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PHARMACOKINETICS OF DOMOIC ACID FOLLOWING INTRAVENOUS ADMINISTRATION IN RATS

A Thesis

Submitted to the Graduate Faculty

in Partial Fulfillment of the Requirements

for the Degree of

Master of Science

in the Department of Anatomy and Physiology

Faculty of Veterinary Medicine

University of Prince Edward Island

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Charlottetown, P.E.I.

August 1990

ISBN 0-315-62408-6

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ABSTRACT

In late 1987 156 cases of acute poisoning were reported following consumption of shellfish contaminated with domoic acid (DOM), an excitatory amino acid. Appropriate therapy for these patients was impaired due to the lack of knowledge of the in vivo effects of DOM, including relevant pharmacokinetic data. The studies described herein were designed to address this need. The primary objectives were: (a) to develop an assay for the detection of DOM in rat serum, and (b) to examine the pharmacokinetics of DOM following intravenous and oral administration in rats.

To measure DOM in serum an appropriate extraction method was developed and coupled to an analytical procedure using high performance liquid chromatography (HPLC). Modifications screened during development of the extraction procedure included: (a) methods of deproteinization, (b) effect of repetitive extraction, (c) modifications of solvent ratios, (d) prior exposure to cation exchange media, and species differences in serum composition. Modifications to the chromatographic conditions included composition of the mobile phase and alterations of the detection wavelength.

Final chromatographic conditions used a C-18 reversed phase column, a mobile phase consisting of 10.5% acetonitrile and 89.5% distilled water (v/v) delivered at 1.0 mL/minute, and UV detection at 242 nm. Calculated extraction efficiencies (N=4) were 89.0±1.73 (S.D.)% for 1.00 μg/mL and 87.3%±5.86 (S.D.)% for 0.50 μg/mL. The minimum detectable concentration was 0.20 μg/mL and the overall coefficient of variation was 6.5%.

This method was used to study the kinetics of DOM extracted from contaminated mussels (1.0 mg/Kg) following intravenous administration in Long-Evans rats (N=18). Over the 80 minute sampling period, DOM was eliminated via a first order process that was best described by a two compartment model. Elimination half lives were 15.6±8.8 min. (α phase) and 32.6±15.0 min. (ß phase) respectively. DOM had an apparent volume of distribution (V_d) of 0.202 L/Kg and a clearance (Cl) of 0.32 L/Kg/hr.

The kinetics of DOM following oral administration were studied in both Long-Evans rats (N=8) and Hartly guinea pigs (N=8). Animals were fasted for 24 hours prior to receiving an oral dose of DOM (1.0 mg/Kg) via a feeding tube. DOM was not detected in the serum of either species at any time during the 80 minute sampling period.

It was concluded that following intravenous administration in rats, DOM distributes minimally to other tissues and is rapidly eliminated. Following oral administration of DOM in both rats and guinea pigs no detectable serum levels of DOM were found, indicating that less than 5% of orally administered DOM is available to the systemic circulation in these species.

DEDICATION

To my family and friends. Although you may be unaware of your contribution to this thesis, it was greatly appreciated and will not be forgotten. Thank-you

ACKNOWLEDGEMENTS

The successful completion of this thesis was made possible with the support of many people.

Sincere appreciated is expressed to Dr. Amend for placing me on the correct path toward my then to be supervisor Dr. R.A.R. Tasker and in general a special thanks to the Anatomy and Physiology Department for making my stay over the past two years an entertaining one. Dr. Tasker over the past two years has provided financial support, encouragement and unlimited time toward answering my questions and dealing with any problems, for this I extend my sincerest gratitude. To describe in one word my opinion of his ability as a supervisor, it would be extraordinary. To Barry Connell, Sandra Strain, Elizabeth Holmes, Orysia Dwydiak, Brian Grimmelt, Wendy Drake, Janice Gillis, Nirmal Sidhu, Mark Powell and the rest of the gang, your smart wit and rye humour helped over the past two years. A special thanks to my supervisory committee: Dr. Amend, Dr. Tasker, Dr. Johnson, Dr. Nijjar and Dr. Burka, your guidance throughout this project was invaluable.

Also I would like to extend a sincere thanks to all those who contributed to this document in the graphics department, especially Michelle Gauthier, Mary Ada Upstone, Glenda Clements and Shelly Ebbett.

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NOTATION

A_0	concentration at time zero
α	alpha
AMPA	alpha amino-3-hydroxy-5-methyl-isoxazole-4-propionate
AP_4	L-aminophosponobutyric acid
APV	DL-amino-5-phosponovalerate
ATP	adenosine triphosphate
AUC	area under the curve
ASP	amnesic shellfish poisoning
B_0	concentration at time zero
ß	beta
BA	bioavailability
BVS	blood vascular system
C_0	concentration at time zero
C°	celsius
Cl	clearance
CNQX	6-cyano-7-nitro-quinoxaline-2, 3 dione
CNS	central nervous system
CPP	3-(2-carboxypiperazin-4-yl) propyl-1-phosphate
di-HQC	6-7-dichloro-3-hydroxy-2-quinoxalinecarboxylicacid
DNQX	6-7-dinitro-quinoxaline-2, 3 dione
DOM	domoic acid
DSP	diarrhetic shellfish poisoning
EAA	excitatory amino acid
gm	gram
HCL	hydrochloric acid
HPLC	high performance liquid chromatography
hr	hour
HVPE	high voltage paper electrophoresis
ip	intraperitoneal
iu	international units
iv	intravenous
KA	kainic acid
K_{el}	rate constant of elimination
Kg	kilogram
L	litre
LC	liquid chromatography
$\log_{10}$	log to the base ten
LTP	longterm potentiation
min	minute
mL	millilitre
mm	millimeter
NMDA	N-methyl-D-aspartate
ODS-3	octadecyi silicate
PCP	phencyclidine
PE_{50}	polyethylene tubing
PSP	paralytic shellfish poisoning
r^2	correlation coefficient

Rt	retention time
SD	standard deviation
SSA	sulfasalycylic acid
T	time
$T_{1/2}$	half life
UV	ultraviolet
V_d	volume of distribution
v/v	volume/volume
μg	microgram
μL	microlitre
%	percentage
±	plus or minus
$[X_0]$	concentration at time zero

Chapter 1

GENERAL INTRODUCTION

1.1. Suspension line mussel culturing

Mussel culturing had its earliest beginnings during the first and second World Wars (1). In Atlantic Canada, the longline system of suspension culturing is most popular for culturing the blue mussel (Mytilus edulis) (2). Following the successful application and development of suspension line mussel culturing on Prince Edward Island in the 1970's, mussel culturing has florished throughout the Maritimes and abroad. The main components of this system include the: (a) backline, (b) buoys, and (c) anchors (Figure 1). The backline, which is connected to the concrete anchor(s), serves to keep the mussel socks separated and suspended at a particular depth above the sea floor. Styrofoam bouys connect the backline for flotation, initially at three meter intervals, with subsequent addition of other buoys as the mussels grow. The back line serves to position the line just below the surface of the water, to keep the mussels suspended in the water column, for optimum feeding and in winter, protection from the ice.

Initially mussels were cultured to market size using similar equipment to that used to collect the immature (motile) mussels or spat. This proved too costly and inefficient for large scale operations. Growing mussels in nylon sleeves increased production and reduced fouling (3).

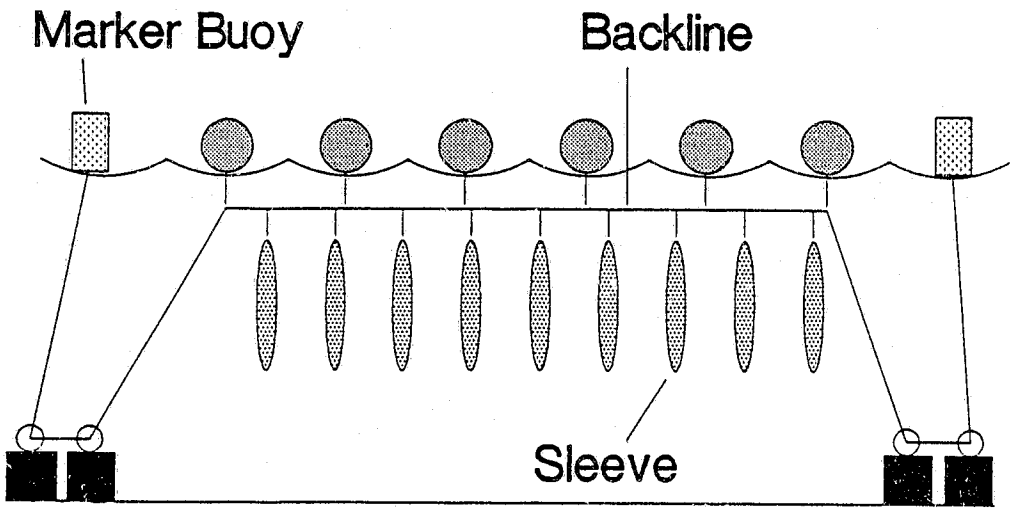

Figure 1. Schematic diagram depicting long line mussel culturing system

For the region of Prince Edward Island, sleeving usually occurs in the fall months due to the irregular spat (seed) size. Italian-mesh sleeve material (40 mm mesh size) is the most popular for growing juvenile mussels to market size (2). Once the mussels have been declumped and graded they are placed into 3-8 meter socks, depending on the water depth, with the diameter of the sock remaining constant. Following this the sleeves are placed in salt water for approximately twelve hours to allow byssal fibers from the mussel to attach to the sock. Harvesting occurs at various times throughout the year depending on when the sleeves are placed in the water and the extent to which the mussels grow.

1.2 Shellfish Toxins

Bivalves (mussels, clams, oysters etc.) are suspension feeders and obtain food by actively filtering particulate matter from the surrounding water. This process is relatively nondiscriminate with particles ranging in size from 1-100μm being filtered. Under optimal conditions, each mussel may filter as much as three liters of sea water every hour (6). This leads to rapid growth, but may allow water borne toxins to be filtered and concentrated in the digestive glands of the shellfish. Although many of the substances concentrated by bivalves are poisonous to humans, they usually have little effect on the bivalve itself. Human illnesses due to shellfish toxins have occurred since the 18th century (4).

Today the increased number of reported cases of shellfish poisoning is related to: (a) greater demand for shellfish in the market place, (b) a global increase in toxin producing algae, (c) increased public awareness of food related diseases and (d) an increased understanding of marine toxins (5).

Marine toxins that appear in shellfish range from those produced by ocean algae to chemical pollutants and heavy metals (5). In the Maritimes the most common form of shellfish contamination is paralytic shellfish poisoning (PSP), with outbreaks of PSP occuring between May and November resulting from several toxins (saxitoxins, neosaxitoxin and gonyautoxin) secreted by the phytoplankton Alexandrium tamarensis (7). More recently a toxin known as domoic acid has been isolated from the diatom Nitzchia pungens F. multiseries. This toxin, responsible for amnesic shellfish poisoning (ASP) (8), was discovered to produce initial clinical signs similar to PSP. This incident is described in greater detail below.

1.3 Outbreak of Human Toxicity

1.3.1 Epidemiology and sequence of events

In the fall of 1987, 156 people became ill following the ingestion of cultured blue mussels (Mytilus edulis) from Prince Edward Island (10). A majority of individuals affected were in Montreal, Quebec. Symptoms (and incidence) ranged from nausea (81%), vomiting (54%), and diarrhea (42%) in the

less severe cases, to memory loss (17%) or death in the most severe situations. Memory loss took two forms depending on the severity of the toxicity. One group of patients incurred partial deficits in their short term memory, while more severly affected patients experienced loss of both short term and long term memory. Of the 156 individuals affected during the incident, about 80% were older than forty years of age (8). On average gastrointestinal effects appeared at 6 hours after ingestion, while central nervous system (CNS) effects were noted approximately 16 hours following ingestion of contaminated mussels (22). Those individuals most severely affected were admitted to hospital intensive care units and three elderly patients died.

To restrict the consumption of poisonous mussels, Health and Welfare Canada initially issued a public warning on December 1st against the consumption of P.E.I. mussels. Mussels from the Magdalene Islands were also restricted from the market on December 5th based on a recommendation of a government task force, composed of provincial and federal officials and local experts on the mussel culturing industry. Approximately one week following the formation of the task force, PSP was ruled out as the toxin responsible for mussel contamination. This meant that the toxin in Prince Edward Island mussels was a compound not previously detected in the region, leading to widespread concern about the nature and extent of the contamination. On December 11th a second

warning was issued by Health and Welfare Canada and a total recall, from 50,000 establishments in the U.S and Canada, was made for live or cooked clams, oysters and quahaugs from New Brunswick, Prince Edward Island, Nova Scotia and Newfoundland.

1.3.2 Identification and Source of Domoic Acid

Once human intoxication had been linked to cultured mussels from Prince Edward Island, the National Research Council of Canada began an intense effort to identify the mussel toxin (11).

Initially it was necessary to isolate the toxic fraction of the mussel for subsequent purification and structural identification. Both high performance liquid chromatography (HPLC) and high-voltage paper electrophoresis (HVPE) were employed in the isolation. Following electrophoresis of toxic aqueous extracts, toxicity was associated with an acidic band that stained yellow with ninhydrin and migrated very closely with L-glutamate. Suspecting the compound was acidic and possibly a peptide, the content of clean and contaminated mussels was compared using HPLC. Since most organic acids and peptides absorb some ultraviolet light at a wavelength of 210 nm, this wavelength was chosen for initial screening of samples. The chromatogram for the toxic fraction showed a unique peak at 12.1 minutes with a maximum absorption at 242 nm. No other components of the fraction produced toxicity when injected into mice. When the toxic fraction from the

HPLC analysis was subjected to HVPE, a yellow band was observed on the electrophoretic paper, which corresponded with the position that had earlier produced toxicity. Subsequent bioassays of these fractions showed that only the yellow band was toxic and conversely this band produced the same retention time (12.1 min) and displayed the same UV spectrum (242 nm) as the previous toxic extract. At this point NRC announced that the toxin had been isolated. Subsequent, structural analysis using mass spectrometry indicated that domoic acid (DOM), a structural analog of glutamic acid, was responsible for human intoxication (11).

Along with the isolation of DOM by the NRC, both the NRC and Dr. L.A. Hanic of the University of P.E.I. were involved in identifying the source of DOM in the water column. Plankton tows performed by Dr. Hanic in the affected area showed an intense plankton blooms, that were latter shown to contain the diatom Nitzchia pungens (personal communication). Identification of Nitzchia as the source of DOM, was further supported by three main lines of evidence: (a) extracts of the plankton tow produced the same toxicity in mice as the contaminated mussel extracts (11), (b) digestive glands of mussels during the toxin episode contained large concentrations of Nitzchia (11) and, (c) cultures of Nitzchia are capable of de novo synthesis of DOM in an isolated laboratory environment (8).

1.3.3 Chemistry of Domoic Acid

Domoic acid, the structure of which is shown in Figure 2, is an excitatory amino acid (EAA) originally isolated in 1956 from the red algae Chondria armata by Takemoto et al. (12). DOM is an amino acid that is reasonably hydrophilic at any pH and exhibits a structure very similar to that of both glutamate and kainic acid (KA). Domoic acid has been previously shown to have both insecticidal and neuroexcitatory effects in vitro (13-16) and in vivo (17,18).

Domoic acid has come to the forefront of EAA research in Atlantic Canada following the mussel toxin outbreak. Since the toxic outbreak, research seems to indicate that DOM is creating its neurotoxicity through the kainic acid receptor subtype (19) and this neurotoxicity seems to reflect the distribution of kainate receptors in the brain. Although the structures of KA and DOM are very similar, DOM has greater affinity for the KA receptor and is about three times more potent on isolated cells (16) and 8 times more potent following systemic administration (20). Both DOM and KA produce similar signs of toxicity in rodents following intraperitoneal (i.p.) administration, these include excitement, scratching, "wet dog" shakes and convulsions which were considered "atypical" of PSP poisoning and therefore helped eliminate PSP from the list of possible toxins (9).

Although DOM has both insecticidal and neurotoxic effects (13-16), a partially purified form, has been employed as an

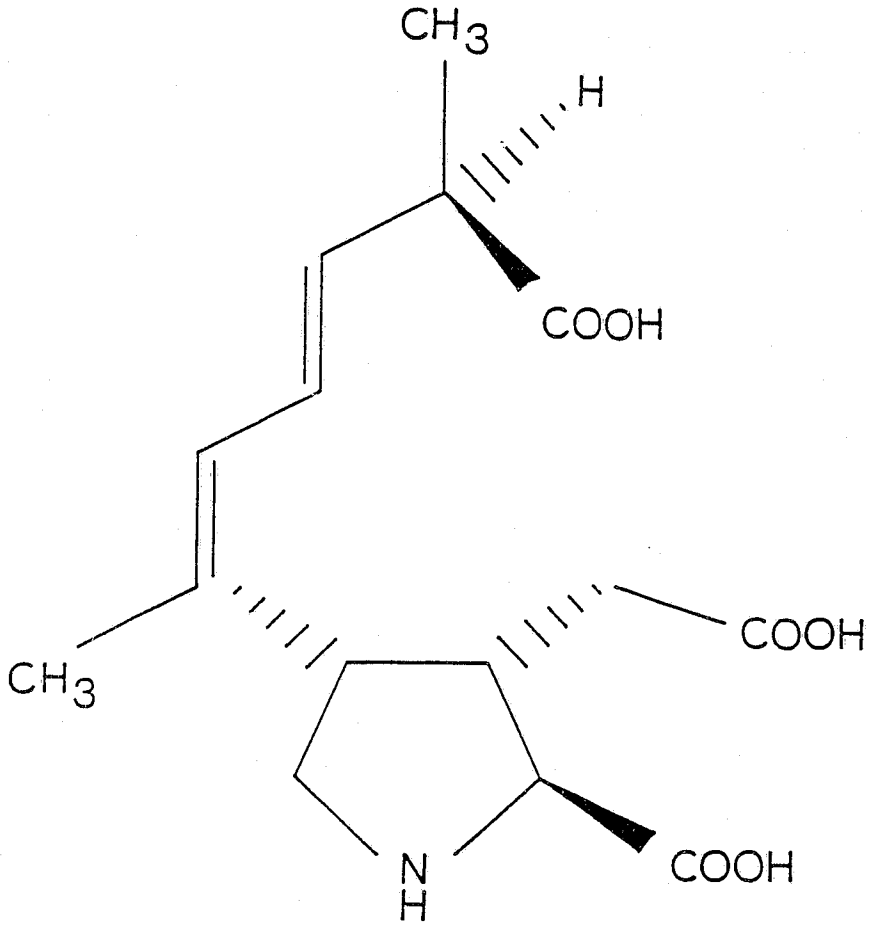

Figure 2. Structure of domoic acid

anthelmintic in Japan (21) with oral doses of 20 mg/Kg being administered with no ill effects (i.e. a 30 Kg child receives 600 mg DOM). Presently little is known, about the neurotoxic mechanisms, receptor interactions and absorption kinetics of DOM in humans. On going research is slowly enhancing the physiological and pharmacological profile of DOM in vivo.

1.3.4 Clinical Findings

Of the 156 confirmed cases of DOM poisoning, a majority of the patients who were admitted to the hospital experienced nausea, vomiting and varying degrees of confusion. Although some children were admitted, 80% of the patients were greater than forty years of age and those over 70 years were admitted to intensive care units and later died (8).

As described above, apparently elderly patients were the most severely affected. Possibly due to any number of factors including renal insufficiency or decreased blood brain barrier (BBB) integrity. Upon admission to the hospital, the condition of these individuals was characterized by: (a) severe uncontrollable convulsions, (b) self mutilation, (c) decreased sensitivity to pain, (d) abnormal facial expressions, (e) profound hyper/hypotension, (f) serious gastrointestinal bleeding and (g) memory loss (22).

It has been estimated that three individuals for every thousand portions of mussels served became ill enough to report to hospital (23). This seems to suggest that a segment

of the population was either predisposed to mussel toxicity and/or consumed considerably more domoic acid than others. Alternatively they may have ingested other products that increased the absorption of DOM. To date no productive correlations have been described.

1.3.5 Animal Data

Over the past three years establishing the major target sites for DOM in the brain, the mechanism of action, and if dose or route of administration alter the pattern of neuronal toxicity, are areas deserving attention.

The most extensively damaged neuronal structure, following intravenous administration of DOM in rats and mice, is the hippocampus (CA_1, CA_3 and CA_4) (20,24), a structure which has been implicated in long term and short term memory (25). Both dose and route of adminstration affect the extent to which DOM produces neuronal degeneration (23). Following oral administrations of DOM, both rats and mice excrete 98% or more of the toxin through the feces, while no detectable levels are seen in the urine (23). Also Iverson et al. (1990) had indicated that oral doses of 70 mg/kg created negligible behavioral changes in rodents (23), while 5 mg/kg administered orally to primates (cynomolgus monkey) promoted vomiting, mastication, yawning and fine tremors (25), thus reflecting a degree of species differentiation with respect to absorption. With regard to the elimination of DOM, a recent

report by Preston and Hynie (1990) indicated that the kidneys play a critical role in elimination of i.v. DOM and that following nephrectomy in rats, concentrations of DOM in cerebrospinal fluid increased by a factor of two (26).

Based on the results of these and other studies one can speculate on several features of DOM pharmacology namely that: (a) the molecule is not well absorbed from the gut in humans, (b) once in circulation the hippocampus is the major neuronal target site, (c) diffusion across the cerebrovascular barrier is relatively slow, and the integrity of this barrier is critical, and (d) the kidneys play a prominent role in DOM elimination.

1.4 Pharmacology of Excitatory Amino Acids

For years scientists have been aware of the various amino acid pools in the central nervous system (CNS), but unaware of their unique function in regulating synaptic transmission within the CNS. It was in 1951 that Okamoto and colleagues first discovered that systemic injections of glutamate, an endogenous amino acid, produced convulsions in the rat (27). Later Curtis and Watkins (1960) showed, via intracellular recording, that glutamate was able to depolarize individual neurons in the CNS.(28). From these initial experiments developed the class of EAA's, along with an ever diverging field of research into the structure and function of other exogenous and endogenous EAA's.

Excitatory amino acids such as glutamate, aspartate and others are found throughout the mammalian CNS. Both glutamate and aspartate are involved in the majority of excitatory neurotransmission in the CNS (29). These two comprise a majority of excitatory amino acid pools in the CNS, and satisfy many of the criteria required for classification as neurotransmitters (27). They are :

(1) present in appropriate concentrations within the brain.

(2) released in a calcium dependent manner upon electrical stimulation in vitro.

(3) powerful excitatory effects on neurons when iontophoresed in vivo.

(4) their concentrations are partially controlled by high affinity uptake systems in nerve endings in a number of neuronal pathways.

(5) they possess selective binding sites in vitro and active receptor sites in vivo.

These putative neurotransmitters are primarily ionotropic, meaning their receptors function to open ion gates on postsynaptic (or presynaptic) membranes, causing large conductance changes that result in excitatory actions. Although no competitive antagonist has been found for glutamate, most investigators are increasingly confident, that glutamate and aspartate are the predominant excitatory neurotransmitters within the CNS.

After the initial discovery of endogenous EAA's, efforts were directed toward establishing the classification of their respective receptors. The evolution of the receptor theories that are accepted today, have comprised more than thirty years of research and have evolved from the initial one receptor theory to the widely accepted four (or more) receptor theory.

The major receptors for EAA's are separated into classes via binding and electrophysiological studies (30-32) and consist of N-methyl-D-aspartate (NMDA), quisqualate (AMPA), kainate (KA) and L-aminophosponobutyric acid (AP_4) receptors.

NMDA Receptors

The most extensively studied EAA receptor is the NMDA receptor; so named because NMDA was the first agonist to produce selective activation (29). Currently the activity of this receptor can be altered at five pharmacologically distinct sites (29):

1. a transmitter binding site, that binds L - glutamate
2. a regulatory or coactivator site, that binds glycine
3. a site within the channel that binds phencyclidine (PCP) and related compounds.
4. a voltage dependent magnesium binding site
5. an inhibitory divalent cationic site that can bind zinc

In addition ligand binding studies indicate that there are two distinct binding sites associated with the transmitter

recognition site, one of which predominantly binds agonists and the other predominantly antagonists (33). Some of the most common agonists for the NMDA receptor include, NMDA, glutamate, aspartate and ibotenate (27), while the prominent antagonists include DL-L-Amino-5-phosphonovalerate (APV) and D-alpha-amino adipate among others (27).

As mentioned previously, NMDA receptors may contain either antagonist or agonist preferring binding sites, and this reflects a differential distribution of NMDA receptors in the mammalian brain. The agonist preferring sites, receptors labelled by L-[H^3] glutamate, concentrate in regions of the medial striatum and cerebellum (29), while the antagonist sites ([^{3}H] 3-(2-carboxypiperazin-4yl) propyl-1-phosphate [CPP] labelled receptors) mainly exist in the lateral thalamus (29) and cerebral cortex (29). Both sites are present to a lesser extent in other regions of the CNS.

NMDA receptors are dynamic serving many functional roles in the mammalian nervous system. They appear to be integral to the phenomenon of long - term potentiation (LTP) (34), thus NMDA receptor agonists may play a role as nootropic drugs in the future (35). Both competitive and non-competitive antagonists of NMDA receptors are effective against epileptic activity in various animal models (36-39) and NMDA antagonists have been shown to be effective against neurodegeneration related to hypoxia (40).

Quisqualate Receptors

The quisqualate receptor system was identified by responses produced by quisqualic acid (35) a compound originally isolated from the seeds of the plant Quisqualis fructus (42). Other known agonists of quisqualate receptors include L - glutamate (43) and AMPA (43). AMPA, an analog of ibotenic acid, was initially synthesized in 1980 by Krogsgaard - Larsen (43) and today is the ligand of choice in identifying quisqualate receptors (43). Although quisqualic acid has a higher affinity for the quisqualate receptor (44), AMPA induces a depolarization analogous to quisqualic acid but has very little affinity for the NMDA or kainate receptors, indicating a higher degree of selectivity. This makes AMPA a more useful ligand for identification of quisqualate receptors.

One major problem in the characterization of this receptor class is that specific antagonists have not yet been developed for the quisqualate receptor. The earliest antagonists, exhibited effects on NMDA, quisqualate and kainate receptors which made differentiation difficult. Presently the most potent non - selective quisqualate antagonists include : 6-cyano-7-nitro-quinoxaline-2,3-dione (CNQX) (45) and 6-7-dinitro-quinoxaline-2,3 -dione (DNQX) (45). It should be kept in mind that these antagonists also block kainate induced responses (45), thus illustrating the need for more selective antagonists for definitive receptor classification.

Although characterization of the quisqualate receptor has proven difficult, its distribution throughout the brain has been well documented. Autoradiographic analysis of [^{3}H] AMPA binding sites indicates that quisqualate receptors are localized mainly in the telencephalic regions of the brain (29), particularly the CA_1 (stratum radiatum) region of the hippocampus, outer layers of the cerebral cortex, lateral septum and the molecular layer of the cerebellum (46-48).

Kainate Receptors

The third class of EAA receptors, kainate receptors, respond primarily to alpha-kainate (49), a rigid glutamate analogue originally isolated from the seaweed Digenea simplex. Kainate receptor agonists include domoic acid (50), kainic acid (51) and L-glutamate (51). Until recently only two relatively nonspecific antagonists existed for kainic acid receptors, namely CNQX (45) and DNQX (45). Characterization of kainate receptors, has therefore been plagued by the same problems discussed earlier for the quisqualate receptor. In 1988 Frie et al. (52) reported that 6,7 - dichloro - 3 - hydroxy - 2 - quinoxalinecarboxylic acid (diCl - HQC), was a potent and selective antagonist of kainate receptors. If the selectivity of diCl - HQC is confirmed, it may become a valuable tool for further studies in EAA receptors.

As with quisqualate receptors, kainate receptors appear localized mainly in the telencephalic regions of the brain

(29). Using [^{3}H] kainate as a radioligand, high concentrations of kainate receptors have been found in the hippocampal CA_3 striatum lucidum, deep cerebral cortical layers, striatum, reticular nucleus of the thalamus and granule cell layer of the cerebellum (53, 54). Thus kainate and quisqualate receptors exhibit a similar but not identical distribution. Whether this implies some common functional relationship and/or interaction between these receptors is yet to be determined.

AP_4 RECEPTORS

The method of identifying AP_4 receptors is unique in the classification scheme of EAA receptors. Unlike the receptor classes discussed previously, the L -aminophosphono-butyric acid (AP_4) receptors were identified through their antagonistic actions at glutaminergic synapses (29). Other agonists of this receptor class include the endogenous ligand L - glutamate and L - serine - O - phosphate (29). Unfortunately no antagonists for this receptor have been discovered to date.

Only preliminary studies have been performed on the characterization of this receptor. This work has shown that AP_4 is a potent antagonist in both spinal cord (55) and perforant path synapses (55), that appear to use glutamate as their neurotransmitter. NMDA antagonists are moderately effective at these sites and specific kainate and quisqualate

responses are altered by t. e presence of AP_4 (56). Thus the AP_4 receptor, represents an intriguing addition to the EAA receptor classification scheme and research has yet to determine its physiological importance.

1.5 Pharmacokinetics of Amino Acids

1.5.1 Amino Acid Absorption

Absorption of an amino acid in the gastrointestinal tract can take place via either an active or passive transport. Passive absorption involves the energy independent transport from the lumen into the cell depending on the concentration gradient. Active transport is a carrier mediated event requiring an energy source (usually ATP) and amino acids (neutral, basic or acidic) and this amino acid transport may or may not be dependent on cotransport with sodium. (57)

Analysis of amino acid transport systems is complicated by the presence of multiple transporters with overlapping specificity. At least 12 different classes of transporters have been described for vertebrates (58). Both proline and glutamate are structurally similar to DOM, and their absorption across the jejunal border has been specifically characterized (59, 60). Since the two molecules share certain structural features, perhaps this Na^+/K^+ dependent transport system will aid in the elucidation of DOM absorption from the gastrointestinal tract in humans.

1.5.2 Distribution and Elimination of Amino Acids

Following the GI absorption of an amino acids, they enter the portal circulation and may either remain in the amino acid pool of the liver, undergo transamination/deamination or enter the circulation to be either eliminated or used as precursors for proteins and hormones. Depending upon the structure and related charge of the molecule the distribution into the tissues may be extensive or restricted largely to the blood vascular system for rapid elimination via the kidney or liver. Amino acids that are in excess in the body or that are nonessential are transported to the liver and are broken down into ammonia and keto acids (61), the latter of which may be used for amino acid synthesis. A majority of the ammonia produced from deamination in the liver is converted to urea for excretion via the kidneys (61).

1.6 Statement of the Problem

Following the outbreak of domoic acid toxicity, a great deal of work was concerned with the pathological consequences of exposure to DOM, the possible receptor interactions involved and the specific target sites for DOM in the CNS. Unfortunately these data do not yet give any indication to a clinician what action should be taken, in the event of receiving a patient immediately following consumption of DOM contaminated shellfish. Information regarding the rate of absorption, route of elimination, or possible drugs available

to enhance the elimination of DOM are all questions deserving of attention. A critical analysis of the pharmacokinetics of DOM is therefore required for examining these questions and predicting therapeutic strategies.

As in many novel *in vivo* pharmacokinetic studies both absorption and elimination kinetics of DOM need to be examined. The studies described in this thesis are concerned with (a) developing an appropriate method of measuring concentrations of DOM in a biological matrix (serum) and (b) using this method to determine critical kinetic variables such as: rate constant of elimination (K_e), half life ($T_{1/2}$), volume of distribution (V_d) and clearance (Cl).

Chapter 2

DEVELOPMENT OF AN ASSAY FOR DOMOIC ACID IN SERUM

2.1 Introduction

In November and December of 1987 between 100 and 150 people became ill after ingesting cultured blue mussels (Mytilus edulis) harvested from eastern Prince Edward Island. Within three weeks researchers at the Atlantic Canada Division of the National Research Council of Canada isolated the molluscan toxin and identified it as domoic acid (DOM) (9, 62) (see Chapter 1).

Following the outbreak much of the research was directed toward producing qualitative and quantitative procedures for the detection of domoic acid in both mussel tissue and sea water. These procedures included a mouse bioassay previously used to detect other molluscan toxins (63), reversed-phase high performance liquid chromatography (HPLC) with UV detection of domoic acid extracted from mussels (62) and more recently a fluorescence derivitization technique used for detecting low concentrations of domoic acid in sea water (10).

To date, however there have been no reports of an assay that would be applicable to the detection of DOM in body fluids. A suitable method for quantifying DOM concentrations in body fluids, especially blood, is essential to studies on the pharmacokinetics and fate of domoic acid in vivo in

different species. This was therefore, a necessary prerequisite for studies described later in this thesis.

2.1.1. Statement of Problem

To provide a method of DOM analysis that would be appropriate to studies of DOM pharmacokinetics, it was necessary to develop an extraction procedure and chromatographic conditions for DOM in rat serum. Initial objectives for this method include minimal within day and between days variability, good extraction efficiency (>75%), adequate sensitivity (< 0.25 ug/mL) and an overall coefficient of variation of less than 10% (see Appendix A).

2.2 General Methods and Materials

2.2.1 Apparatus, Chemicals and Solvents

All samples were analyzed using a Perkin-Elmer (Montreal, Canada) HPLC system consisting of an SEC-4 environmental control chamber, ISS-100 autoinjector, Series 410 LC pump, LC 90 UV spectrophotometric detector and an LC-100 computing integrator. Analytes were separated using a reversed-phase column (250mm X 4.1mm I.D.) containing 10-μm Partisil^{R} ODS-3 packing.

HPLC-grade acetonitrile, phosphoric acid, chloroform and methanol were purchased from Fisher Scientific (Halifax, Canada).

2.2.2 Biologicals

Blood samples were collected from both Long-Evans rats and Hartly guinea pigs with the aid of a jugular catheter. All blood samples were allowed to clot and centrifuged to obtain the serum, which was frozen at -20 degrees celcius until analysis. Domoic acid was obtained by acid extraction of contaminated mussels as described by Grimmelt et al. (1989) (65). Concentration and purity of DOM were verified by analytical HPLC (65).

2.3 Results and Discussion

2.3.1 Development of Extraction Procedures

2.3.1.1 Chromatographic conditions

To develop a method for measuring DOM in serum, it was first necessary to establish chromatographic conditions for detecting aqueous DOM.

Chromatographic conditions for aqueous DOM have been described elsewhere (65) and are listed in Table I. The method employs isocratic elution of DOM on a reversed-phase column with subsequent detection by UV absorbance (242nm). Once these conditions had been established, aqueous standard curves were prepared to determine if there was a linear relationship between peak height and concentration of DOM and to assess the degree of variability in this method for DOM detection. Linearity was desirable because it facilitated

Table I: Chromatographic conditions for analysis of DOM extracted from mussels.

Sample	20 μl injection (Perkin-Elmer) 155-100 autosampler.
Pump	Perkin-Elmer series 410.
Solvent	87.5/12.5 (v/v) (H_2O/acetonitrile) pH 3.0
Flow Rate	1.0 mL/min.
Column	Perkin-Elmer C-18 (250mm x 4.1mm I.D.)
Detector	Perkin-Elmer LC-90 UV absorbance (242 nm)
Recorder	Perkin-Elmer L-100 Integrating Recorder

interpolation of the standard curve, and knowing the degree of variability assessed the need for an internal standard prior to beginning work with DOM spiked serum samples.

Aqueous standard curves were linear throughout the concentration range tested (0.1-2.0 μg/mL) (r^2 = 0.999), with no significant within day (p=0.12) and between day variability (p=0.24). A composite (n=2) aqueous standard curve was described by the equation y=0.0425x + 0.0012.

These data confirmed that the previously described chromatographic conditions were reliable for the detection of DOM in water.

2.3.1.2 Existing method of analysis

The next question that needed to be answered, was whether DOM could be reliably detected in serum using an extraction procedure that had been developed for isolating DOM from mussels

The extraction used was a modification of a procedure originally described by Bligh and Dyer (64) for separation of lipids from tissue samples and later by Grimmelt (65) for DOM detection. Blank or spiked serum was placed in a culture tube (3.0 mL, 10x75mm). An aliquot (80μl) of serum was removed and added to a second test tube containing chloroform (100μl) and methanol (200μl). This solution was then mixed using a vortex mixer for 1 minute. The remaining 100 μl of serum and an additional 200 μl of chloroform were added to the same test

tube. Following an additional 1 min. on a vortex-mixer the tubes were centrifuged (Model TJ-6, Beckman Instruments, Toronto, Canada) at 350xg for 12 minutes at ambient temperature. From the resultant triphasic suspension a 200 μl aliquot of the top phase (DOM/methanol mixture), was removed for subsequent HPLC analysis (duplicate 20 μl injections).

Following this extraction, chromatograms of blank rat serum produced peaks at 10.67 and 12.87 minutes. Chromatograms of serum spiked with 2.00 μg/mL of DOM show that DOM eluted at 10.72 min. under these conditions, but could not be reliably quantified due to the interfering peak at 10.67 min. (Table II.A.)

2.3.1.3 Effects of deproteinization with sulfosalicylic acid or boiling

Although the organic solvents in the Bligh and Dyer extraction procedure (64) partially deproteinized the serum, it is primarily designed for selective extraction of lipids. It was possible, therefore that the interfering peak at 10.67 min. could be a soluble protein, peptide or amino acid that was not denatured.

To test this possibility, the top phase (200μl) of the triphasic suspension following extraction (preceeding text) was transferred to a clean test tube and 20 μl of sulfosalicylic acid (SSA)(50/50 w/v), a known deproteinizing

TABLE II: Development of a serum extraction method. Modifications to the procedure for extracting DOM from mussels

MODIFICATION TO EXTRACTION PROCEDURE			
A)	Basic Procedure min. protocol: STEP 1: 80 µL/100 µL/200 µL (serum/$CHCL_3$/MeOH) STEP 2: 100 µL/100 µL (serum/$CHCL_3$)	A)	Rt for DOM: 10.72 Serum blank peaks Rt: 10.67 and 12.87 min. CONCLUSION: Peak interference at 10.67 min.; therefore unacceptable.
Re-extraction			
B)		B)	Rt: for DOM Serum blank:
		Unresolved Peak Rt	Peak Height
a)	Protocol: Step 1: 80 µL/100 µl/200 µl) (serum/$CHCL_3$/MeOH) Step 2: 100 µl/100 µl (serum /CH3 CL)	a) 10.67 min.	125 mm
b)	Repeat using supernatent from (a)	b) 10.26 min.	64 mm
c)	Repeat using supernatent from (b)	c) 10.22 min.	70 mm
		Conclusion: the re-extraction procedure did not satisfactorily reduce any peak interference at 10.67 min.	

C)	Volume Ratio Alteration Protocol: Step 1: 180 μl/200 μl/400 μl (serum/$CHCl_3$/MeOH) Step 2: 200 μl/200 μl ($CHCL_3$/H2O)	C) Rt for DOM: 11.75 min. Blank peak Rt: 10.28 min., 11.66 min. and 12.38 min. <u>Conclusion</u>: Poor resolution.
D)	Substitution With Isopropanol Protocol: STEP 1: 180 μl/200 μl/400 μl (serum/$CHCL_3$/isopropanol) Step 2: (180 μL/200 μL) (serum/$CHCL_3$)	D) Rt for DOM: 11.0 min. Blank peak Rt: 8.04 min. and 9.48 min. <u>Conclusion:</u> peak in the blank sample coeluted with DOM to produce one broad peak at 11.14 min.

agent (65) was added to the test tube. This solution was then mixed, centrifuged (850 x g) (Model TJ-6, Beckman Instruments Ltd., Mississauga, Ont.) and the supernatant was adjusted to pH 3.0 with concentrated phosphoric acid. Samples were analyzed by HPLC as described above (Table I., Section 2.3.1.1).

Using these conditions a serum sample spiked with DOM (1.0 ug/mL) produced a number of peaks around the elution time of DOM (9.7 min.). This lack of resolution made modification of the extraction procedure with SSA unacceptable for practical use [Table III(A)].

During the early stages of the mussel toxin incident established procedures for PSP detection were used to isolate DOM from mussel tissue (63). A crucial step in these procedures, was to boil the tissue homogenate in either water or acid, prior to filtration and chromatographic analysis. Although deproteinization with SSA was unacceptable, it was postulated that deproteinization by boiling might remove the contaminant from the serum.

Both blank and spiked (10 μg/mL) samples (200 μl) were prepared in culture tubes and later placed in boiling water for 1 minute. Following this an attempt was made to separate the denatured proteins, by centrifugation (850xg. Model TJ-6, Beckman Instruments Ltd.), from the rest of the serum components. Unfortunately there was such an extensive amount of denaturing that it was not possible to separate the

Table III: Development of an assay for DOM in serum, effect of select modification in extraction, animals or chromatographic conditions.

	Modification	Result
A)	Addition of sulfasalycilic acid to supernatant.	Increased number of interfering peaks.
B)	Boiling.	Extensive denaturing did not allow for phase separation.
C)	Cation exchange column.	Domoic acid undetected.
D)	Fasted rat.	Interference still present.
E)	Extraction of guinea pig serum.	Interference unaltered
F)	Detection modification. (238-248nm)	None satisfactorily removed interference
G)	Mobile phase composition	(i) 87.5/12.5 (v/v) (H_2O/acetonitrile)
		Interference present
		(ii) 89.5/10.5 (v/v) (H_2O/acetonitrile)
		Rt > 30 min for interfering peak

various phases [Table III(B)].

2.3.1.4. Effect of repetitive extraction and varying solvent composition.

To test the possibilities that either the extraction efficiency for DOM was greater than that of the interfering peak, or that substitution of another solvent would produce greater selectivity for DOM, both repetitive extractions and alterations of solvent composition were performed.

To establish baseline values for the HPLC analysis blank and spiked (2.00 μg/mL) serum samples were prepared and extracted as previously described (section 2.3.1.2) and injected on the HPLC. This produced a typical output for blank serum in which an interfering peak eluted off the column at 10.67 minutes.

Since the extraction procedure was able to reduce many interfering components in the serum, further extraction of the top phase with chloroform and methanol, may significantly reduce the concentration of the contaminant. To test this hypothesis, a blank sample was extracted as before. The resultant top phase was then re-extracted a second and third time with the identical protocol, except that serum was replaced by the top phase of the preceding extraction [Table II, (B)].

In all three extractions the DOM interfering peak was present (Rt = 10.67, 10.26 and 10.22 min respectively).

Although there was a reduction in the peak height from 125 mm to 70 mm, a peak of this size would still be a major confounding variable in quantifying low concentrations of DOM (0.20-1.00μg/mL).

The next hypothesis tested was that altering the volume ratio of sample and solvent would reduce interference during the elution time of DOM. If the extraction solvents were saturated with the respective serum components, an increase in the volume of the solvents may make it possible to reduce the interference.

Blank and spiked (2.00 μg/mL) serum samples were prepared and extracted as previously described (2.3.1.2), with the exception that the volume of extraction solvent in each step was doubled and the entire serum sample (180 μl) was used in step 1. Following extraction the samples were analyzed by HPLC. Chromatograms from all samples had a sizeable peak that eluted at approximately the same time as DOM (Rt=11.75) [Table II (C)]. This approach was, therefore, unsuitable for quantifying DOM in serum.

Because it was well established that the interfering serum component dissolved in methanol, an attempt was made to substitute a different alcohol, isopropanol, in the extraction. Isopropanol is less polar and a weaker eluent than methanol. Both blank and spiked (2.00 μg/mL) serum samples were prepared and extracted using the ratios (serum/chloroform/isopropanol) listed in Table II D. Following

injection on the HPLC, the peaks that were present in blank serum (Rt=8.04, 9.48) eluted with the DOM (Rt=11.14) to produce a single large peak. This implied that serum components not soluble in methanol were extracted into the isopropanol and the use of this solvent was therefore unacceptable.

2.3.1.5. Effect of cation exchange

Ion exchange chromatography using anion or cation exchange columns is a common approach to selectively purifying complex mixtures. Because DOM is a zwitter ion it remains charged over a wide pH range. By passing the top supernatant phase (DOM/Methanol/ interfering component) through a cation exchange column, it was possible that DOM would remain on the column (to be eluted off at a latter time), while the undesirable components would elute off immediately. It had been reported that this technique was useful as a cleanup step for mussel tissue homogenate prior to chemical derivitization of DOM (62).

The procedure tested utilized a solid phase, strong cation exchange cartridge (LC-SCX/1mL) affixed to a vacuum manifold. Following the preconditioning of the column (6.0 mL methanol/6.0 mL 0.1M HCL), the aqueous methanolic top phase (200μl) of the previously described extraction (5.0 μg/mL DOM in serum) was poured onto the column and left for 1 min. The column was then washed with distilled water (3.0 mL). The

aqueous eluent, was collected for analysis and the column was dried by aspiration (14 min. at 14mm Hg). The dried column was then treated with 0.6mL of 0.5M HCL (5 min.). Eluent was collected under vacuum for subsequent analysis. The column was then treated with a second 0.6 mL volume of 0.5M HCL and this fraction was collected for both analysis and subsequent purification (note: all eluents were adjusted to approximately pH 3.0 prior to final purification).

For the next step of the purification an octadecyl silane (C-18) cartridge was conditioned with 6.0 mL of methanol, 6.0 mL of distilled water and 6.0 mL of 0.5M HCL. The combined acid fractions from the previous two elutions were passed through the column at 2.0 mL/min and the cartridge was then washed with 3.0 mL of distilled water. On the premise that DOM remained on the C-18 column, the column was treated with two 0.4 mL washes of 20% acetonitrile, to elute DOM for subsequent analysis.

HPLC analysis of both acidic fractions from the cation exchange column showed that no detectable DOM eluted off the column at any time [Table III (C)]. The C-18 purification step, was successful in eliminating one of the peaks found in the blank and spiked samples (8.47/8.76 min.), but was unable to eliminate the major interfering peak (Rt approximately 10.00 minutes). An additional peak was seen at 6.55 minutes following the cation exchange procedure. Subsequent addition of DOM (2.00 ug/mL) to this sample followed by HPLC analysis,

indicated that this peak was probably not DOM since the addition of the spike produced a peak at 11.00 minutes that was very close to the elution time of aqueous DOM (10.50 min.). To confirm that the 6.55 min peak was indeed not DOM, an aqueous DOM standard (2.00 μg/mL) was processed using the cation exchange column (identical protocol) and again no DOM was detected following HPLC analysis. This confirmed that DOM was remaining on the column and the various environments were not sufficient to elute DOM.

Although this procedure was adequate for eliminating a single peak from the serum samples, the fact that it is difficult to elute DOM from the column and the major interfering peak was still present makes this approach impractical.

2.3.1.6. Effects of fasting on serum composition

Dietary consumption plays a large role in determining the composition of the blood with respect to proteins, amino acids and various metabolic by products. It was possible therefore that fasting the animals would reduce the size of the interfering peak.

Prior to serum collection, rats were provided water but denied food for 24 hours. Blank serum samples were prepared and extracted as described (section 2.3.1.2) and injected on the HPLC. Following analysis, the chromatograms indicated that the interfering serum peak (9.94 min.) was marginally

reduced. However the amount of reduction was not sufficient to allow for reliable quantification of DOM concentrations [Table III (D)].

2.3.1.7. Extraction of guinea pig serum

Guinea pigs are also a common rodent model for pharmacokinetic studies. To determine if the interfering peak was a function of the species used, guinea pig serum was collected by cardiac puncture, extracted (section 2.3.1.2) and analyzed (section 2.3.1.1) on HPLC. The chromatographic profile of the guinea pig serum was similar to the rat serum, with the interfering component eluting at approximately the same time as DOM (Rt=9.98 min) [Table III (E)].

2.3.2 Modification of Chromatographic Conditions

2.3.2.1 Effect of wavelength variation

All compounds that can be detected by UV absorption have their own characteristic spectrum. They exhibit a maximum absorption at one particular wavelength, but will also absorb at other wavelengths. On this basis, it was postulated that there is a wavelength at which the absorption ratio between the compound of interest (DOM) and the interfering compound is increased. Ideally this wavelength would eliminate the interfering peaks if it was outside the absorption spectrum for the interfering compound.

This rationale was used as a basis to establish whether the

peak that was interfering with DOM could be eliminated from the HPLC chromatogram, by varying the detection wavelength about the absorption maximum for DOM (242nm) (Figure 3).

All samples [blank/spike (5μg/mL)] were extracted in the regular methanol/chloroform extraction (section 2.3.1.2) and the top phases were removed for analysis at various wavelengths. Considering the UV spectrum of DOM (Figure 3) and the level of sensitivity required for the assay (5% of the administered dose), only wavelengths from 238-246 nm were suitable for determining if the interfering peak could be significantly reduced or eliminated [Table III (F)].

The absorbance of the interfering peak was not, however, substantially reduced at any of the wavelengths tested. Thus this was not a viable option to be used in the assay of DOM in serum.

2.3.2.2 Effect of mobile phase modification

The tendency for a compound to partition between the mobile phase and the stationary phase (column packing) in HPLC is dependent on the relative lipophilicity of the compound, the solvent and the column. Thus alterations in mobile phase composition may alter the elution times of the respective compounds. Initially an acetonitrile: distilled water (12.5 : 87.5 v/v) mixture adjusted to pH 3.0 with phosphoric acid was used for the mobile phase and produced unacceptable results (Table I) Section 2.3.1). It was decided therefore

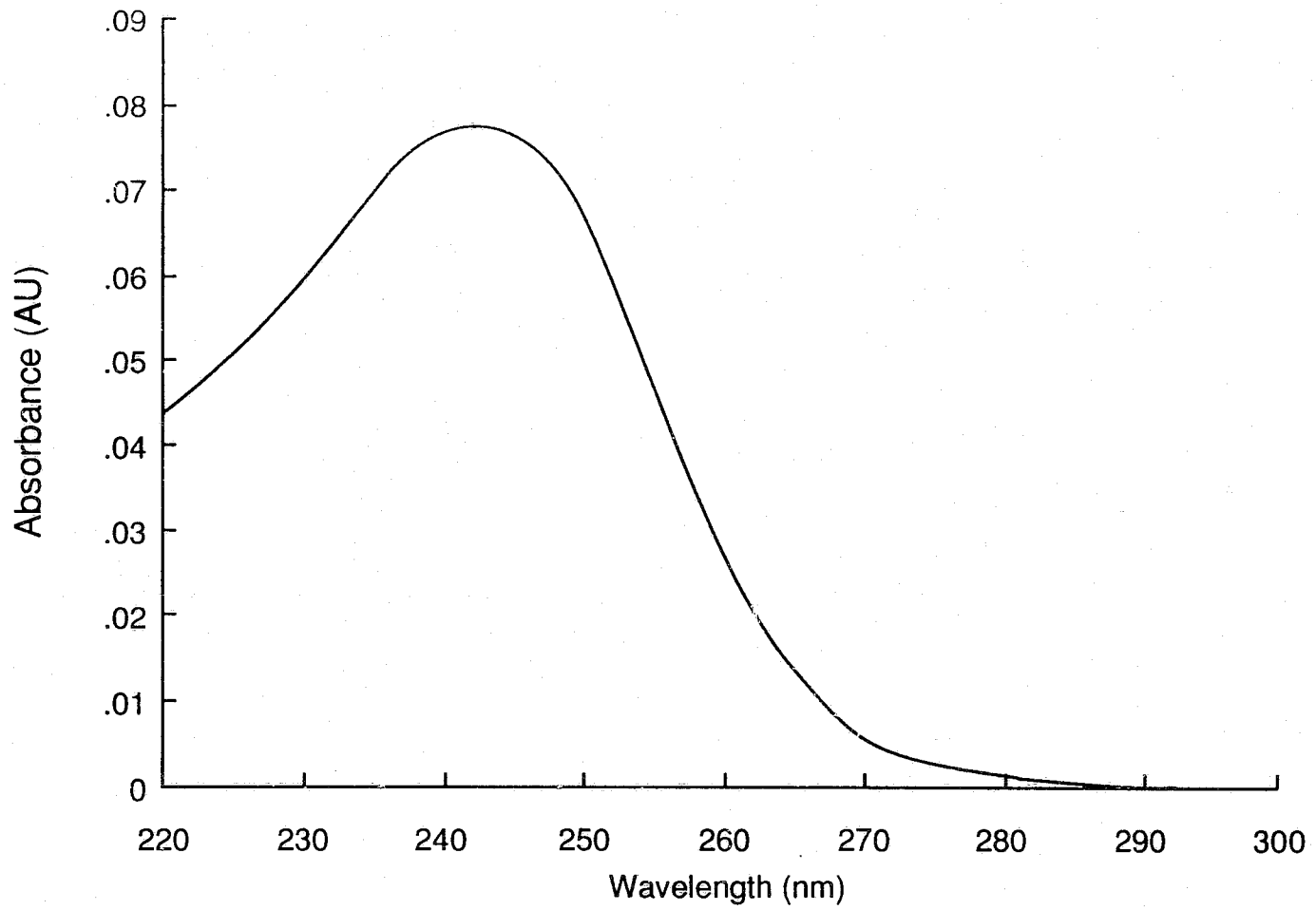

Figure 3. Ultraviolet spectrum of pure domoic acid (1.00 ug/mL)

to alter the elution time of either the interfering peak or DOM by altering the percentage of acetonitrile in the mobile phase. Since the lipophilicity of the interfering substance was unknown, both increasing and decreasing percentages of acetonitrile were used.

Blank and spiked (2.00 μg/mL) samples were prepared (Section 2.3.1.2) and analyzed using the original chromatographic conditions, to establish a baseline from which the mobile phase modifications could be interpreted [Table III (G)]. Use of a mobile phase composed of 10.5% acetonitrile in water, resulted in no interference from serum components during the elution phase of DOM (Rt=12.57 min). Following verification, the chromatography was deemed acceptable for quantifying DOM in serum (Table IV).

2.3.3 Final assay

The final chromatographic conditions listed in Table IV. were used for the remainder of the studies. Sample chromatograms for both blank and spiked (1.00 ug/mL) serum samples can be seen in Figure 4. Peaks produced by interfering serum components eluted during either the initial 7.8 minutes or after approximately 14.0 minutes (Figure 4A). No interfering peaks were seen in any sample at or near the elution time for DOM (11.0 min) (Figure 4B). To determine extraction efficiency, blank serum samples spiked with DOM (0.50 μg/mL and 1.00 μg/mL) (N=3 of each) were prepared and injected on the HPLC. The peak height for each sample was

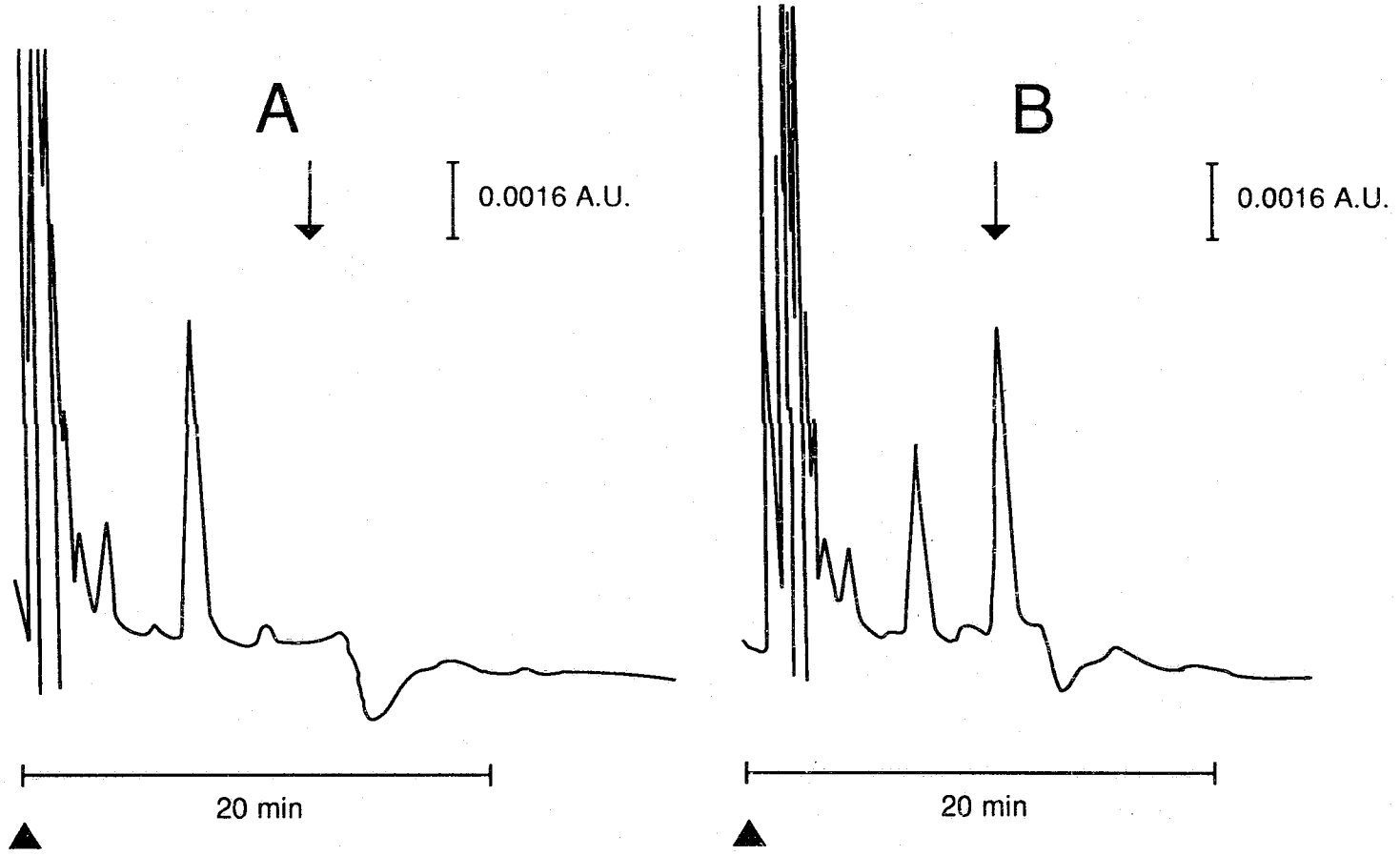

Figure 4. Chromatograms of (A) blank rat serum, and (B) blank rat serum spiked with 2.0 μg/mL DOM. Conditions are described in text (see section 2.5). Arrow indicates normal retention time of DOM.

Table IV Chromatographic conditions for analysis of DOM extracted from serum.

Sample	20 μl injection (Perkin-Elmer) 155-100 autosampler
Pump	Perkin-Elmer series 410.
Solvent	89.5/10.5 (H_2O/acetonitrile) pH 3.0.
Flow Rate	1.0 mL/min.
Column	Perkin-Elmer C-18 (250mm x 4.1mm I.D.).
Detector	Perkin-Elmer LC-90 UV absorbance.
Recorder	Perkin-Elmer L-100 Integrating Recorder

measured and the concentration extrapolated from an aqueous standard curve of peak height versus concentration. These respective concentrations were compared with the theoretical concentrations (100% recovery) to establish an extraction efficiency of 89.0±1.73 (S.D.)% for 1.0 μg/mL and 87.3±5.86 (S.D.)% for 0.50 μg/mL. The minimum concentration that could be reliably detected using this extraction method and these chromatographic conditions was determined to be 0.20 μg/mL.

Several serum standard curves were prepared with various concentrations of DOM (0, 0.25, 0.50, 1.00, 2.00 μg/mL) to produce a composite curve described by the equation y=0.0344x + 0.0032 (y:concentration/x:peak height). All serum standard curves were linear throughout the concentration range tested (0.25 to 2.0 μg/mL) and showed a high correlation coefficient (r^2 0.999). A high degree of reproducibility was evident following an analysis of variance on the respective serum standard curves. The respective peak heights for the individual replicates or samples at each concentration used in the standard curves, were compared within (replicates) and between (samples) each group using a Student's T-test. No significant variation was found for either within day variability (p=0.27) or between day variability (p=0.17) making an internal standard unnecessary (overall coefficient of variation=6.5%).

2.3.4 Other Species

To establish if this assay was applicable to other species both frozen rainbow trout (Oncorhycus mykiss) serum and fresh guinea pig serum, collected via cardiac puncture, were tested. Extracted blank guinea pig serum produced no interfering peaks after 8.4 minutes while trout serum produced peaks during the first 8.1 minutes and after 17.6 minutes. The elution times for DOM were 11.3 and 11.5 minute respectively.

Chapter 3

KINETICS OF DOMOIC ACID FOLLOWING ORAL AND INTRAVENOUS ADMINSTRATION

3.1 Introduction

To produce its effects a drug or toxin must be present in adequate concentrations at its sites of action. For an ingested compound such as DOM, the active concentration is dependent on the amount of toxin consumed and the extent and rate of absorption, distribution and elimination.

3.1.1 Factors affecting absorption, distribution and elimination of toxins

The manner in which toxins are absorbed after ingestion is of considerable importance. The rate of absorption of a toxin will determine both the intensity and duration of its effects. Following oral ingestion of mussels contaminated with DOM various factors will contribute to the absorption of DOM into the systemic circulation. Absorption of anything from the intestinal tract depends on some universal factors such as: (a) surface area of the GI mucosa, (b) blood flow to the site of absorption, (c) the physiological properties of the toxin and (d) the concentration of toxin at the site of absorption. Absorption may be due to passive diffusion, passive transport

or active transport.

Following absorption of a toxin into the blood stream, distribution into interstitial and cellular fluids may occur. Depending upon the physiological state of the individual and the physicochemical properties of the toxin, there may be rapid distribution to the heart, liver, brain and kidneys followed by elimination, or there may be additional phases of distribution (redistribution) into other sites such as body tissues and fat stores. Higher order distribution kinetics will usually prolong the elimination of the toxin from the body.

In general chemical compounds are eliminated from the body either unchanged or as metabolites. Primary excretory organs such as the lungs and kidneys usually eliminate relatively polar compounds, but eliminate more slowly those compounds that are lipophilic. Lipophilic toxins,therefore are usually not eliminated from the body until they are metabolized to more polar compounds mainly in the liver or kidney with minor contributions made by blood or other sites of enzymatic activity.

For most c ounds the two main elimination routes are the kidney and the liver. Active secretion, glomerular filtration and passive tubular reabsorption are the main components of kidney function, while many liver metabolites are excreted via the kidney or are secreted into the bile as conjugates. Biliary conjugates either undergo reabsorption and excretion

in the urine or direct excretion in the feces (71).

Again in both situations regional blood flow, kidney and liver function, blood volume, chemical properties of the toxin and concentration at the site of elimination are some of the parameters that will determine the elimination rate.

3.2 Pharmacokinetic Parameters and Their Significance

3.2.1 Absorption parameters

Bioavailability (BA) is closely related to the absorption of a toxin in that it is an indication of the amount of toxin which enters the systemic circulation in an intact form. Bioavailability is usually expressed as a percentage or proportion, of the administered dose. Under normal physiological conditions this proportion is fairly constant, thus giving the clinician an idea as to the amount of toxin absorbed following ingestion. Factors that affect the BA of a toxin are similar to those of absorption, such as: (a) chemistry of the compound and pH of the intestinal tract, (b) surface area for absorption, (c) any first pass effect through the GI mucosa or liver, and (d) regional blood flow.

BA is expressed as a ratio between the area under the serum concentration versus time curve (AUC) following intravenous administration (100% BA), and the equivalent AUC following oral administration.

This plus other parameters are important in the clinical situation in estimating the amount of toxin which the

individual has been exposed to, for proper treatment.

3.2.2 Distribution and elimination parameters

Following the administration of a toxin in a experimental setting the profile of the serum concentration versus time may be described using either a one, two or even multi-compartment model. For the purpose of this discussion a two compartmental model will be used.

In dealing with a two compartment model, following absorption the compound undergoes an initial phase of distribution and elimination (α phase) and a secondary phase that is primarily due to elimination (ß phase). The apparent volume of distribution (V_d), extrapolated from the ß phase, is the best indicator of the extent and location(s) to which the compound distributes throughout the body. V_d is defined as the volume into which the toxin appears to distribute, and is calculated using the administered dose and the plasma concentrations. The more extensive the distribution, the smaller will be the concentration of the compound in the plasma. For each species there is a "reference" V_d (0.65 L/Kg in humans) which is equal to total body water. The experimental V_d relative to the reference V_d is used to determine in what compartment the compound predominates. For those compounds that have a high affinity for plasma proteins or are very polar, restriction to the blood vascular compartment, would be expected, and these compounds would

therefore have a small V_d with respect to the reference value. Conversely, those compounds with high affinity for tissues would have a large V_d and those with extremely high tissue affinity may have a V_d very much larger than total body water. For many compounds, elimination from the body requires that the drug or toxin be in the blood vascular system. Thus if the V_d for a particular toxin is very large the amount available to be eliminated is reduced, thereby increasing the time it takes to eliminate the toxin from the body and vice versa.

Compounds may be eliminated according to zero order kinetics (a constant amount per unit time), but elimination is more commonly a first order process. In such a case some indicators of toxin elimination are (a) K_{el}, that indicates the proportion of drug that is eliminated per unit time; (b) Cl, determines the amount of V_d that is cleared of drug per unit time and (c) $T_{1/2}$, the time it takes for the concentration of toxin to decrease by 50%. (Appendix A)

Some important characteristics of a first order process are: (a) the amount of toxin eliminated at any time is directly proportional to the concentration of the toxin at that time, (b) the $T_{1/2}$ is independent of dose, and (c) the concentration of the toxin in the plasma and the tissues decreases at a constant fraction per unit time (K_{el}) (66). Conversely during a zero order process the rate or amount of toxin eliminated from the body is constant (eg: 30 mg/hr) and is independent of dose or amount of toxin present (66).

It should be kept in mind that there are instances in which both zero order and first order processes are required to eliminate a toxin from an individual. In those situations where large amounts of a toxin have been absorbed, the elimination mechanisms may be saturated, causing elimination to occur initially according to a zero order process; subsequently when the concentration is decreased, a first order elimination would predominate.

3.3 Materials and Methods

3.3.1 Experimental animals

Mature male Long-Evans rats (250-300gm), (N=18) and Hartly guinea pigs (300-350gm) (N=8), (Charles River Canada, Laval Quebec) were used in this study, in accordance with Canadian Council of Animal Care guidelines. For the duration of the experiments the animals were group housed in the animal care facility of the Atlantic Veterinary College, with food and water available ad lib.

3.3.2 Apparatus and conditions

All samples were analyzed using a Perkin-Elmer (Montreal, Canada) HPLC system (see section 2.2.1). Analytes were separated and quantified using a reversed-phase HPLC assay described in detail elsewhere (67) (see Chapter 2)

Body temperature was monitored with a rectal thermometer (Model 42SC, Yellow Springs, Ohio) and all oral

administrations were with the aid of an oral feeding tube purchased from Biomedical needles (Popper and Sons Inc., New Hyde Park, New York.)

3.3.3 Anesthetic and solvents

The inhalational anesthetic was halothane (M.T.D. Pharmaceuticals, Cambridge, Ontario), delivered by an anesthetic machine via a piggyback bain circuit (Medigas Ltd., Dartmouth N.S.). HPLC-grade acetonitrile, phosphoric acid, chloroform and methanol were purchased from Fisher Scientific (Halifax, Canada). Domoic acid was obtained by acid extraction of contaminated mussels and concentration and purity were verified by analytical HPLC (65).

3.4 Experimental

3.4.1 Anesthesia

Halothane, an inhalational anesthetic, was used due to its low blood/gas partition coefficient (3.6) (68). With such a low partition coefficient, body concentrations quickly followed changes in the inspired concentration, thus the level of anesthesia could be increased or decreased very rapidly (68).

Initially rats were induced with halothane (2.0 volume percent) and a fresh gas flow of oxygen at 1.5 liters/minute. Following induction (1-2 min.), the animals were maintained on 1.5 volume percent halothane and pure oxygen, delivered at

1.1 liters/minute (69). Body temperature was monitored with the aid of a rectal thermometer and maintained at 36±2 °C, with a ventrally located heating unit. Before proceeding with the surgery, both respiration level (diaphramatic) and a toe pinch test were used to determine the level of anesthesia.

3.4.2 Surgical procedures

Under anesthesia, animals were shaved and an incision made in the ventral surface of the neck. The right jugular vein was exposed and two suture threads were positioned beneath the vessel. One suture (cranial) was tied to ligate the vein. Using iris scissors a small incision was made in the vein and a catheter [PE_{50} tubing (Intramedic, Becton Dickinson and Company, New Jersey)] containing heparinized saline (100i.u./mL) was inserted such that the tip came to rest in the right atrium of the heart. The second suture thread was then tied around the catheter, at the site of the incision, to prevent any leakage.

3.4.3 Intravenous Administration of DOM and Serial Blood Sampling

Once the catheter was in place and the animal stabilized, a 0.5 mL blood sample was withdrawn to serve as a time=0 control. Mussel extract containing DOM (dose:1.0 mg/Kg) or vehicle control (clean extract) was then infused in a volume of 1.0 mL over 2.0 minutes. Serial blood sampling with a

total of eight 0.5 ml samples were taken at designated time intervals (0, 10, 20, 30, 40, 50, 60, 80 min.) and replaced with an equal volume of Ringer's solution containing lactate. Samples were then centrifuged (Beckman microfuge model TJ-6, Beckman Instruments Ltd., Mississauga, Ontario) and the supernatant (serum) was withdrawn for extraction (section 2.5) and subsequent HPLC analysis.(section 2.5).

3.4.4 Oral administration of domoic acid and serial blood sampling

For this study all animals (Long-Evans rats/Hartly guinea pigs) were fasted 24 hours prior to the administration of DOM (1.0 mg/Kg) or vehicle control (clean extract) through an oral feeding tube. Following administration serial blood sampling was the same as described above for the i.v. administration study. DOM extraction from serum and subsequent HPLC analysis was also consistent with Sections 2.5.

3.5 Results:

3.5.1 DOM kinetics following intravenous administration in the rat

Following the administration of DOM, mean circulating blood concentrations (N=6) ranged from 5.34 μg/mL at 10 min to 0.65 μg/mL at 80 min (Figure 5). As illustrated in Figure 5 the data points indicated a nonlinear elimination of DOM, this made it difficult to determine if elimination was

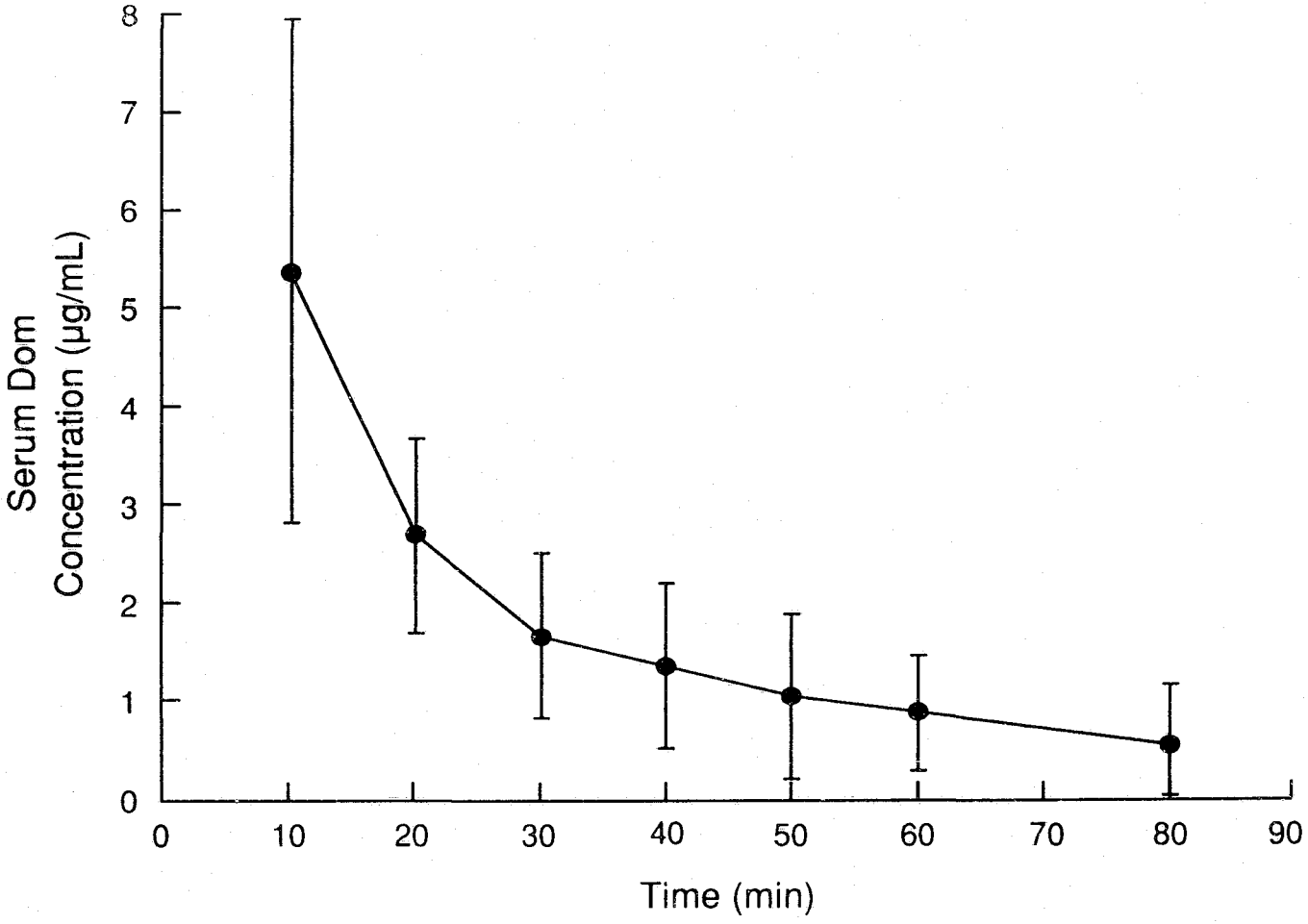

Figure 5. Concentration versus time curve for DOM in rat serum following intravenous administration (1.0 mg/Kg).

occuring in 1, 2 or 3 phases. Knowing the appropriate number of phases (compartments) is crucial to subsequent analysis. To clarify the profile, therefore, an earlier time point was chosen at 5 minutes. For the second group of animals (N=4), samples were withdrawn at 5, 10, 20, 30, and 40 minutes. HPLC analysis yielded mean concentrations at the 5 min time point of 10.6 μg/mL (Figure 6). DOM concentration at 10, 20, 30, and 40 min. were not significantly different from those obtained in the original group ($p>0.01$). Data from the second group of animals were combined with those shown in Figure 5 to produce a composite curve (Figure 6). This enhanced elimination profile had concentrations ranging from 10.6 μg/mL (5 min) to 0.65 μg/mL (80 min). Technical limitations made sampling at less than five minutes impractical. A two compartment model for DOM elimination is illustrated in Figure 7. DOM elimination is described by both an α and a ß phase, with theoretical concentrations at time zero of 11.74 μg/mL (A_0) and 4.96 μg/mL (B_0) and half lives of 15.6±8.8 min and 32.6±15.0 min, respectively. The volume of distribution (V_d) and clearance (Cl) as determined by the ß phase (Appendix A) were 0.202 L/Kg and 0.32 L/Kg/hr, respectively (These values are summarized in Table V).

3.5.2 Determination of the appropriate compartmental model

In determining the kinetic parameters (section 3.1) shown in Table V, a best fit compartmental analysis was performed

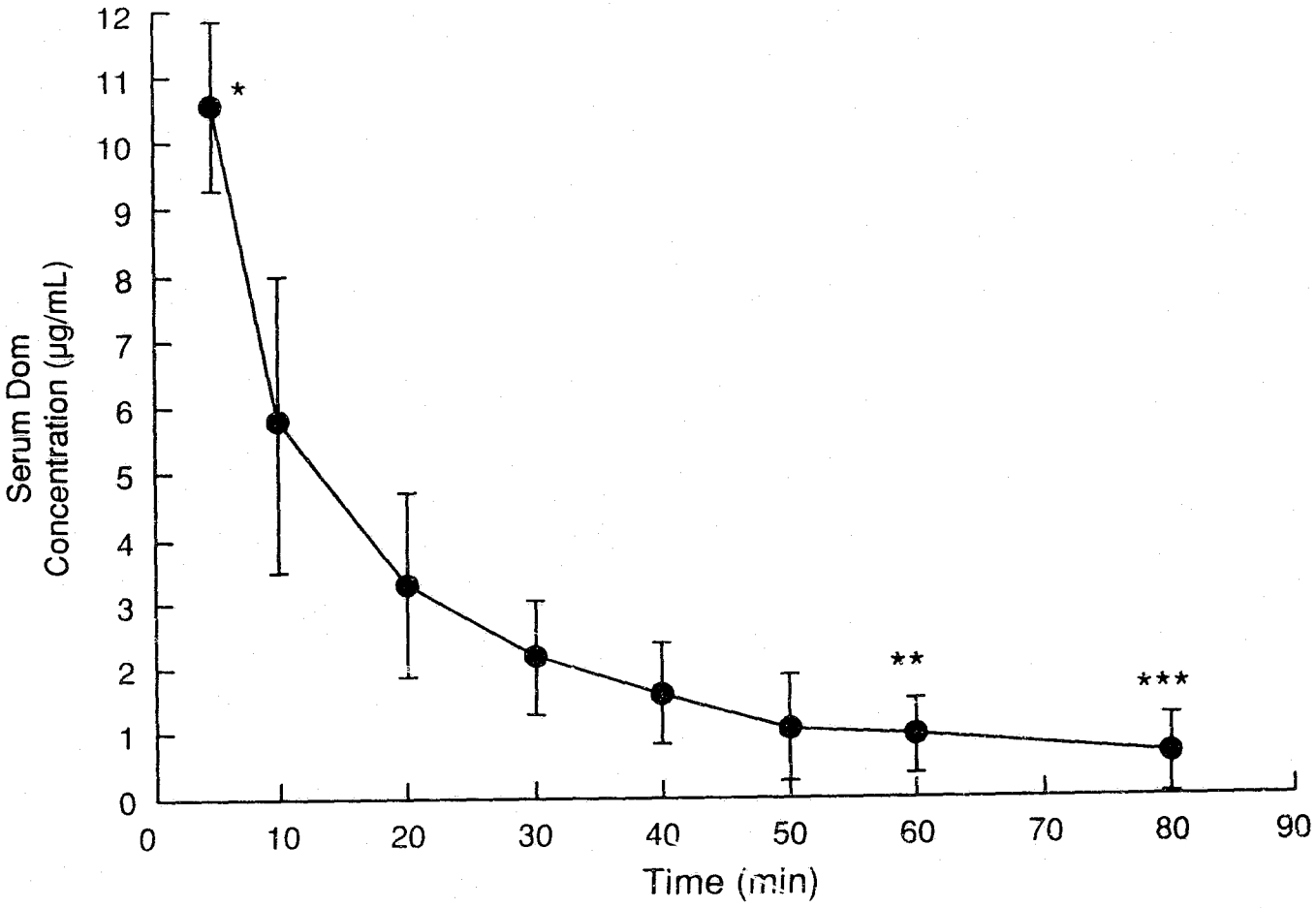

Figure 6. Expanded concentration versus time curve for DOM in rat serum following intravenous administration (1.0 mg/Kg). Data shown are mean ± standard deviation. N=10 except (N=3), **(N=6), ***(N=5).

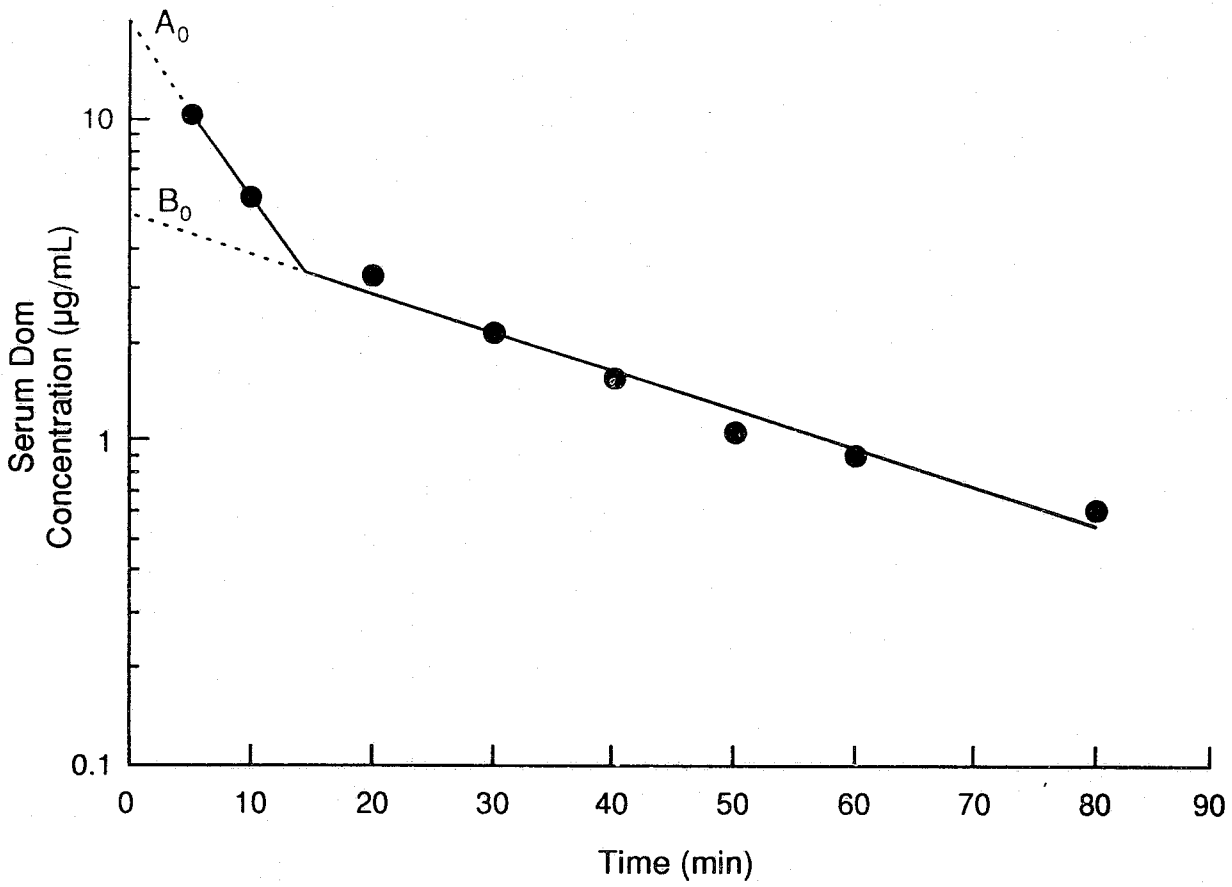

Figure 7. Semilogarithmic transformation of data presented in Figure 6, with error bars omitted for clarity. Regression lines based on two compartment kinetic analysis. (Slidewriter®). Theoretical intercepts at time zero are shown by A_0 (11.74 µg/mL) and B_0 (4.96 µg/mL) for the α and ß compartments respectively.

Table V: Kinetic parameters for serum DOM following i.v. administration. Analysis is based on two compartment model. Dose of DOM = 1.0 mg/Kg. Data derived from Figure 3.

	α	β
$[X_0]$	11.74 μg/mL	4.96 μg/mL
$T_{1/2}$	15.6±8.8 min.	32.6±15.0 min.
V_d	N/A	0.202 L/Kg
Cl	N/A	0.32 L/Kg/hr

N/A: not applicable
Values calculated from elmination phase data only

on the raw data obtained from 10 rats (Table VI) (Figure 6).

In a one compartment model, kinetic parameters are determined mathematically from a single regression line, fitted to the logarithm of the concentration versus time (exponential decline). In a two compartment model however only the second (ß) phase is used in the calculations. In the event the wrong compartmental model is chosen, the calculations could be artificially high or low, thus giving an inaccurate representation of DOM elimination. For this reason an accurate determination of the appropriate compartmental model is of primary importance.

During the analysis, the raw data was fitted to several compartment models using a commercially available software package (Slidewriter^{R}). The difference between the observed concentrations and the concentrations predicted by the model were quantified in the form of a percent deviation. The percent deviation and the r^2 were used to determine the model that best fit the data.

A one compartment model analysis proved to be less than satisfactory as indicated by the large deviations and an r^2 value of 0.767 (Table VI). Both the two and three compartmental analyses had relatively little deviation of the observed data from the values estimated by either model. The correlation coefficients were also much greater than for the one compartmental analysis, being 0.997 and 0.999 for 2 and 3 compartmental analysis respectively (Slidewriter^{R}). Since

Table VI: Best fit compartmental analysis of serum DOM concentration following i.v. administration in rats. DOM dose=1.0mg/Kg

			COMPARTMENTS					
			I		II		III	
TIME(min)	N	OBSERVED [DOM]μg/ml	ESTIMATED [DOM]μg/ml	% DEV	ESTIMATED [DOM]μg/ml	% DEV	ESTIMATED [DOM]μg/ml	% DEV
5	3·	10.60	6.44	-39.24	10.60	0	10.60	0
10	10	5.76	5.45	-5.40	5.76	0	5.76	0
20	10	5.26	3.91	19.79	3.01	-7.65	3.24	-0.72
30	10	2.16	2.80	29.58	2.20	1.82	2.20	1.69
40	10	1.64	2.01	22.32	1.72	4.71	1.63	-0.57
50	6	1.10	1.44	30.71	1.35	23.16	1.30	18.08
60	6	0.96	1.03	7.34	1.07	11.50	1.09	13.32
80	5··	0.81	0.53	-34.65	0.67	-17.45	0.83	2.51
r^2				0.767		0.997		0.999

· One sample not recovered

·· One animal died prematurely

there was little difference between the two fits and generally kinetic data is analyzed using the least number of compartments, the two compartment model was employed in determining the kinetic parameters (Figure 7) (Table V).

3.5.3 Dom kinetics following oral administration in rats.

Following the oral administration of DOM (1.0 mg/Kg) serial blood samples were withdrawn (0-80 min) extracted and analysed by HPLC. The serum sample taken at time zero (Figure 8A) showed no interference at the elution time of the DOM (12.8 min) as determined using a spiked sample (2.00 μg/mL) sample (Figure 8B). Serum samples withdrawn from animals that received an oral dose of the vehicle control (clean extract) (N=4) produced HPLC profiles, at all time points (5-80 min.) that were very similar to the blank sample taken at time zero (see Figure 9A). Following the administration of DOM (1.0 mg/Kg) (Figure 9B) contaminated mussel extract (N=4), the chromatograms were similar to those shown in the preceding Figures (8A, 8B and 9A). Thus within the limit of sensitivity of the assay, DOM is not detected in serum following oral administration in rats.

3.5.4 Dom kinetics following oral administration in guinea pigs

Analysis of control (T=0) guinea pig serum revealed no indication of any interference at the elution time of DOM

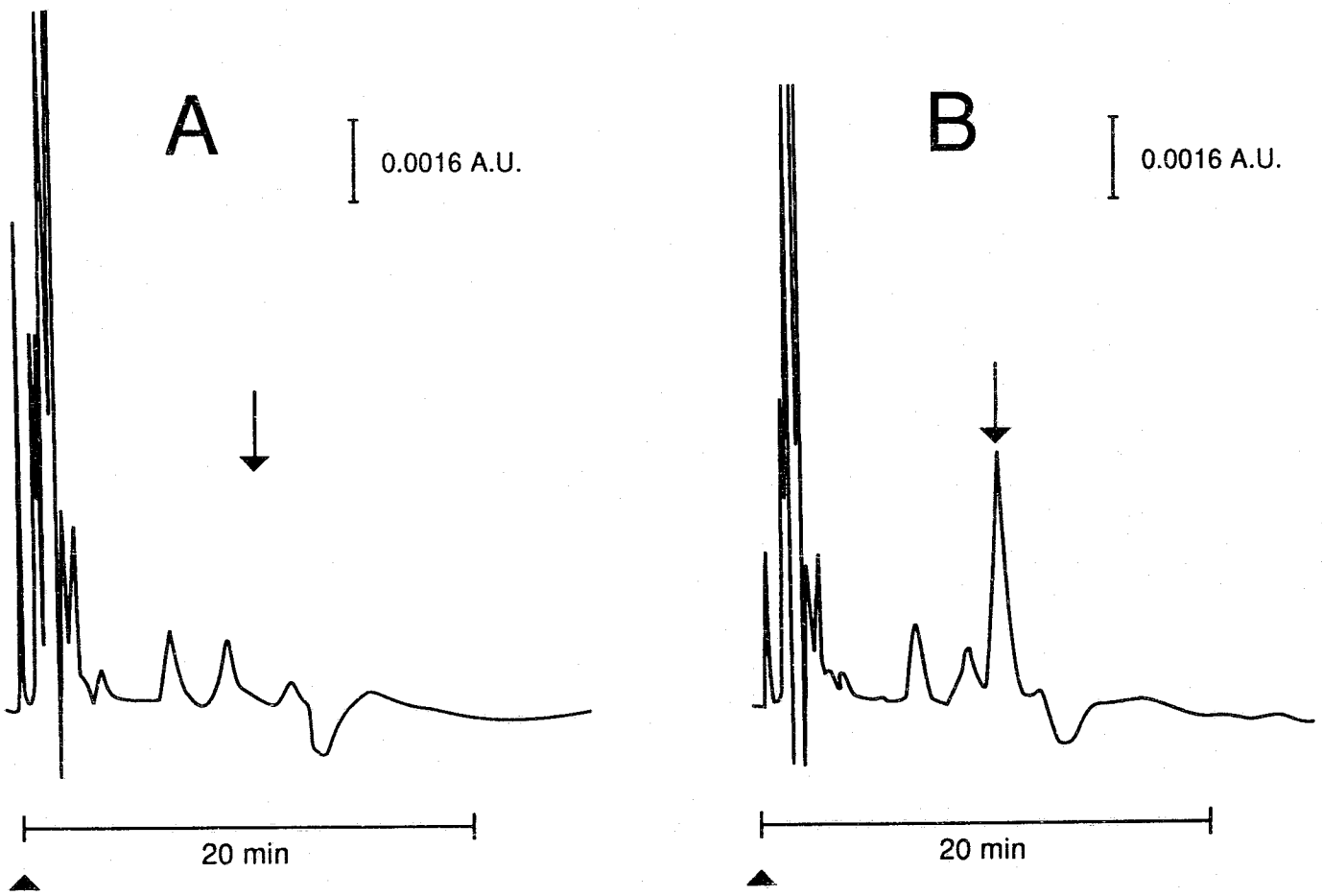

Figure 8. Chromatograms for rat serum at (A) time=0 and (B) time=0 spiked with DOM (2.00 μg/mL). Conditions as described in text (see section 2.5). Arrow indicates normal retention time for DOM.

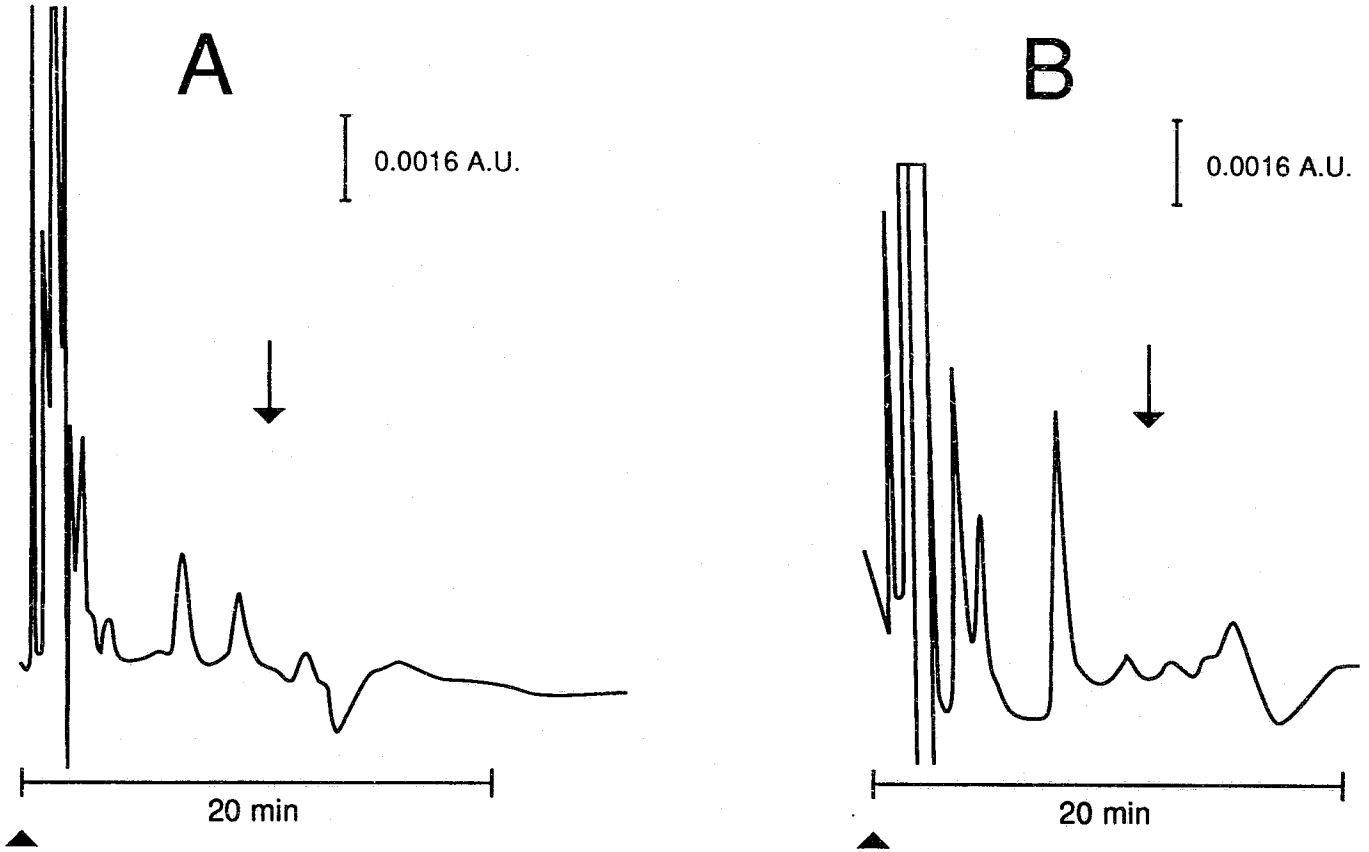

Figure 9. Chromatograms for rat serum 80 minutes after oral administration (A) of clean extract, (B) mussel extract contaminated with DOM (1 mg/Kg). Conditions as described in text (see section 2.5). Arrow indicates normal retention time for DOM.

(13.45 min) (Figure 10A) as determined using serum spiked with DOM (2.00 μg/mL) preparation (Figure 10B). Administration of the vehicle control (clean extract) (N=4)led to a changed HPLC profile, as shown in Figure 11A. Throughout the sampling period a peak with a retention time of 11.5 min. (Figure 11A) progressively increased in amplitude. A similar profile over the 80 minute period was also produced following administration of mussel extract contaminated with DOM (1.0 mg/Kg) (Figure 11B). As shown in Figure 11B this peak was distinct from that produced by DOM (RT=13.4 min.), thus indicating that this peak represents a component of the mussels extract, that was absorbed over time, rather than that of DOM. Peaks corresponding to the retention time for DOM were not seen in samples any time following oral administration of DOM. It was concluded that guinea pigs like rats (see section 3.3.2), do not absorb detectable amounts of DOM following oral administration.

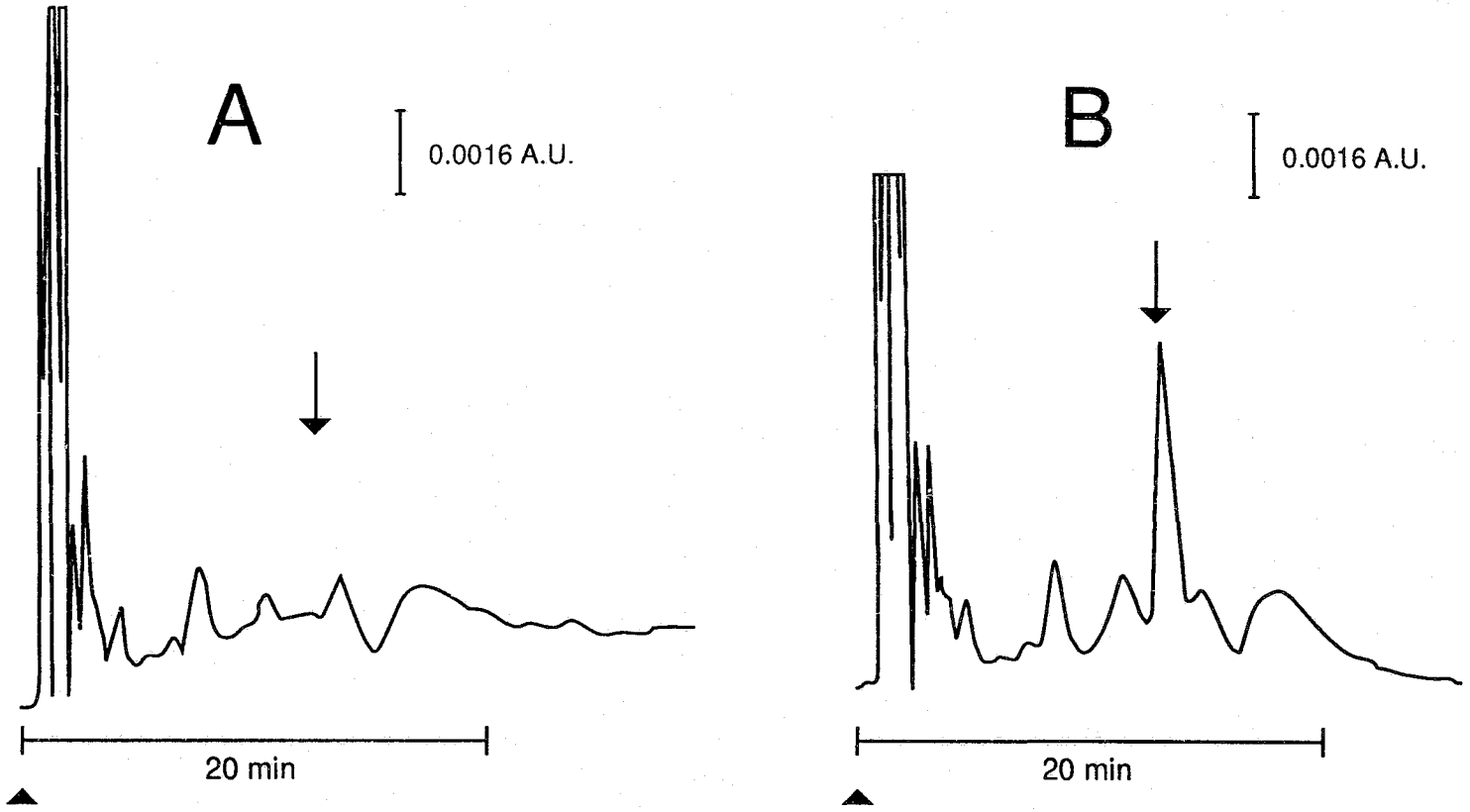

Figure 10. Chromatograms of (A) blank guinea pig serum and (B) blank guinea pig serum spiked with 2.0 μg/mL of DOM. Conditions as described in text (see section 2.5). Arrow indicates normal retention time for DOM.

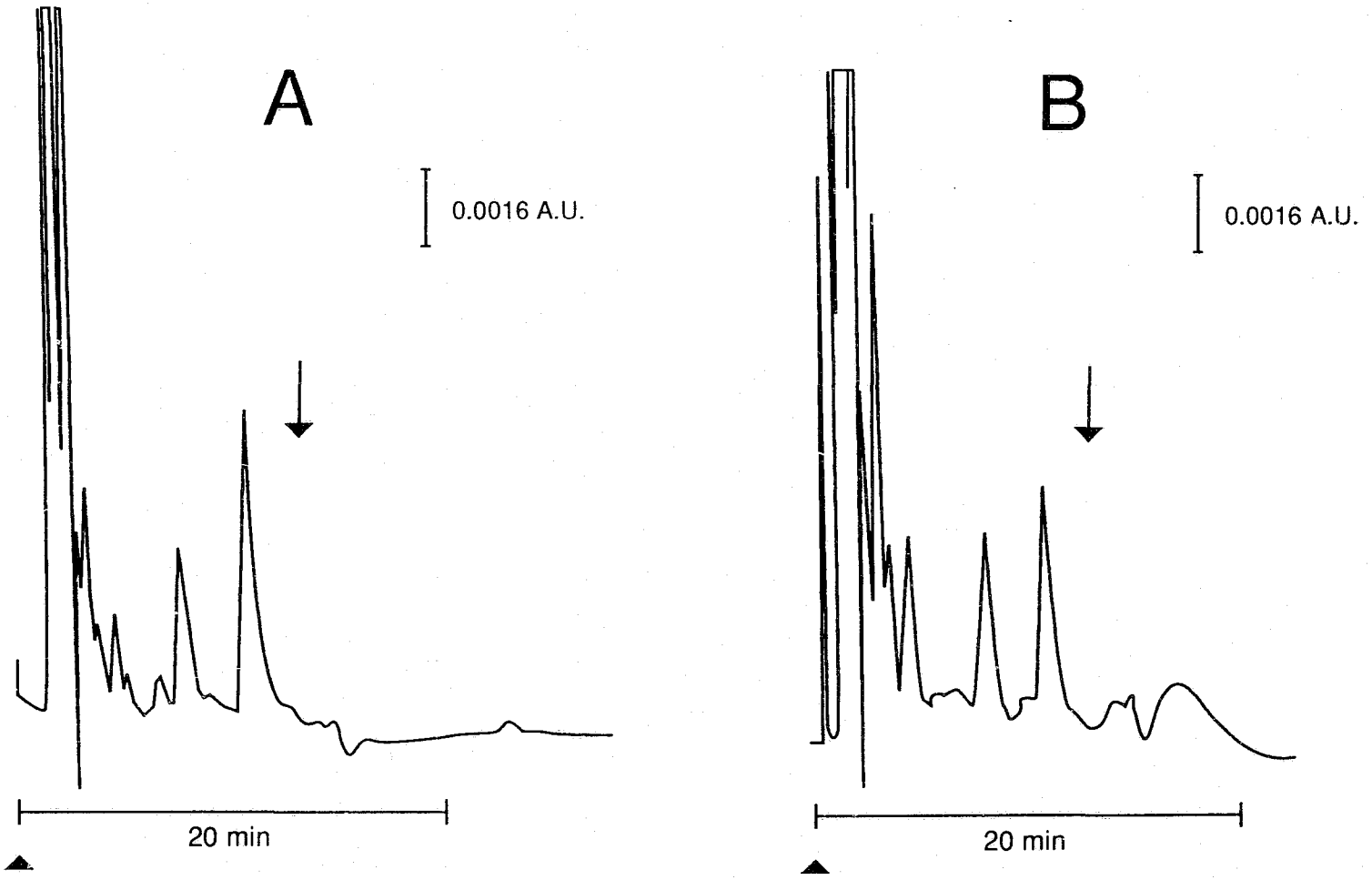

Figure 11. Chromatograms for guinea pig serum 80 minutes after oral administration (A) of clean extract, (B) mussel extract contaminated with DOM (1 mg/Kg). Conditions as described in text (see section 2.5). Arrow indicates normal retention time for DOM.

3.6 Discussion:

3.6.1 Kinetics of DOM elimination following intravenous administration

Following the administration of DOM, initially high concentrations appeared to undergo a rather rapid non-linear elimination. The raw data were therefore linearized and graphed by semilog transformation (Figure 7) and the ß phase was used to determine the in vivo kinetic values for DOM (Table V).

From the linear representation (Figure 7) and the results of the compartmental analysis (see Section 3.5.2) DOM elimination occurs in two phases (compartments). Normally these phases are assumed to represent distribution plus elimination (α phase) and elimination (ß phase). Thus DOM distribution following intravenous (i.v.) administration appears to be rapid, occurring in a matter of minutes. This implies that DOM remained primarily in the blood vascular system (bvs) with little distribution into tissues. This premise was further supported by the rather small V_d (Table V).

The V_d is determined by dividing the dose of DOM administered by the concentration at time zero (C_0), as determined by extrapolation of the ß phase of elimination. As indicated in the Introduction, each species has its own "reference V_d" through which it is possible to determine where the compound of interest predominates (eg: tissue/plasma).

The less extensive the distribution of the compound into other compartments, the higher will be blood C_0, therefore making the V_d smaller than total body water. This was the case for DOM, although most amino acids undergo more extensive distribution.

This rather limited distribution of DOM might be expected following an examination of its structure (Figure 2, Section 1.3.3). DOM, is a zwitterion, containing both amine and carboxyl groups. The major implication of this is DOM should be charged at both an acidic and basic pH. This will limit passive diffusion from the plasma to the tissues. Although there is a possibility of an active transport system, the fact that the V_d was so small in the current study, would indicate that if present the effects are negligible or DOM elimination is extremely rapid. Concurrent with the small V_d was the limited $T_{1/2}$ (32±15.0 min) of the terminal elimination phase (ß) (Table V). Because a large amount of the administered dose is restricted to the circulation (small V_d), the rapid elimination of a hydrophilic compound via the liver or kidneys may have been expected. Whether this elimination is due to metabolism and/or renal excretion is yet to be determined. If renal excretion is largely responsible, as implied by a recent communication by Preston and Hynie (1990) (26), this may help to explain why only a few of the people who ingested contaminated mussels became seriously ill. It is now believed that many or all of these victims also had some form of renal

impairment, and may therefore have been predisposed to toxicity. Impairment of a major elimination mechanism that normally gives rise to a very short $T_{1/2}$, would have a significant effect on circulating DOM concentrations, but this requires experimental verification.

3.6.2 Absorption kinetics of DOM in both rat and guinea pig models

In an attempt to determine the absorption kinetics of DOM (dose:1.0mg/Kg) following oral administration both the rat and guinea pig model were used. DOM was not detected in the serum of either species throughout the 80 minute sampling period. Because the assay used was capable of detecting 4% of the theoretical concentration (B_0), at least 96% of the orally administered DOM remained in the gastrointestinal (G.I.) tract for 80 minutes. These data are in agreement with those of Health and Welfare Canada, which indicate that 98% or greater of an oral dose, of DOM could be recovered in feces of both rats and mice (25). No detectable levels were seen in urine during the same time frame (25). As well Iverson et. al. (25) indicated in the same study that an oral dose (70 mg/Kg) of contaminated mussel extract was required to produce initial signs of toxicity in rats, while a 35 mg/Kg oral dose of pure DOM in saline was necessary to produce scratching behaviour (25). In short, the fact that: (a) no detectable levels of DOM are seen in serum following oral administration in rats

and guinea pigs (present study), (b) 98% or greater of an orally administered dose, in rats and mice can be recovered in the feces (25), (c) DOM was not detected in the urine of rats or mice (25) and (d) an oral 70 mg/Kg dose of contaminated mussel extract is required to produce initial clinical signs of toxicity in rats (25), indicates that DOM absorption is negligible in these animal models.

In contrast to the preceding rodent studies, Iverson et al. (In press) have also reported that following oral administration of DOM in cynomolgus monkeys, a 5 mg/Kg dose promotes vomiting, mastication, yawning and fine tremors (25). Absorption of DOM after oral administration may, therefore, be unique to both primates including some individuals in the human population, which makes them susceptible to DOM toxicity. It is interesting to note that those GI transport systems which may be involved in the absorption of compounds that are chemically (acidic amino acids) and structurally similar to DOM, such as (a) Na^+/K^+ dependent glutamate transport (60), (b) aspartate transport (60) or (c) proline transport (59) are all found in both rat gut as well as human gut. This implies that these transport systems may not be the major contributor to the absorption of DOM. Other possibilities include: (a) there is a unique transport system in primates, (b) the integrity of the G.I. tract may be suspect, due to age and social factors (stress), or (c) the affected individuals consumed considerably more toxic mussels

than others. It is also possible that the affected individuals had other physiological abnormalities (eg. reduced integrity of the blood brain barrier) that made them more susceptible to DOM toxicity.

Of those reported cases of poisoned patients, greater than 80% were 40 years of age or older (8), and of these cases the most seriously affected were 70 yrs of age or older (8), probably had renal insufficiency and may have had some G.I. bleeding either prior or subsequent to consumption of mussels, that would have circumvented normal G.I. absorption (5).

Considering for every 1000 portions of mussels shipped there were only 3 reported cases of toxicity (23) and on average each person received a dose of 5 mg/kg (50 Kg person) (23), the combination of both epidemiological and animal research seems to support a common theme. There may be a select portion of the population which were predisposed to toxicity, by perhaps having some form of G.I. or blood brain barrier insufficiency or as indicated by Preston and Hynie 1990 (26) renal insufficiency.

Chapter 4

GENERAL DISCUSSION

Since the outbreak of amnesic shellfish poisoning in 1987, a great deal of work has been completed on DOM with respect to determining the mechanism of neurotoxicity, the major target sites in the CNS, and establishing new directions for research and therapy. This study examines the importance of determining the in vivo kinetics of DOM.

DOM, once ingested is absorbed from the human gut under some circumstances, but the exact mechanism is yet unknown. Both the present study and others (Iverson et al. In press) have failed to duplicate this absorption in other species. This study demonstrates that once in the blood, DOM undergoes minimal distribution to other tissues and is rapidly eliminated.

In 1989 it was reported by Iverson et al. (25) that the kidneys play a major role in the elimination of DOM from the BVS. Unfortunately no published research is available to give any indication as to the mechanism of elimination by the kidney. Whether DOM is being eliminated via glomerular filtration or active secretion, and whether the molecule undergoes tubular reabsorption, are all possibilities that must be examined. Since DOM is an organic acid and charged at virtually any pH, elimination by an organic acid secretory

mechanism seems an attractive possibility. To determine if elimination occurs via this route it should be possible to alter excretion by competitively inhibiting active secretion, using a commonly known organic acid, such as probenecid. Probenecid is used clinically to prolong the half life of some drugs that are organic acids (eg. penicillin G). To examine the role of glomerular filtration the effect of administering an osmotic diuretic or surgically reducing renal blood flow could be examined. In both of these situations the object would be to determine if there was any alteration in the rate of elimination of DOM from the body. This could be established either by (a) determining the normal serum kinetic profile of DOM and observing if there was any alteration following administration of the drugs/surgery, or (b) establishing control and experimental groups using direct measurements of DOM in urine over a predetermined time frame.

Although DOM is a highly charged molecule it does have the capacity to cross the blood brain barrier, possibly via an active transport mechanism. Once in the brain, DOM causes extensive damage in a variety of regions, especially the hippocampal CA_3 region (24). Increasing evidence indicates kainic acid receptors mediate the neurotoxic effect of DOM and the pattern of destruction in the hippocampus reflects somewhat the distribution of KA receptors in this region. A number of possibilities exist as to the exact mechanism by which this occurs. Possibly DOM is acting directly on the

post synaptic KA receptors causing an over-stimulation thereby "exhausting" the resources of the cell. Although this is an attractive idea, other possibilities also exist including: (a) DOM acts presynaptically to enhance calcium influx, causing excess release of glutamate into the synaptic cleft, (b) DOM acts intracellularly altering the biochemical process that may or may not be linked to post-synaptic receptors or (c) a possible interplay between these postulated mechanisms.

Evidence for this presynaptic action mentioned above, has recently been obtained by studying the massive input to the CA_3 region of the hippocampus from the mossy fiber system, originating from the granular cells of the dentate gyrus. Debonnel et al. (17), indicate that lesioning of this pathway significantly reduces neuronal responses to iontophoretically applied DOM, in the CA_3 region of the hippocampus. This seems to implicate KA or related receptors located on the mossy fiber terminals, that subsequently lead to the release of glutamate, aspartate or other unknown EAA(s). Ultimately the identification of the KA ligand and the EAA(s) released from the mossy fiber terminals may lead to the development of a treatment for future intoxicated victims.

Although the work to date represents an intense effort by those involved, it is yet to be completed, to gain a more complete understanding of this molecule <u>in vivo</u>. Some of this work will include: (a) determining the kinetics of DOM in cerebrospinal fluid following i.v. and oral administration,

(b) establishing the mechanism by which DOM crosses the blood brain barrier (c) determining the mechanism of elimination and the degree to which the liver may contribute, (d) determining where along the GI tract DOM is absorbed and the mechanism of absorption (e) establishing the mechanism by which DOM creates its neurotoxicity in the brain and (f) developing an antagonist against neuronal degeneration.

Not only was the consumer affected by the P.E.I. mussel toxin incident, decreased demand for mussels also affected the mussel culturing industry. From 1978 until 1986, just prior to the incident, the market demand for Prince Edward Island mussels was at an all time high. In 1986, 1220 tonnes of mussels were marketed generating a total revenue of $ 1,712,000 (70). Following the DOM scare landings dropped by approximately 185 tonnes (70) and sales dropped by $ 168,000 (70). Obviously the public was somewhat apprehensive about purchasing and consuming this product. Eventually as more monitoring programs were put in place and the publicity of the incident decreased the narket for this product began to increase within months. From 1987-89 the total landings increased by 1409 tonnes (70) and are at an all time high for the mussel culturing industry, with the same relative contributions being made by each province [PEI (78%) and Queb, NB, NFLD, NS (22%)] (70) . This renewal of consumer confidence is largely due to the establishment of a routine system of monitoring commercial shellfish for the presence of

toxins, including those toxins responsible for ASP, PSP, DSP and other syndromes.

Although this incident has come to a conclusion for the consumer, DOM levels continue to be detected in the Maritime water ways. Occasionally this has resulted in the closure of some river systems for a period of time, such as the Brudenell River in 1990. Thus research must continue in the event that we or other communities are affected by this toxin in the future.

APPENDIX A

Formulae for select variables

1. Coefficient of Variation (C.V.)

$$C.V.=\frac{S.D.}{X}$$

where S.D.=standard deviation
X=arithmetic mean

2. Apparent Volume of Distribution

$$V_d=\frac{Dose}{C_0}$$

where C_0=theoretical concentration in serum at time zero.

3. Clearance (Cl)

$$Cl=V_d*K_{el}$$

where K_{el}=elimination rate constant

4. Elimination Half-Life ($T_{1/2}$)

$$T_{1/2}=\frac{0.693}{K_{el}}$$

REFERENCE

1. Lutz RA. Mussel culture and harvest. In: Lutz RA ed. Mussel culture and harvest a North American perspective. Amsterdam, Elsevier, 1980.

2. Boghen AD. Culture of the mussel mytilus edulis. In: Mallet AL ed. Cold-water aquaculture in Canada. Canadian Institute of Research on Regional Development 1989:179-207

3. Inze LS, Lutz RA. Mussel culture: An east coast perspective. In: Lutz RA ed. Mussel culture and harvest: A North American perspective. Amsterdam, Elsevier. 1980:99-140

4. Gillfillan ES, Page DS, Vallas D, Gonzales L, Pendergast E, Foster JC, Hansen SA. Vernberg FJ ed. Marine pollution and physiology: Recent advances. Mystic, Conn. 1983

5. Yentsch T, Inze LS. In: Lutz RA ed. Mussel culture and harvest: A North American perspective. Amsterdam, Elsevier. 1980:223-246

6. MacNair N, Wagner S. Introduction. In: Domoic acid toxicity in the blue mussel mytilus edulis, of eatsern Prince Edward Island. 1980:1-10

7. Trish P. Human health aspects. In: Bates SS, Worms J eds.Proceedings of the first Canadian workshop on harmful marine algae gulf fisheries centre, Moncton, N.B. 1990;No.1712

8. Perl TM, Bedard L, Rosatsky T, Hockin J, Remis RS. Gastrointestinal and neurological illness related to mussels from P.E.I.: a new clinical syndrome associated with domoic acid. Presented at: Epidemic Intelligence Service Conference, Centres for Disease Control, Atlanta GA. 1988

9. Bates S, Bird CJ, DeFrietas ASW, Foxall R, Gilgan M, Hanic LA, Johnson GR, McCulloch AW, Odense P, Pocklington R, Quiliam AW, Sim PG, Smith JC, Subba Roa DV, Todd ECD, Walter JA, Wright JLC. Pennate Diatom Nitzschia pungens as the primary source of domoic acid toxin in shellfish from eastern Prince Edward Island. Can. J. Fish. Aquat. Sci. 1989;46:1203-1215

10. Addison RF, Stewart JE. Domoic acid and the eastern Canadian mussel shellfish industry. Aqua. 1989;77:263-269

11. Wright JLC, Boyd Pk, DeFrietas ASW, Falk M, Foxall, RA, Jamieson WD, Laycock, MV, McCulloch AW, McInnes AG, Odense P, Pathak VP, Quilliam MA, Ragan MA,Sim PG, Thibault P, Walter JA, Gilgan M, Richrad DJA, Dewar D, Identification of domoic acid, a neuroexcitatory amino acid, in toxic mussels from eastern Prince Edward Island. Can. J. Chem. 1989;67:481-490

12. Takemoto T. Isolation and structural identification of naturally occurring excitatory amino acids. In: McGeer EG, Olney JW, McGeer PL eds. Kainic acid as a tool in neurobiology. New York, Raven press. 1978:6-8

13. Castle F, Evans RH, Kirkpatrick JNP: A comparison of the effect of kainate and some related amino acids on locomotor activity in cockroaches and electrical activity recorded from locust ventral nerve cord. Comp. Biochem. Physiol. 1984;77:399-402

14. Shinozaki H, Ishida M. Inhibition of quisqualic acid by domoic acid of kainic acid in crayfish opener mussel. Brain Res. 1976;109:435-439

15. Shinozaki H. Shibuya I. Effect of kainic acid analogues on crayfish opener muscle. Neuropharm. 1976;15:145-147

16. Tackeuchi H, Watanabe K, Nomoto K, Ohfune Y, Takemoto T. Effects of α-kainic acid, domoic acid and other derivatives on a molluscan giant neuron sensitive to ß-hydroxy-L-glutamic acid. Eur.J.Pharm. 1984;102:325-332

17. Debonnel G, Weiss M, DeMontigny C. Reduced neuroexcitatory effect of domoic acid following mossy fiber denervation of the rat hippocampus: further evidence that toxicity of domoic acid involves kainic acid receptor activation. Can. J. Physiol. Pharm. 1989;67:904-908

18. Meldrum B. Excitatory amino acid antagonists as potential therapeutic agents. In: Neurotoxins and their pharmacological implications. New York, Raven Press. 1987:33-53

19. Debonnel G, Beauchesne L, DeMontigny C. Domoic acid the alleged "mussel toxin" might produce its neurotoxic effect through kainate receptor activation: an electrophysiological study in the rat hippocampus. Can. J. Physiol. Pharm. 1989;67:29-33

20. Tasker RAR, Connel BJ, Strain SM. Pharmacoligical, behavioral and morphological studies of domoic acid in vivo. Eur. J. Pharm. 1990;183:959

21. Daigo K. Studies of the constituents of Chondria armata. Isolation of an anthelmintic constituent. J. Jap. Pharm. Ass. 1959;79:353-357

22. Teitelbaum J, Zattore R, Carpenter S, Gendron D, Cashman N. Clinical presentation of acute domoic acid poisoning. Symposium on domoic acid toxicity. Ottawa, Ont. 1990. Supplement to Canadian Disease Report, in press

23. Iverson F, Truelove J, Nera E, Tryphonas L, Cambell J, Lok E. Domoic acid poisoning and mussel-associated intoxication: preliminary investigations in the response of mice and rats to toxic mussel extract. Fed. Chem. Tox. 1989;27:377-384

24. Tryphonas L, Truelove J, Nera E, Iverson F. Acute neurotoxicity of domoic acid in the rat. Tox. Path. 1990;18:1-10

25. Iverson F, Tryphonas L, Truelove J. The toxicology of systemically administered domoic acid to rodents and primates. Symposium of domoic acid toxicity, Ottawa, Ont. Suppl. Canada Disease Weekly. In press

26. Preston E, Hynie I. Transfer constants for blood brain barrier permeation of the neuroexcitatory shellfish toxin, domoic acid. Proc. Can. Fed. Biol. Soc. 1990;33:89

27. Okomoto S. Epileptogenic action of glutamate directly applied into the brain of animals and inhibitory effects of proteins and tissue emulsions on its action. J. Physiol. Soc. Jpn. 1951;13:555-562

28. Curtis DR, Watkins JC. The excitation and depression of spinal neurones by structurally related amino acids. J. Neurochem. 1960;6:117-141

29. Monaghan DT, Bridges RJ, Cotman CW. The excitatory amino acid receptors: their classes, pharmacology and distinct properties in the function of the central nervous system. Annu. Rev. Pharm. Tox. 1989;29:365-402

30. Watkins JC, Evans RH. Excitatory amino acid transmitters. Ann. Rev. Pharm. Tox. 1981;21:165-204

31. McLennan H. Receptors the excitatory amino acids in the mammalian central nervous system. Prog. Neuro. 1983;20:251-271

32. Hicks TP, Hall JG, McLennan H. Ranking the excitatory amino acids by the antagonists glutamic acid diethyl ester and D-α-aminoadipic acid. Can. J. Physiol. Pharm. 1978;56:901-907

33. Monaghan DT, Olverman HJ, Nguyen L, Watkins JC, Cotman CW. Two classes of N-methyl-D-aspartate recognition sites: Differential distribution and regulation by glycine. Proc. Natl. Acad. Sci. 1988;85:9836-9840

34. Coan EJ, Collindrige GL. Characterization of an N-methyl-D- aspartate receptor component of synaptic transmission in rat hippocampal slices. Neuro. 1987;22:1-8

35. Johnson RC, Koerner JF. Excitatory amino acid neurotransmission. J. Med. Chem. 1988;31:2057-2066

36. Croucher MJ, Collins JF, Meldrum BS. Anticonvulsant action of excitatory amino acid agonists. Science. 1982;216:899-901

37. Clineschimdt BV, Martin GE, Bunting PR. Anticonvulsant activity of MH-801, a substance with potent anticonvulsant, central sympathomimetic, and apparent anxiolytic properties. Drug. Dev. Res. 1982;2:123-134

38. Meldrum B. Possible therapeutic applications of antagonists of excitatory amino acid neurotransmitters. Clin. Sci. 1985;68:113-122

39. Chapman AG, Meldrum BS, Nanji N, Watkins Jc. Anticonvulsant action and biochemical effects in DBA/2 mice of CPP(3-((±)-2-carboxypiparizin-4-yl)-proyl-1-phosphate), a novel N-methyl-D-aspartate antagonist. Eur. J. Pharm. 1897;139:91-96

40. MacDonald JW, Silverstein S, Michael VJ. MK-801 protects the neonatal brain from hypoxic-ischemic damage, Eur. J. Pharm. 1987;140:359-361

41. Coan EJ, Collindrige GL. Characterization of an N-methyl-D-aspartate receptor component of synaptic transmission in rat hippocampal slices. Neuro. 1987;22:1-8

42. Takemoto T. Isolation and structural identification of naturally occurring excitatory amino acids. In: McGeer EG, Olney JW, McGeer PL eds. Kainic acid as a tool in neurobiology. New York, Raven press. 1978:12-14

43. Krogsgaard-Larsen P, Honore T, Hansen JJ. New class of glutamate agonists structurally related to ibotenic acid. Nature. 1980;284:64-66

44. Honore T. Excitatory amino acid receptor subtypes and specific antagonists. Med. Res. Rev. 1989;9:1-23

45. Honore T, Davies SN, Drejer J, Fletcher EJ, Jacobsen P. Lodge D, Nielsen FE. Quinoxalinediones: Potent competitive non-NMDA glutamate receptor antagonists. Science. 1988;241:701 703

46. Monaghan DT, Yao D, Cotman CW. Distribution of ^{3}H-AMPA binding sites in rat brain as determined by quantitative autoradiography. Brain. Res. 1984;324:160-164

47. Olsen RW, Szamiaj O, Houser CR. [^{3}H]-AMPA binding the glutamate receptor subpopulations in rat brain. Brain. Res. 1987;402:243-254

48. Rainbow TC, Weizorek CM, Halpain S. Quantitative autoradiography of binding sites for^{3}H-AMPA, a structural analogue of glutamic acid. Brain, Res. 1984;309:173-177

49. Hicks TP, Lodge D, McLennan H. In: McLennan H. ed. Excitatory amino acid transmission. Cumosby Company. 1983:743-769

50. Biscoe TJ, Evans RH, Headly PM, Martin MR, Watkins JC. Structure activity relations of excitatory amino acids on frontal cortex and rat spinal neurones

51. Watkins JC, Pook PCK, Sunter DC, Davies J, Honore T. Experiments with kainic acid and quisqualic acid agonists and antagonists in relation to the subclassification of "non-NMDA" receptors. In: Ben A ed. Excitatory amino acids and synaptic plasticity. New York, Plenum Press. 1990

52. Frei P, Berney D, Herrling PR, Mueller W, Urwyler S. 6-7-dichloro-3-hydroxy-2-quinoxyline carboxylic acid is a related potent antagonist at NMDA and kainic acid receptors. Neuro. Let. 1988;91:194-198

53. Monaghan DT, Cotman CW. Distribution of ^{3}Hkainic acid binding sites in rat central nervous system as determined by autoradiography. Brain. Res. 1982;252:91-100

54. Unnerstall JR, Wamsely JK. Autoradiographic localization of high affinity ^{3}H-kainic acid binding sites in the rat forebrain. Eur. J. Pharm. 1983;86:361-371

55. Koener JF, Cotman CW. Micromolar L-2-amino-4-phosphonobutyric acid selectively inhibits perforant path synapses from lateral entorhinal cortex. Brain Res. 1981;216:192-198

56. Davies J, Watkins JC. Actions of D and L-forms of 2-amino-5-phosphonovalerate and 2-amino-4-phosphonobutyrate in the cat spinal cord. Brain Res. 1982;235:378-386

57. Berne RM, Levy MN. Gastrointestinal motility. In: Berne RM, Levy MN ed. Physiology 1983;742-762

58. Christensen HN. On the strategy of kinetic discrimination of amino acid transport systems. J. Membr. Biol. 1885;84:97-103

59. Wright EM, Schell RE, Stevens BR. Specificity of intestinal brush border proline transport: cyanine dye studies. Biochem. Biophys. Acta. 1985;818:271-274

60. Rajendran VM, Hairg JM, Adams MB, Ramaswany K. Transport of acidic amino acids by human jejeunal brush border membrane vesicles. Am. J. Physiol. 1987;252:G33-G39

61. McDonald P, Edwards RA, Greenhalgh JFD. Digestion. In: Animal nutrition. New York, John Wiley and Sons. 1988:130-158

62. Lawrence JF, Charbonneau CF, Page BD, Lacroix GMA. Confirmation of domoic acid in molluscan shellfish by chemical derivitization and reversed phase liquid chromatography J. Chromatogr. 1989;462:419-425

63. Helrich K. Official methods of analysis of the association of official analytical chemists. 15th edition. Helrich ed. Association of official analytical chemists, Arlington VA. 881-882

64. Bligh EG, Dyer WJ. A rapid method of total lipid extraction and purification. Can. J. Became. Physiol. 1959;37:911-917

65. Grimmelt B. M.Sc. Thesis, University of Prince Edward Island, Charlottetown 1989

66. Klaasen CD. Distribution, excretion and absorption of toxicants. Klaassen CD, Amdur MO, Doull J eds. In: Toxicology: the basic science of poisons. 1980:33-64

67. Blanchard JRT, Tasker RAR. High performance liquid chromatographic assay for domoic acid in serum of different species. 1990;526:546-549

68. Marshall BE, Wollman H. General anesthetics. Gilman AG, Goodman LS, Gilman A eds. In: The pharmacological basis of therapeutics. New York, MacMillan. 1980:276-299

69. Green CJ. Orders lagomorpha, rodentia, insectivora and chiroptera. Green CJ ed. In: Animal anesthesia, Spottiswood Ballantyne. 1982:131-161

70. Barb Williams. Prince Edward Island Department of Fisheries and Aquaculture. Annual Report 1990.

71. Mayer SE, Melmon KL, Gilman AG. Introduction; The dynamics of drug absorption, distribution and elimination. In: Gilman AG, Goodman LS, Gilman A (eds) 6th edition. The Pharmacological Basis of Therapeutics. Collier MacMillan Canada Ltd. 1980:1-27