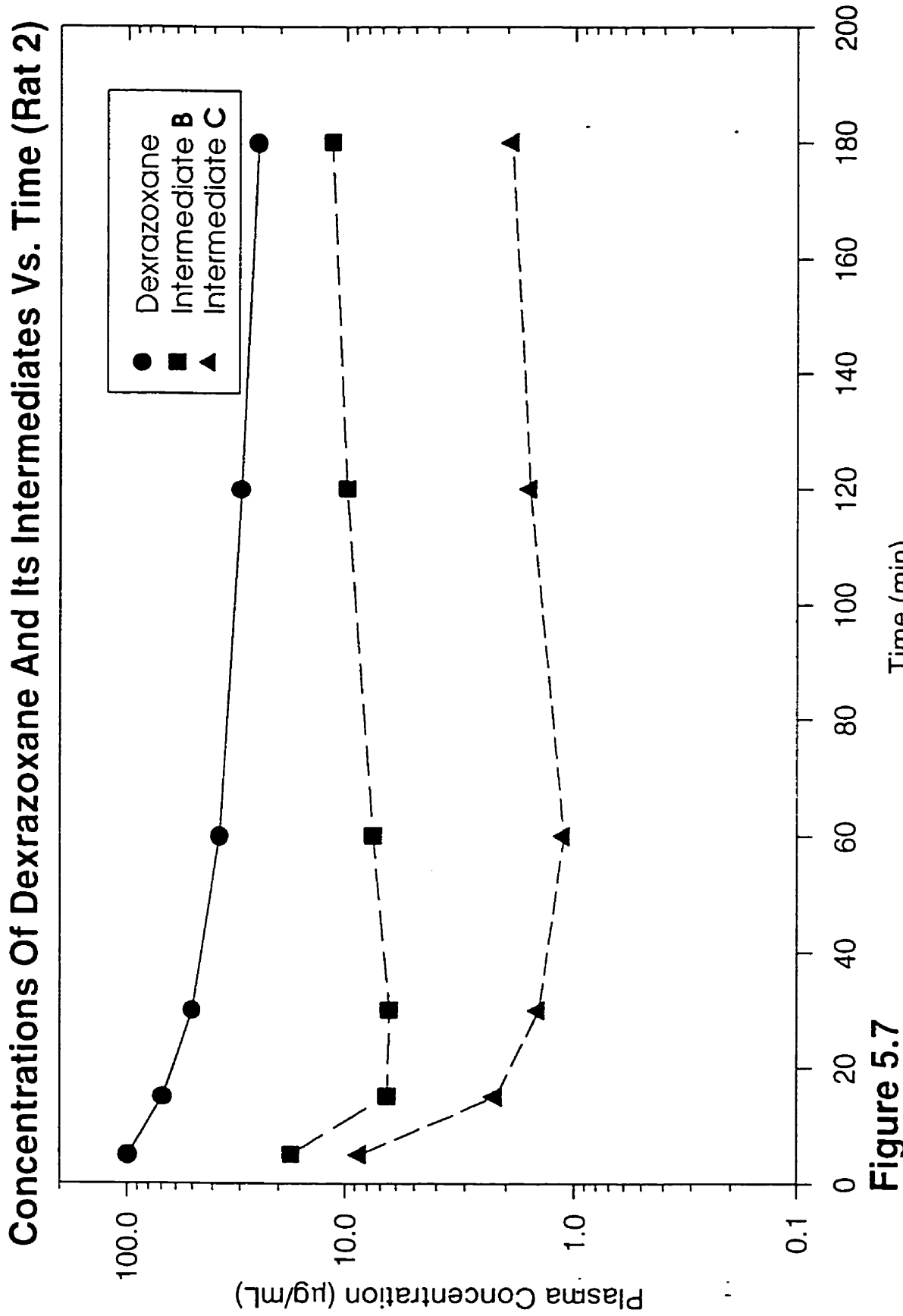

Concentrations Of Dexrazoxane And Its Intermediates Vs. Time (Rat 2)
Dexrazoxane
Intermediate B
Intermediate C
Plasma Concentration (µg/mL)
100.0
10.0
1.0
0.1
0
20
40
60
80
100
120
140
160
180
200
Time (min)

Figure 5.7

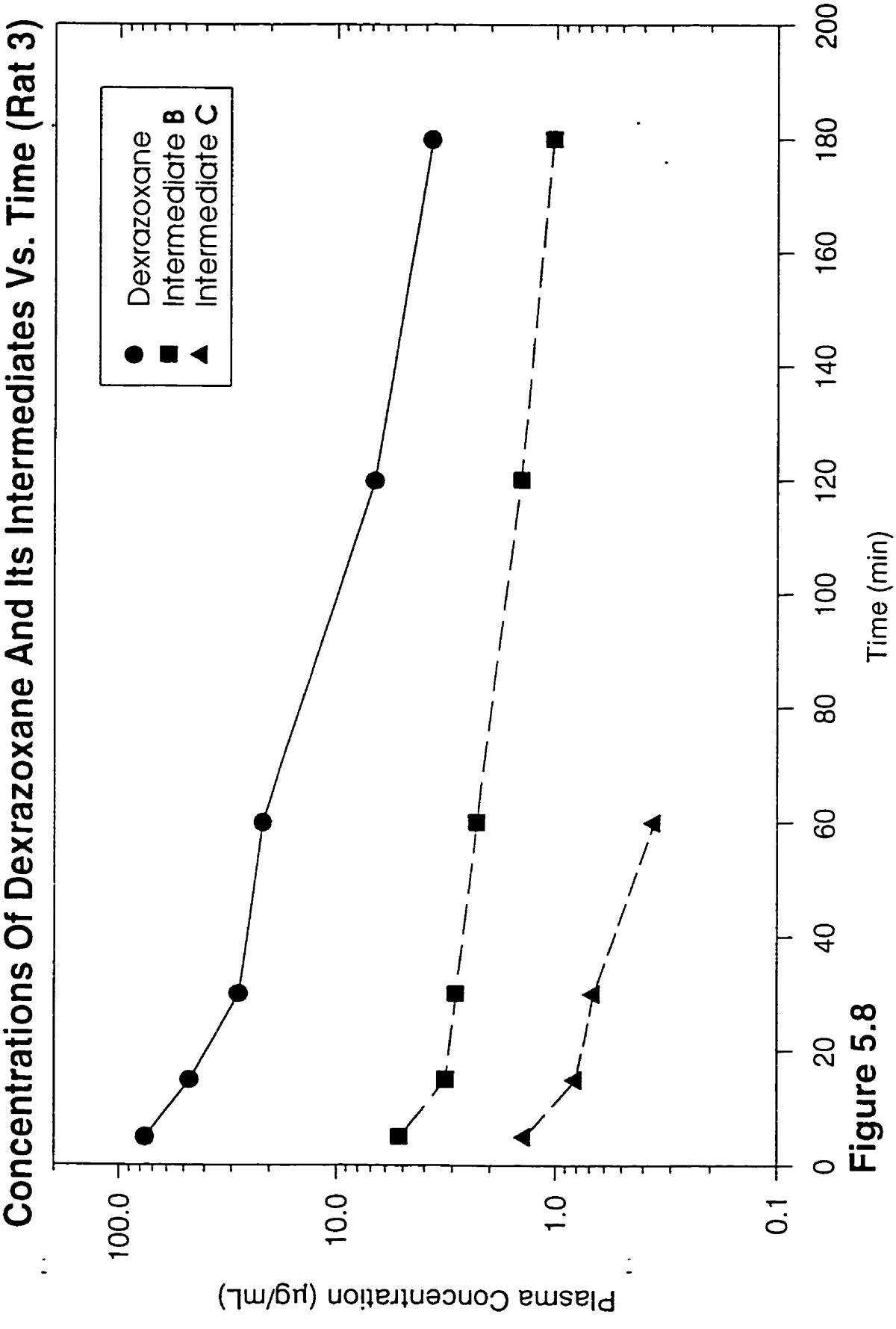

Concentrations Of Dexrazoxane And Its Intermediates Vs. Time (Rat 3)
Dexrazoxane
Intermediate B
Intermediate C
Plasma Concentration (µg/mL)
100.0
10.0
1.0
0.1
0
20
40
60
80
100
120
140
160
180
200
Time (min)

Figure 5.8

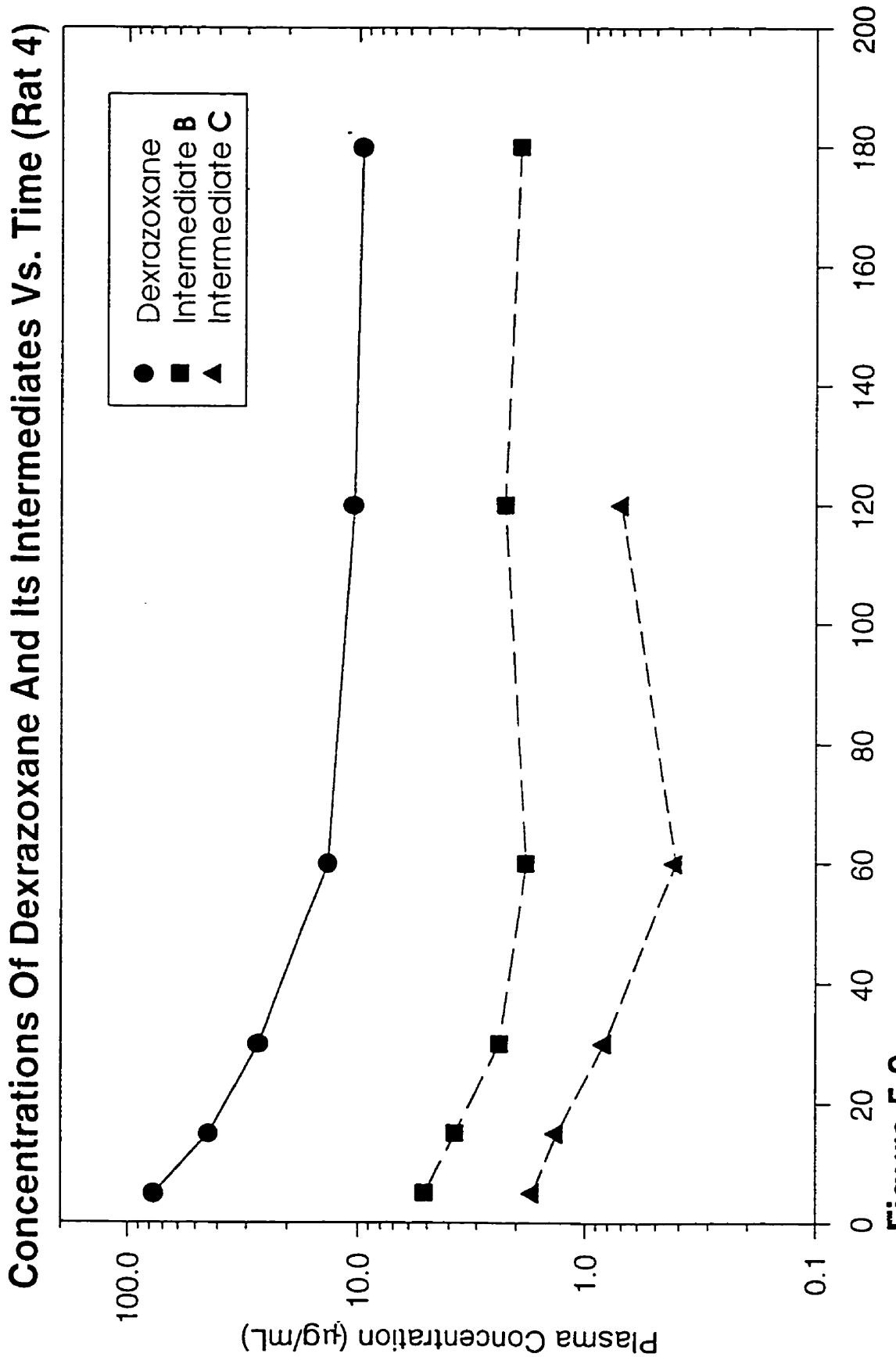

Concentrations Of Dexrazoxane And Its Intermediates Vs. Time (Rat 4)
Dexrazoxane
Intermediate B
Intermediate C
Plasma Concentration (µg/mL)
100.0
10.0
1.0
0.1
0
20
40
60
80
100
120
140
160
180
200
Time (min)

Figure 5.9

The average plasma concentrations of intermediates **B** and **C**, and dexrazoxane are listed in tables 5.4, 5.5, and 5.6, respectively. The average plasma concentrations of intermediates **B** and **C** and dexrazoxane were plotted against time in figure 5.10. Mean peak plasma concentrations were at 5 min with 8.94 μg/mL for intermediate **B**, 3.55 μg/mL for intermediate **C**, and 84.55 μg/mL for dexrazoxane.

Table 5.4 Average Plasma Concentration For Intermediate **B** (4 rats)

Time (min)	Intermediate **B** (μg/mL)	SEM (μg/mL)
5	8.94	2.97
15	4.59	0.73
30	3.80	0.91
60	3.48	1.37
120	3.87	2.02
180	3.99	2.55

Table 5.5 Average Plasma Concentration For Intermediate **C** (4 rats)

Time (min)	Intermediate **C** (μg/mL)	SEM (μg/mL)
5	3.55	1.69
15	1.49	0.29
30	0.98	0.17
60	0.54	0.19
120	0.85	0.39
180	bloq	na

*bloq: below limit of quantitation *na: value not available

Table 5.6 Average Plasma Concentrations For Dexrazoxane (4 rats)

Time (min)	Dexrazoxane (µg/mL)	SEM (µg/mL)
5	84.55	5.43
15	48.23	7.41
30	31.86	6.23
60	21.54	5.56
120	13.96	5.36
180	11.20	4.80

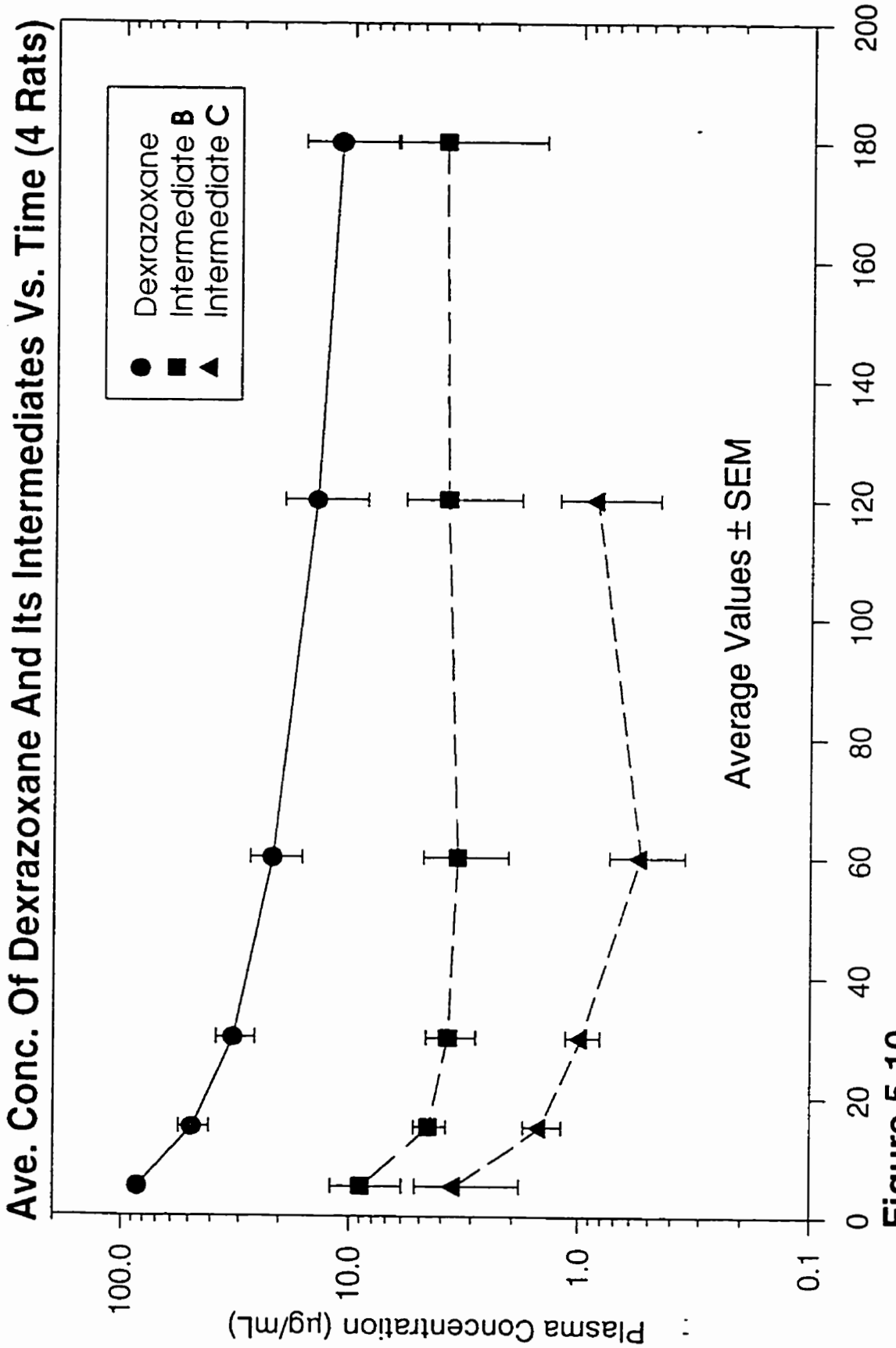

Ave. Conc. Of Dexrazoxane And Its Intermediates Vs. Time (4 Rats)
Dexrazoxane
Intermediate B
Intermediate C
Average Values ± SEM
Plasma Concentration (µg/mL)
100.0
10.0
1.0
0.1
0
20
40
60
80
100
120
140
160
180
200
Time (min)

Figure 5.10

An experiment was performed to test if intermediates **B** and **C** present as contaminants in the dexrazoxane i.v. solution affected the plasma levels of the intermediates seen in the previous study. Two rats were administered a 40 mg/kg dose of dexrazoxane from a new lot. Lot 91N03A contained 0.056% (wt/wt) intermediate **B** and 0.021% (wt/wt) intermediate **C** in a 10 mg/mL solution of dexrazoxane dissolved in 0.9% (wt/vol) saline. Lot 91D23FY was used in the previous study of 4 rats and contained over 10 times more of the intermediates. Lot 91D23FY had 0.577% (wt/wt) intermediate **B** and 0.378% (wt/wt) intermediate **C** in a 10 mg/mL solution of dexrazoxane dissolved in 0.9% (wt/vol) saline.

The plasma concentrations of dexrazoxane and its hydrolysis intermediates for rats 5 and 6 are listed in Table 5.7. The plasma concentrations for rat 5 and 6 were plotted with the average plasma concentrations from the previous study of 4 rats as a comparison and are shown in figures 5.11 and 5.12.

Table 5.7 Plasma Concentrations of Intermediates **B**, **C** And Dexrazoxane After i.v. Dose of 40 mg/kg of Dexrazoxane in 0.9% (wt/vol) Saline (Dexrazoxane lot 91N03A)

Rat 5

Time (min)	Intermediate **B** (μg/mL)	Intermediate **C** (μg/mL)	Dexrazoxane (μg/mL)
5	6.75	2.72	99.68
15	2.99	1.21	41.72
30	4.44	1.52	26.62
60	4.80	0.83	21.23
120	2.53	bloq	13.47
180	2.31	bloq	11.33

*bloq: below limit of quantitation

Rat 6

Time (min)	Intermediate B (µg/mL)	Intermediate C (µg/mL)	Dexrazoxane (µg/mL)
5	7.51	3.77	106.35
15	4.50	1.42	56.92
30	2.72	0.94	24.08
60	3.71	0.95	17.61
120	3.28	0.61	14.74
180	3.16	0.46	11.87

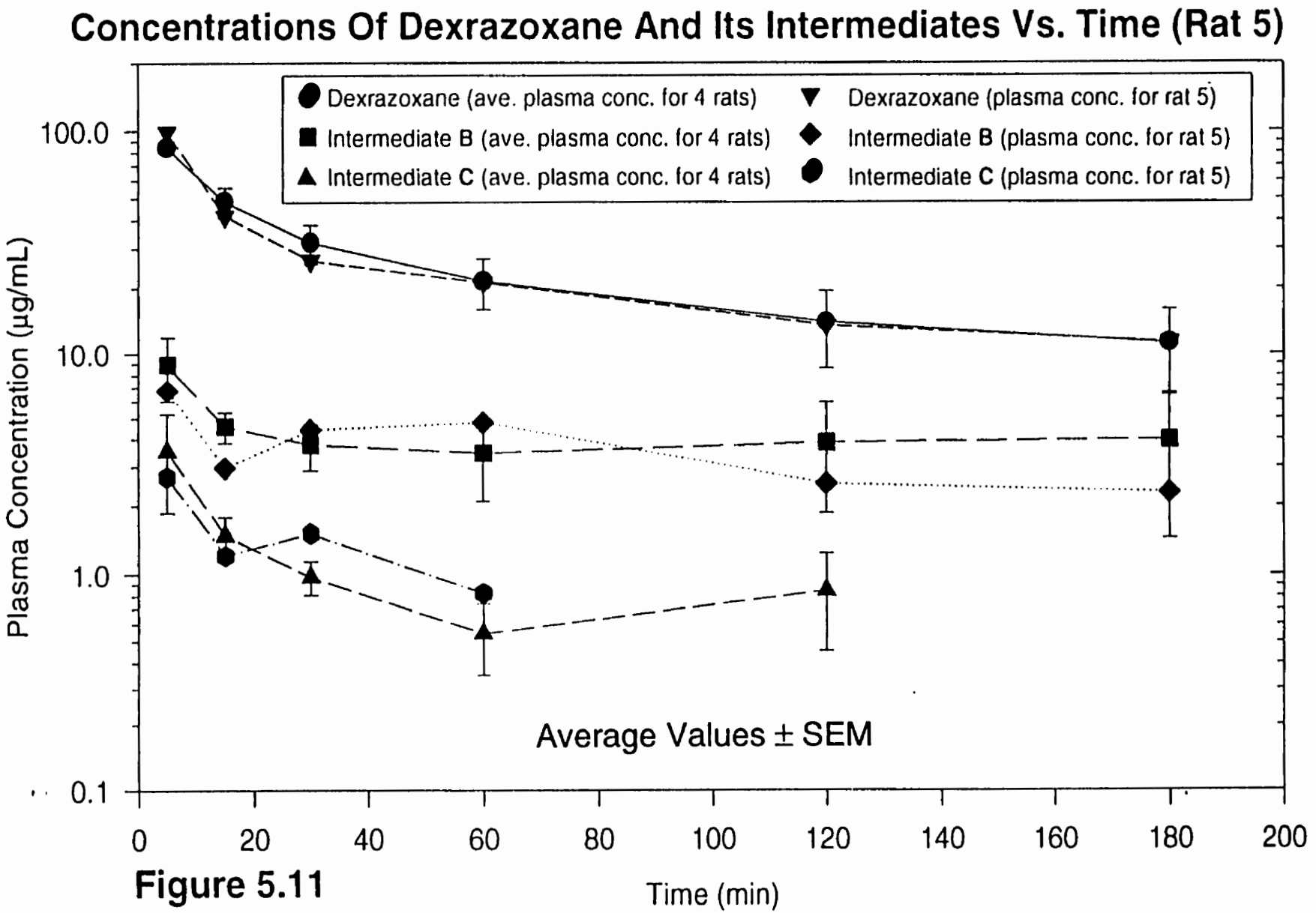

Concentrations Of Dexrazoxane And Its Intermediates Vs. Time (Rat 5)
Dexrazoxane (ave. plasma conc. for 4 rats)
Intermediate B (ave. plasma conc. for 4 rats)
Intermediate C (ave. plasma conc. for 4 rats)
Dexrazoxane (plasma conc. for rat 5)
Intermediate B (plasma conc. for rat 5)
Intermediate C (plasma conc. for rat 5)
Plasma Concentration (µg/mL)
100.0
10.0
1.0
0.1
Average Values ± SEM
0
20
40
60
80
100
120
140
160
180
200
Time (min)

Figure 5.11

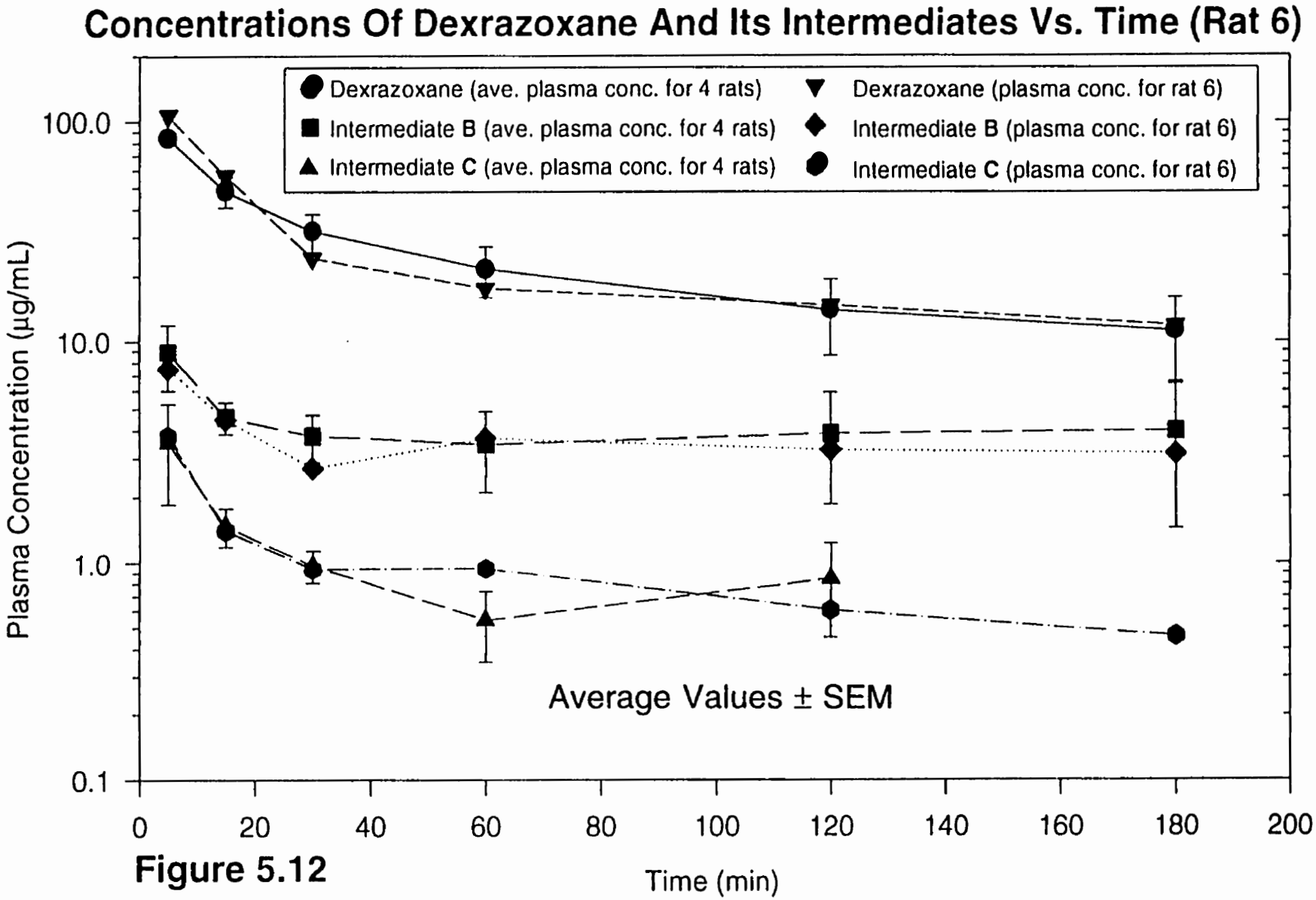

Concentrations Of Dexrazoxane And Its Intermediates Vs. Time (Rat 6)
Dexrazoxane (ave. plasma conc. for 4 rats)
Intermediate B (ave. plasma conc. for 4 rats)
Intermediate C (ave. plasma conc. for 4 rats)
Dexrazoxane (plasma conc. for rat 6)
Intermediate B (plasma conc. for rat 6)
Intermediate C (plasma conc. for rat 6)
Plasma Concentration (µg/mL)
100.0
10.0
1.0
0.1
Average Values ± SEM
0
20
40
60
80
100
120
140
160
180
200
Time (min)

Figure 5.12

For all 6 rats, the average plasma concentrations of intermediates **B** and **C**, and dexrazoxane are listed in tables 5.8, 5.9 and 5.10, respectively. The average plasma concentrations of intermediates **B** and **C** and dexrazoxane were plotted against time in figure 5.13. Mean peak plasma concentrations were at 5 min with 8.34 μg/mL for intermediate **B**, 3.45 μg/mL for intermediate **C**, and 90.71 μg/mL for dexrazoxane.

Table 5.8 Average Plasma Concentration For Intermediate **B** (6 rats)

Time (min)	Intermediate **B** (μg/mL)	SEM (μg/mL)
5	8.34	1.92
15	4.31	0.53
30	3.72	0.62
60	3.74	0.90
120	3.55	1.30
180	3.57	1.64

Table 5.9 Average Plasma Concentration For Intermediate **C** (6 rats)

Time (min)	Intermediate **C** (μg/mL)	SEM (μg/mL)
5	3.45	1.08
15	1.43	0.19
30	0.95	0.11
60	0.56	0.15
120	0.85	0.39
180	bloq	na

*bloq: below limit of quantitation *na: value not available

Table 5.10 Average Plasma Concentrations For Dexrazoxane (6 rats)

Time (min)	Dexrazoxane (μg/mL)	SEM (μg/mL)
5	90.71	5.26
15	48.59	4.09
30	29.69	4.19
60	20.83	3.58
120	14.01	3.39
180	11.33	3.04

The terminal half-lives for dexrazoxane were calculated by finding the slope of the last 3 timepoints and inserted into the formula:

terminal half-life = 0.693/slope

The terminal half-lives for dexrazoxane are listed in Table 5.11.

Table 5.11 Terminal Half-lives For Rats After i.v. Dose of 40 mg/kg of Dexrazoxane in 0.9% (wt/vol) Saline

Rat	Terminal half-life (hr)
1	1.67
2	3.45
3	0.70
4	1.67
5	2.05
6	3.24
Average value for 6 rats ± SEM	2.13 ± 0.43

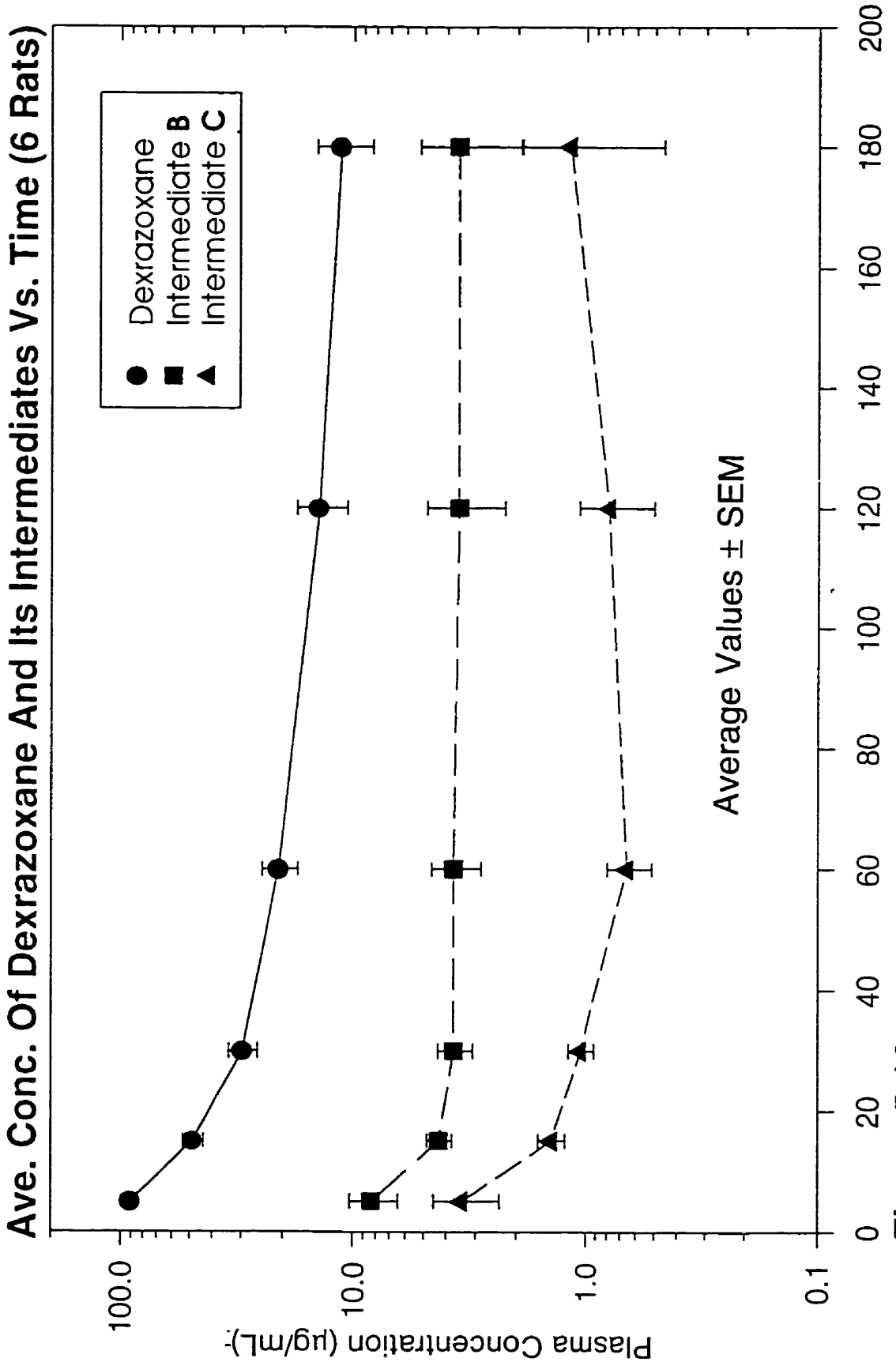

Ave. Conc. Of Dexrazoxane And Its Intermediates Vs. Time (6 Rats)
Dexrazoxane
Intermediate B
Intermediate C
Average Values ± SEM
Plasma Concentration (µg/mL)
100.0
10.0
1.0
0.1
0
20
40
60
80
100
120
140
160
180
200
Time (min)

Figure 5.13

The ratio of the plasma concentrations of the intermediates **B** and **C** were calculated for each rat, listed in Table 5.12 and illustrated in figures 5.14 to 5.19.

Table 5.12 Plasma Concentration Ratio **B/C**

Time (min)	Concentration Ratio **B/C** Rat 1	Concentration Ratio **B/C** Rat 2	Concentration Ratio **B/C** Rat 3
5	3.03	2.06	3.77
15	3.05	2.95	3.99
30	3.62	4.46	4.32
60	7.63	6.79	6.52
120	7.53	6.24	nd
180	nd	6.08	nd

Time (min)	Concentration Ratio **B/C** Rat 4	Concentration Ratio **B/C** Rat 5	Concentration Ratio **B/C** Rat 6
5	3.03	2.48	1.99
15	2.83	2.46	3.18
30	2.88	2.93	2.90
60	4.39	5.81	3.93
120	3.20	nd	5.43
180	nd	nd	6.85

*nd: ratio not determined

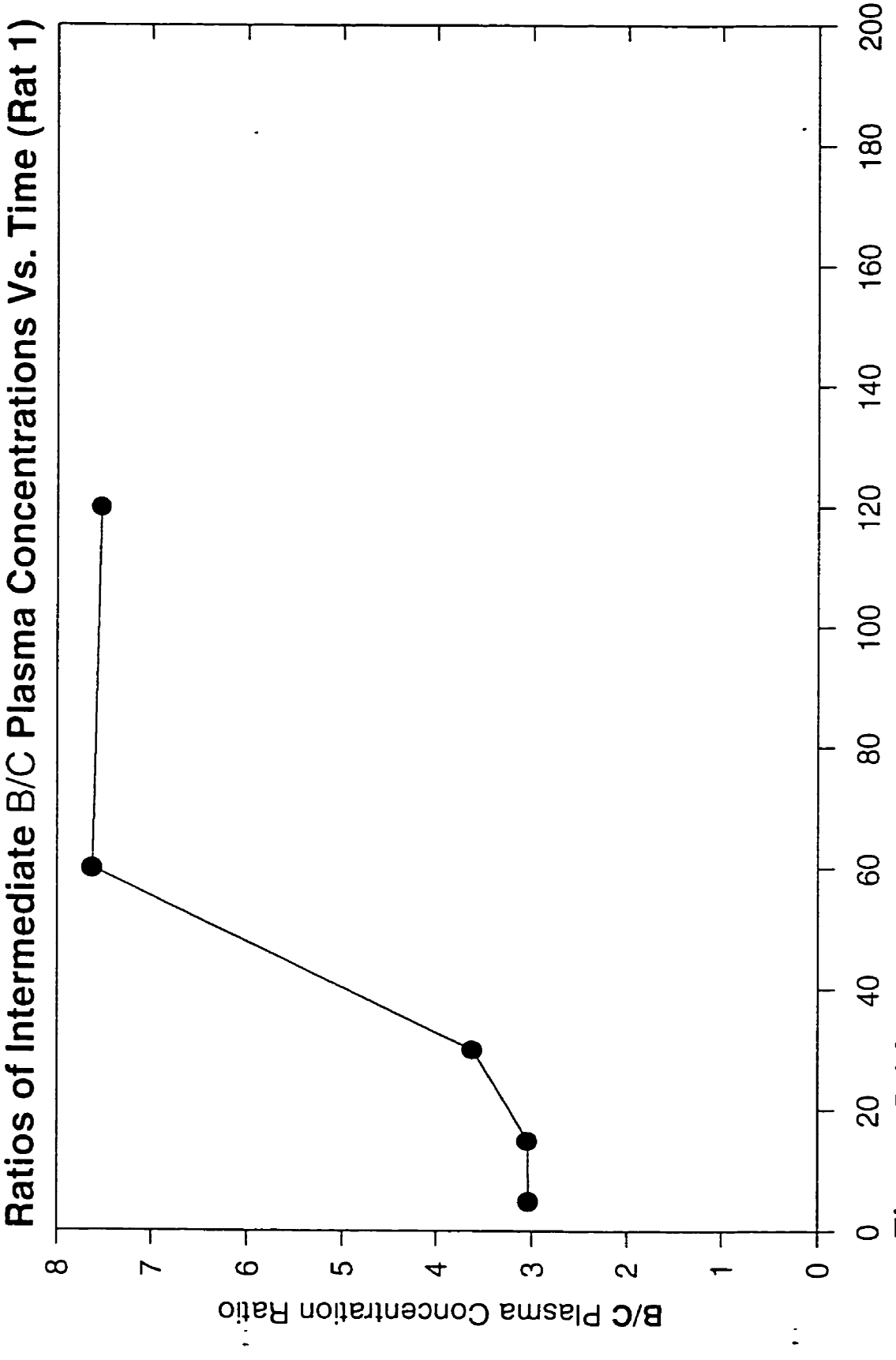

Ratios of Intermediate B/C Plasma Concentrations Vs. Time (Rat 1)
B/C Plasma Concentration Ratio
0
1
2
3
4
5
6
7
8
0
20
40
60
80
100
120
140
160
180
200
Time (min)

Figure 5.14

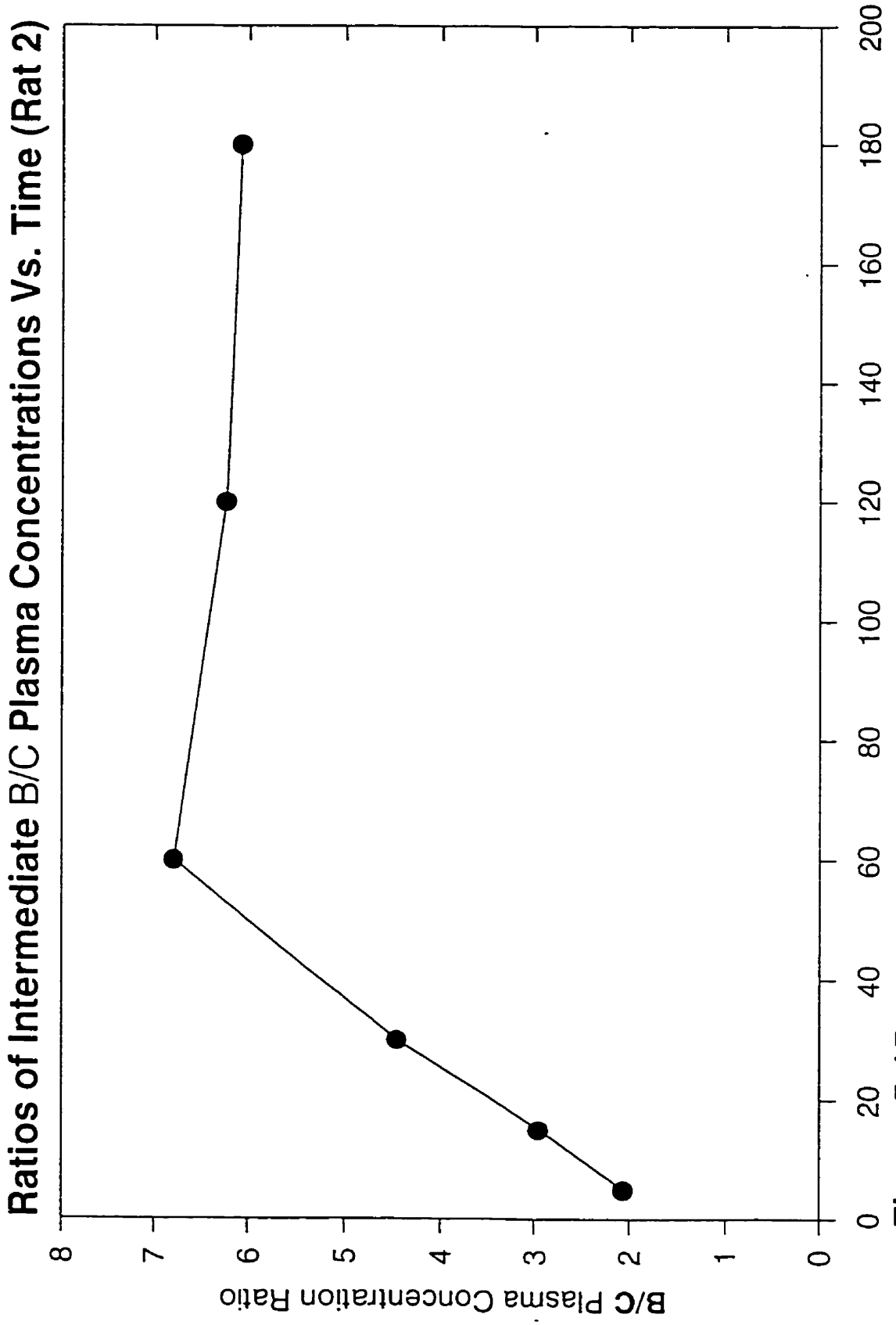

Ratios of Intermediate B/C Plasma Concentrations Vs. Time (Rat 2)
B/C Plasma Concentration Ratio
0
1
2
3
4
5
6
7
8
0
20
40
60
80
100
120
140
160
180
200
Time (min)

Figure 5.15

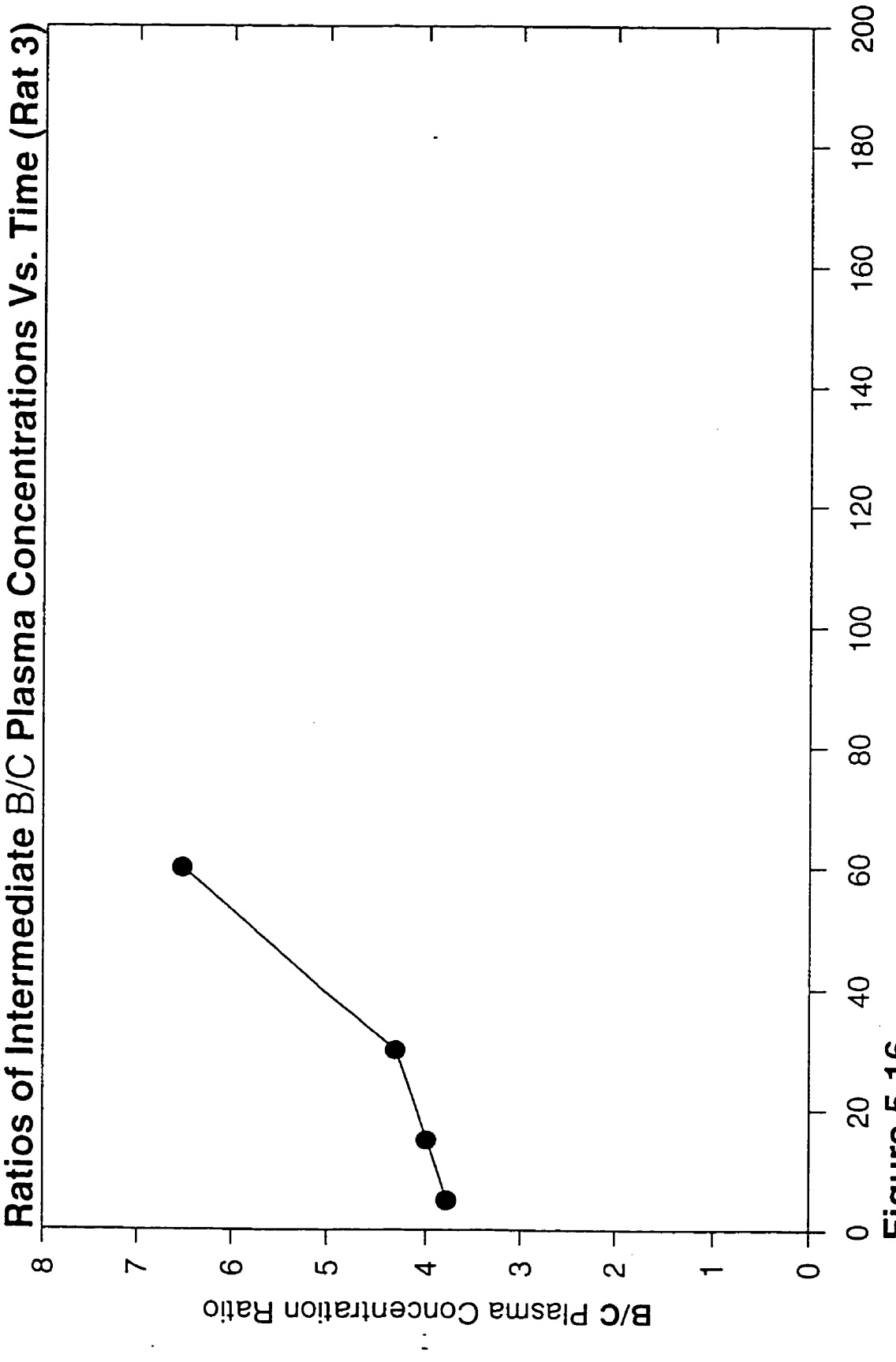

Ratios of Intermediate B/C Plasma Concentrations Vs. Time (Rat 3)
B/C Plasma Concentration Ratio
0
1
2
3
4
5
6
7
8
0
20
40
60
80
100
120
140
160
180
200
Time (min)

Figure 5.16

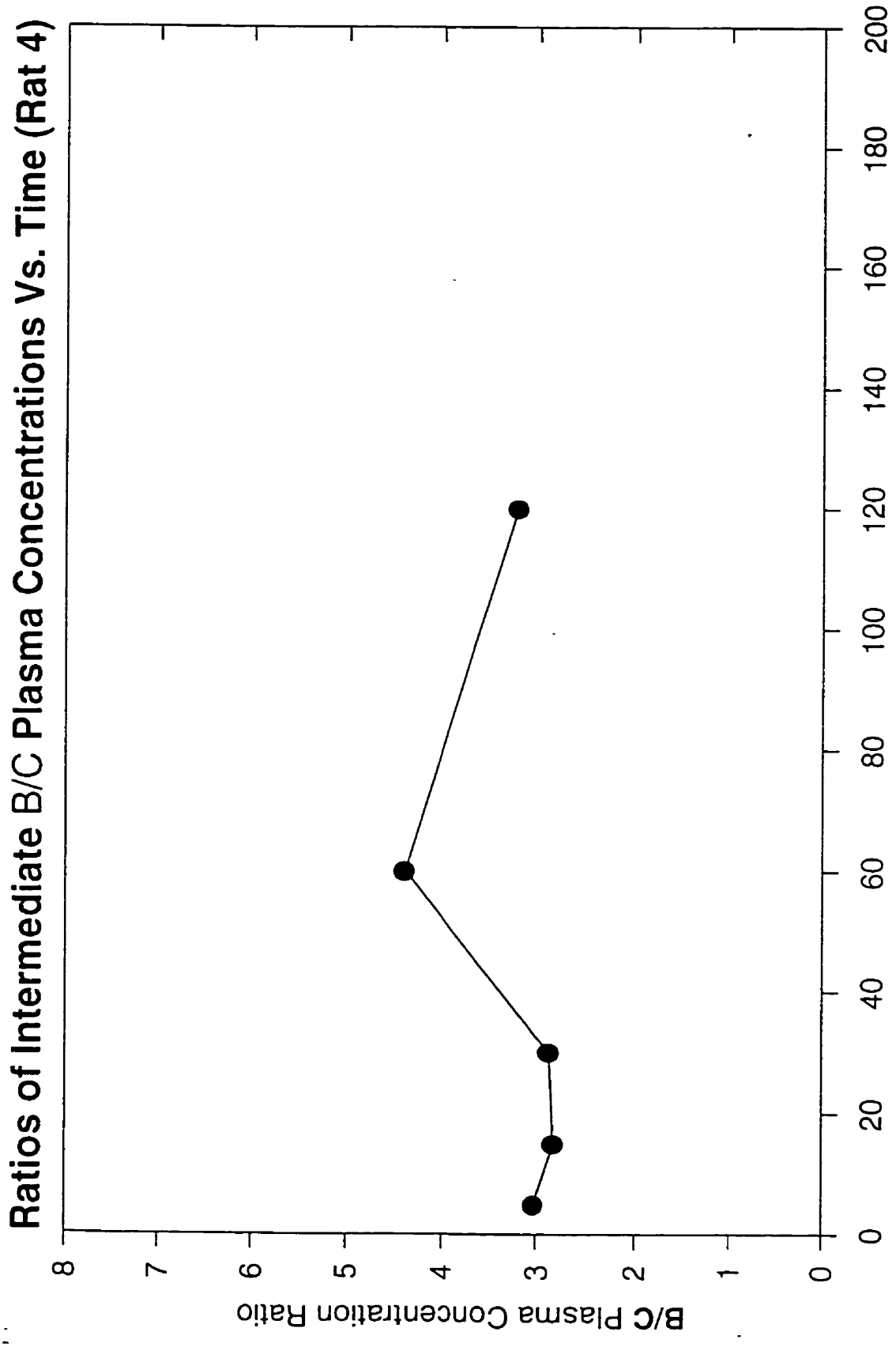

Ratios of Intermediate B/C Plasma Concentrations Vs. Time (Rat 4)
B/C Plasma Concentration Ratio
0
1
2
3
4
5
6
7
8
0
20
40
60
80
100
120
140
160
180
200
Time (min)

Figure 5.17

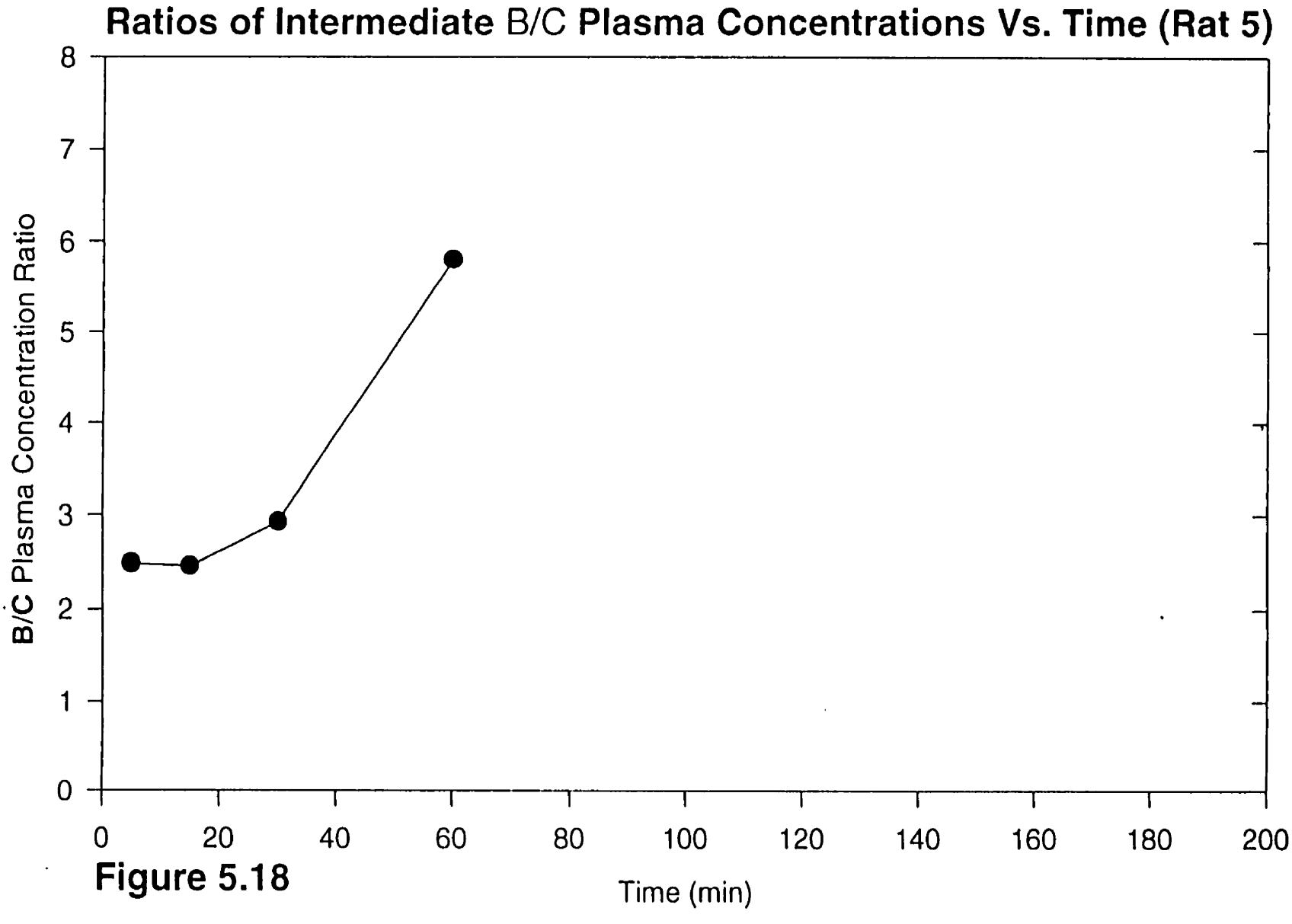

Ratios of Intermediate B/C Plasma Concentrations Vs. Time (Rat 5)
B/C Plasma Concentration Ratio
0
1
2
3
4
5
6
7
8
0
20
40
60
80
100
120
140
160
180
200
Time (min)

Figure 5.18

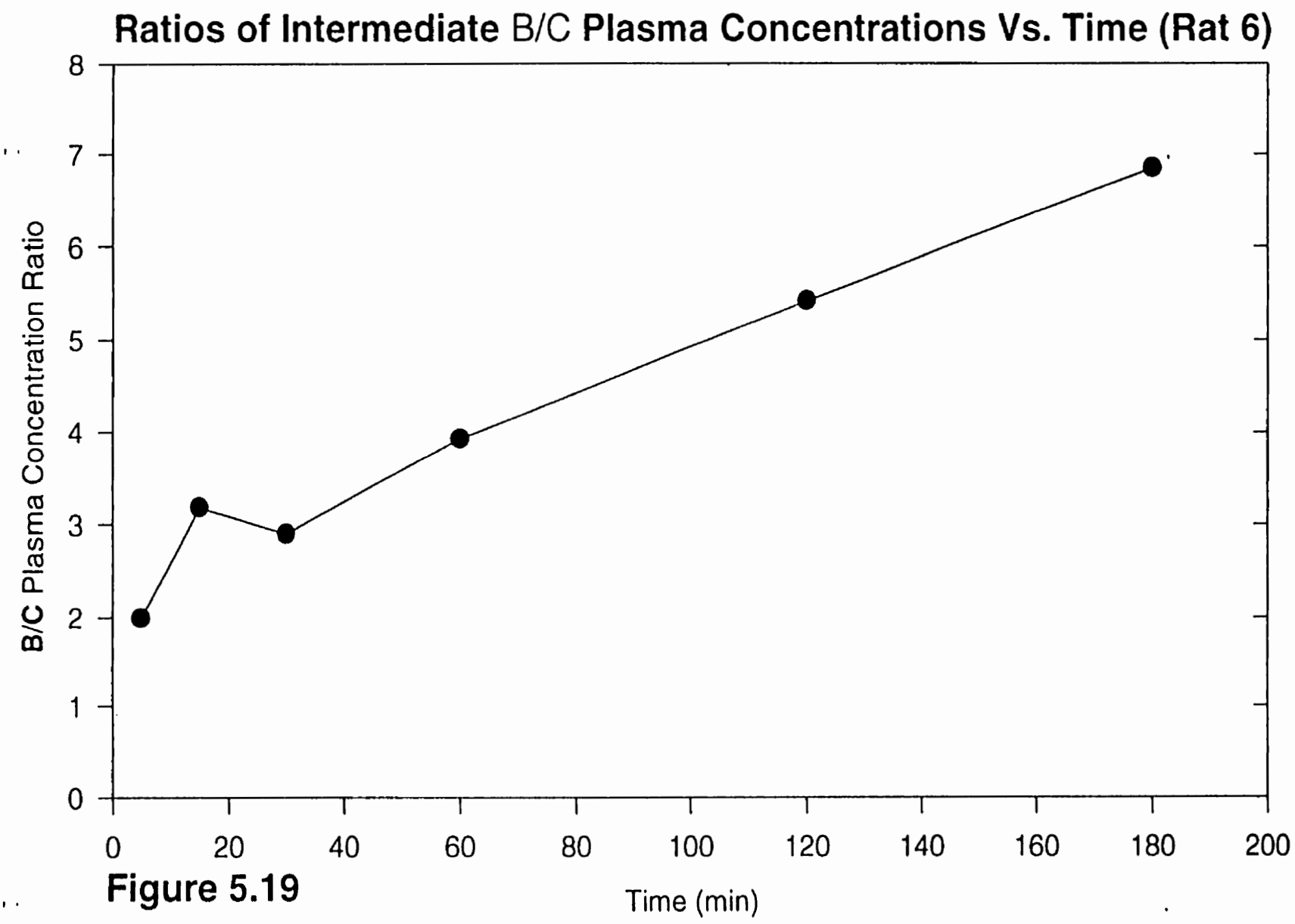

Ratios of Intermediate B/C Plasma Concentrations Vs. Time (Rat 6)
B/C Plasma Concentration Ratio
0
1
2
3
4
5
6
7
8
0
20
40
60
80
100
120
140
160
180
200
Time (min)

Figure 5.19

The average ratio of the plasma concentrations of the intermediates **B** and **C** for all 6 rats were calculated, listed in Table 5.13 and illustrated in figure 5.20.

Table 5.13 Average Plasma Concentration Ratio **B/C** For 6 Rats

Time (min)	Concentration Ratio **B/C**	SEM
5	2.73	0.28
15	3.08	0.21
30	3.52	0.30
60	5.85	0.59
120	5.60	0.91
180	6.47	0.39

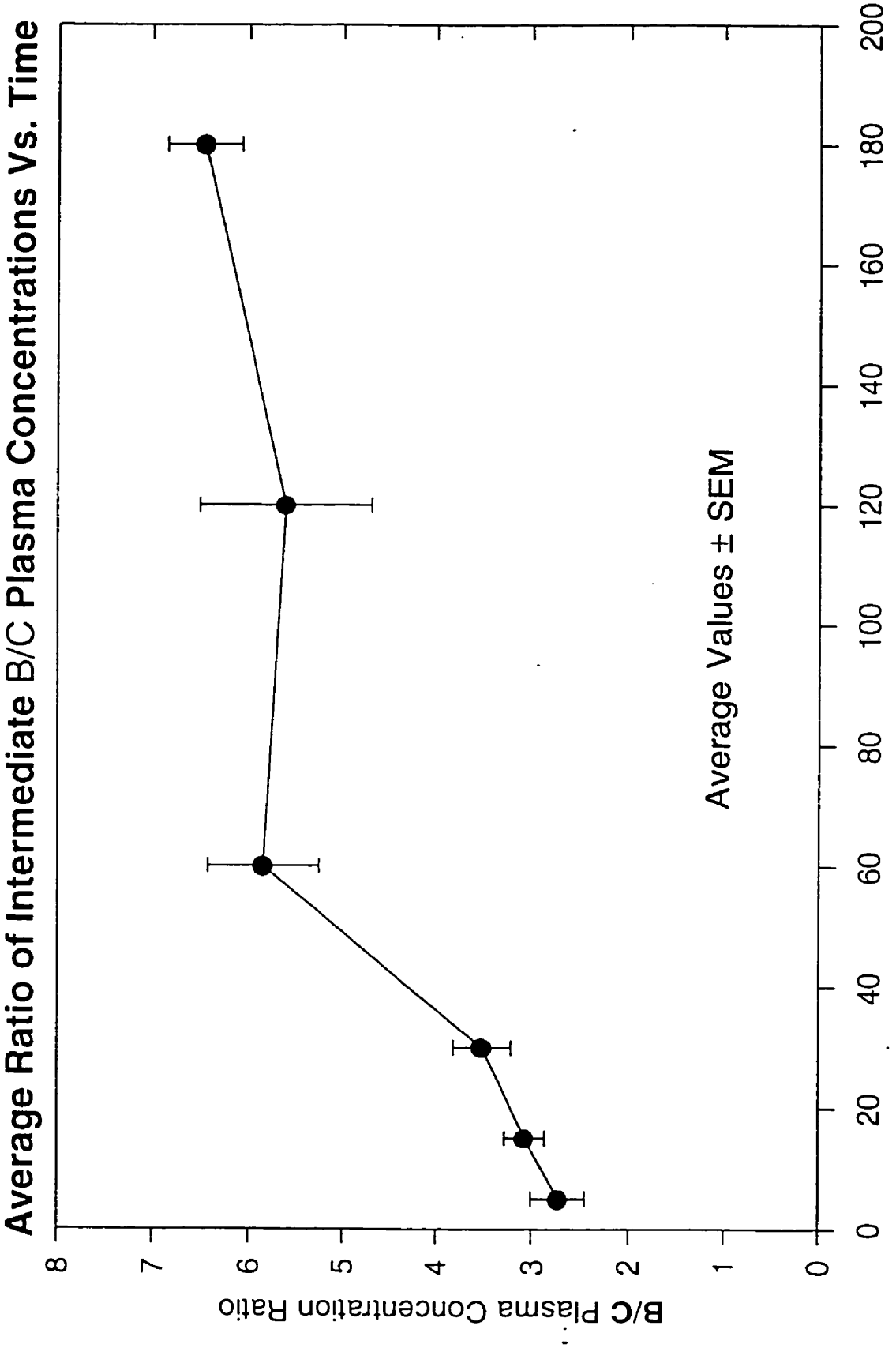

Average Ratio of Intermediate B/C Plasma Concentrations Vs. Time
B/C Plasma Concentration Ratio
0
1
2
3
4
5
6
7
8
0
20
40
60
80
100
120
140
160
180
200
Time (min)
Average Values ± SEM

Figure 5.20

5.3 DISCUSSION

5.3.1 HPLC Quantitation of the Sodium Hydroxide Hydrolysis Intermediate Products of Dexrazoxane

The dexrazoxane intermediates were produced by NaOH hydrolysis at 25°C. Intermediates **B** and **C** were produced in nearly equimolar concentrations in the NaOH solution. These results agree with data from Hasinoff (1993, 1994b).

5.3.2 HPLC Analysis Of Dexrazoxane i.v. Solutions

The dexrazoxane i.v. solution used in the second metabolism study of 2 rats contained less than 10% of intermediates **B** and **C** found in the dexrazoxane i.v. solution used for the first rat metabolism study. If the plasma concentrations of intermediates **B** and **C** determined in the first rat study were due to **B** and **C** as contaminants in dexrazoxane, the use of purer dexrazoxane i.v. solution would have shown a change in rat plasma levels of **B** and **C**. In this case, rat plasma levels of the dexrazoxane intermediates would reflect the concentration of the intermediates already in the dexrazoxane i.v. solution

5.3.3 HPLC Conditions for the Analysis of Dexrazoxane and its Hydrolysis Intermediates

The samples from the metabolism study of dexrazoxane in rats were separated using HPLC conditions described in Chapter 4 and outlined in section 5.1.2.6.2. This procedure modified a method developed by Hasinoff (1993, 1994b) by changing the pH of Na_2EDTA used in the mobile phase from 4.5 to 3.5. Also, 1-heptanesulfonic acid was added to the mobile phase. These changes resulted in better separation between the intermediates **B** and **C**. The improvement in separation is probably due to favourable interactions between the stationary phase in the HPLC column, the 1-heptanesulfonic acid and the carboxylic acid functional group in the intermediates.

Once the concentrated plasma samples from the rat study were separated using the previously discussed HPLC mobile phase conditions, the elutants were

derivatized post-column with NaOH solution. This post-column derivatization method was described in chapter 4 and in section 5.1.1.4.2. The use of this post-column derivatization method and a detection wavelength of 235 nm reduced interference of endogenous compounds present in rat plasma. A large endogenous plasma peak which co-eluted with intermediate **B** was substantially reduced in size using this method. Hasinoff (1990) had found that dexrazoxane maximum absorption peak shifts as the relatively acidic imide group ionizes to its anionic form under basic conditions. It was thought that changing the detection wavelength from 205 nm to other higher values may reduce the interferences from plasma components without reducing the sensitivity of the dexrazoxane and intermediate peaks. A detection wavelength of 235 nm was picked as a compromise between reduced plasma interferences and reduced sensitivity for the intermediates.

5.3.4 Rat Study of The Metabolism of Dexrazoxane

5.3.4.1 Calibration Plot of the Dexrazoxane Hydrolysis Intermediates B and C, and Dexrazoxane

The retention times for intermediate **B**, **C** and dexrazoxane were 6, 7 and 17 min respectively for the HPLC conditions used to analyze these samples and plasma samples from the animal study. An endogenous plasma peak co-eluted with the intermediate **B** peak but a linear calibration plot from 5 to 50 μg/mL was still obtained (r^2 value of 0.9999). Linear calibration plots were obtained for intermediate **C** and dexrazoxane from 1 to 50 μg/mL and for dexrazoxane from 20 to 500 μg/mL (r^2 values of 0.9998, 0.9986 and 0.9963, respectively).

5.3.4.2 Rat Study

The plasma concentrations of intermediates **B**, **C** and dexrazoxane for each of the 4 rats are listed in Table 5.3. The average plasma concentrations for intermediates **B**, **C** and dexrazoxane in 4 rats are listed in tables 5.4, 5.5 and 5.6,

respectively. Peak concentration of intermediate **B** was at 5 min with a value of 8.94 μg/mL. The peak concentration of intermediate **C** was 3.55 μg/mL at 5 min. The peak concentration of dexrazoxane was 84.55 μg/mL at 5 min. For all timepoints, the plasma concentration of dexrazoxane is higher than for intermediates **B** or **C**. Also, the plasma concentration of intermediate **B** is always higher than for intermediate **C**.

Two rats were dosed with a different lot of dexrazoxane which contained less than 10% of levels of intermediate **B** and **C** found in the dexrazoxane used in the previously mentioned study. The plasma concentrations of intermediates **B** and **C** and dexrazoxane for the 2 rats are listed in Table 5.7. The plasma concentrations of intermediates **B**, **C** and dexrazoxane for each rat and the average of the 4 rats in the previous study are illustrated in figures 5.11 and 5.12. Rat 5 and 6 both had plasma concentrations of intermediates **B**, **C** and dexrazoxane similar to those found for the averages of 4 rats in the previous study. If the plasma concentrations of the two intermediates were mostly due to the levels of the intermediates in the dexrazoxane i.v. solution, then rats administered dexrazoxane with less contamination from the intermediates should show a corresponding decrease. This does not appear to be the case for either rat 5 or 6. The rat plasma concentration of the intermediates **B** and **C** is probably not solely due to the amount of intermediates **B** and **C** present in the dexrazoxane i.v. solution. The average plasma concentrations of intermediates **B** and **C** and dexrazoxane for all 6 rats are listed in Table 5.8, 5.9, and 5.10 respectively. The average plasma concentrations of intermediates **B** and **C** and dexrazoxane were plotted against time in figure 5.13. Mean peak plasma concentrations occurred at 5 min with 8.34 μg/mL for intermediate **B**, 3.45 μg/mL for intermediate **C**, and 90.71 μg/mL for dexrazoxane.

Blood samples were collected by the same cannula used to administer the

dexrazoxane. After the dexrazoxane was infused, the cannula was flushed with 100 µL of saline containing heparin. Any residual dexrazoxane that remained in the cannula could affect the results of the first blood sample of each rat. However, following the first blood sample, 1 mL of saline was injected into the cannula after every blood sample collection. The blood samples collected later would not be contaminated. Blood sample collection and fluid replenishment were performed in the same manner for all rats. Any contamination in the first blood sample at 10 min should be consistent from rat to rat.

The terminal half-lives for dexrazoxane were calculated and listed in Table 5.11. The average half-life for dexrazoxane in the rat study was 2.13 ± 0.43 hr (mean ± SEM). This compares to a mean terminal half-life of 40.3 min in plasma for a rat study (Collins, *et al.*, 1983). In beagle dogs, the mean plasma terminal half-life was 1.16 hr (Sisco, 1989). The differences in terminal half-lives between the rats in this study and the two other animal studies may be due to the effects of anesthesia. The two published animal studies were performed on conscious animals. The rats in the current study were rendered unconscious with a mixture of ketamine and xylazine. The differences in terminal half-lives may be due to a species effect. Also, the blood sampling schedule in this study was aggressive. It is possible that the rats were in hypovolemic shock, which could affect the terminal half-lives. The rats were not observed to be in hypovolemic shock during the study. At the end of the study all rats were alive. The terminal half-life of dexrazoxane has also been calculated in some human studies. In one study (Hochter *et al.*, 1992), the terminal plasma half-life was 4.16 ± 2.94 hr (mean ± SD). In another human study (Jakobsen *et al.*, 1994), the terminal plasma half-life was 2.88 ± 1.02 hr (mean ± SD). In a pediatric study (Wexler *et al.*, 1996), the terminal plasma half-life was 1.87 ± 0.37 hr (mean ± SEM).

Terminal half-lives were not calculated for intermediates **B** and **C** as the

slopes for the terminal phase of elimination were positive. Other pharmacokinetic parameters were not calculated as this was beyond the objectives of the study. Due to limits in the sensitivity of the assay, plasma concentrations for intermediate **C** were below the limits of detection for many of the timepoints.

Buss and Hasinoff (1995) studied the hydrolysis of dexrazoxane in the presence of iron. The hydrolysis of 1 mM dexrazoxane in 50 mM Tris Buffer/150 mM KCl (pH 7.40) containing 2 mM Fe^{2+} at 37°C was followed. The intermediates **B** and **C** were not detected in the reaction mixture. The half time for the formation of ADR-925 was decreased from 26.5 hr to 8.9 hr (Hasinoff, 1994a). Further experiments indicated that Fe^{2+} enhances the rate of hydrolysis for **B** and **C** by factors of 2100 and 6000. Once Fe^{2+} binds to either intermediate, the intermediate immediately hydrolyzes to ADR-925. Presumably the low levels of free Fe^{2+} in plasma would not contribute to the hydrolysis of dexrazoxane in the rat. This would explain why intermediates **B** and **C** were observed in plasma in the rat study.

In the absence of added iron, Hasinoff (1994b) followed the hydrolysis of dexrazoxane to the intermediates **B**, **C** and ADR-925 in Tris Buffer/NaCl (pH 7.39) at 37°C. As the dexrazoxane concentration decreases, the concentration of intermediates **B**, **C** and ADR-925 increases. Also, more **B** accumulates than does **C**. At 5 min, the concentration of intermediate **B** was 5.94% (wt/wt) of the dexrazoxane concentration. All values were corrected for the molecular weight difference between dexrazoxane and the two intermediate forms. The concentration of intermediate **C** was 0.76% (wt/wt). At 29 min, intermediate **B** was 10.94% (wt/wt) and intermediate **C** was 2.88% (wt/wt) of dexrazoxane. At 59 min, the intermediate **B** concentration was 14.59% (wt/wt) and the intermediate **C** concentration was 4.19% (wt/wt) of dexrazoxane. At 2 hr,

intermediate **B** was 19.39% (wt/wt) and intermediate **C** was 5.37% (wt/wt) of the dexrazoxane concentration.

These values are smaller than found for the rat study. For the rat study, intermediate **B** was 9.19% (wt/wt) of the dexrazoxane concentration at 5 min. The concentration of intermediate **C** was 3.80% (wt/vol) of dexrazoxane. At 30 min, the concentration of intermediate **B** was 12.54% (wt/wt) and intermediate **C** was 3.19% (wt/wt) of dexrazoxane. At 60 min, the intermediate **B** concentration was 17.94% (wt/wt) and the intermediate **C** concentration was 2.67% (wt/wt) of dexrazoxane. At 2 hr, intermediate **B** was 25.36% (wt/wt) and intermediate **C** was 6.06% (wt/wt) of dexrazoxane.

The faster rates of formation of intermediates **B** and **C** in the rat study may be due to a mechanism other than simple base catalyzed hydrolysis. Hasinoff *et al.*, (1991) suggested that DHPase also hydrolyzes dexrazoxane to its one-ringed opened intermediates **B** and **C**. The enzyme was not able to hydrolyze either intermediate to ADR-925. Opening of the second ring is probably through base catalyzed hydrolysis (Hasinoff, 1993). DHPase catalyzed hydrolysis of dexrazoxane was studied at 4 different pH values between pH 6.0 and 8.28. The product ratio **B/C** ranged from 3.0 at pH 6.0, 6.1 at pH 7.41, 4.3 at pH 7.60 and 2.9 at pH 8.28. If DHPase is the *in vivo* mechanism responsible for hydrolysis of dexrazoxane to intermediates **B** and **C**, the ratio of the plasma concentration of **B/C** from the animal study should be comparable.

The plasma concentration ratio **B/C** for each rat was calculated and listed in Table 5.12 and plotted against time in figures 5.14 to 5.19. The average plasma concentration ratio **B/C** was tabulated in Table 5.13 and plotted against time in figure 5.20. The results were comparable to the product ratio **B/C** in the DHPase study of dexrazoxane by Hasinoff *et al* (1991). In the rat study at 5 min, the average plasma concentration ratio **B/C** was 2.73 and changed slowly until 60

min, where it increased to 5.85. At the last time point of 180 min, the product ratio **B**/**C** was 6.47. The product ratio **B**/**C** increases with time as the concentration of intermediate **B** accumulates in the plasma faster than intermediate **C**. The results suggest that DHPase is the mechanism responsible for the *in vivo* metabolism of dexrazoxane to the intermediates **B** and **C**. Also the presence of intermediates **B** and **C** suggest that Fe^{2+} was not a factor in the hydrolysis of intermediates **B** and **C** to ADR-925.

Chapter 6

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6.0 References

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IMAGE EVALUATION
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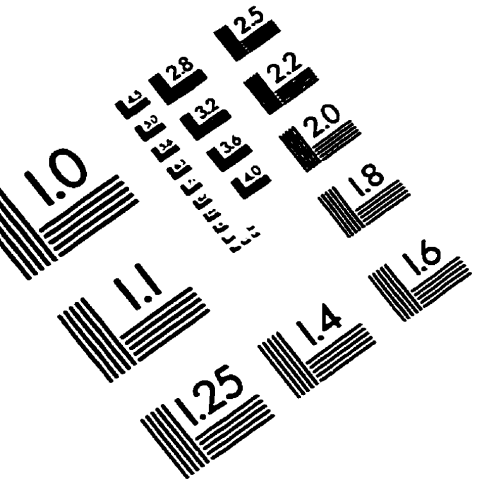

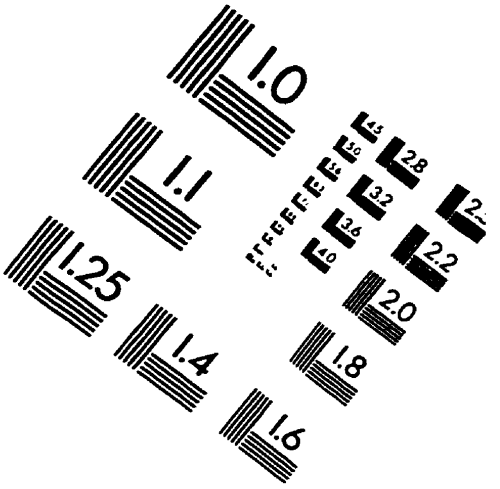

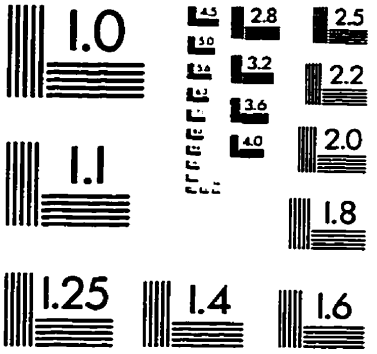

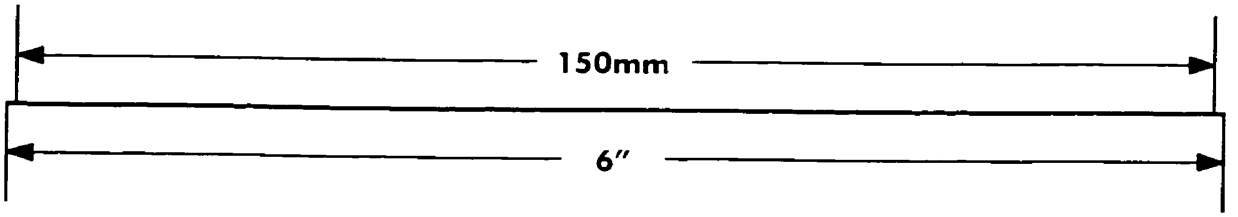

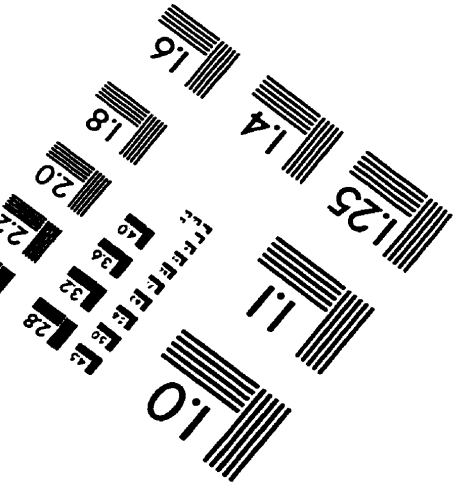

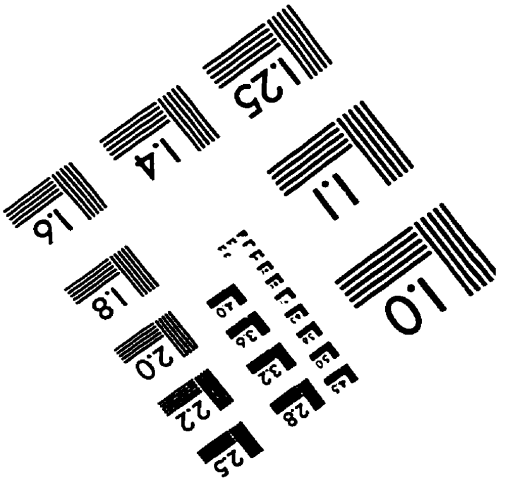

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