

**DEVELOPMENT OF A PHYSIOLOGICALLY BASED
PHARMACOKINETIC (PBPK) MODEL FOR ENDOSULFAN
AND ITS APPLICATION IN HEALTH RISK ASSESSMENTS**

**エンドサルファン生理学的薬動力学モデルの開発
と健康リスク評価への適用**

Melissa Chan Pui Ling

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**Dissertation Submitted for the Degree of
Doctor of Engineering
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ACKNOWLEDGEMENTS

This study was financially supported by the Grant – in – Aid for Scientific Research (B), No. 13555150. I would like to express my deepest gratitude to the following people for all their unfailing guidance, support, patience and constant encouragement throughout my study.

1. Professor Shinsuke Morisawa, Department of Urban and Environmental Engineering (Supervisor and Chief examiner)
2. Professor Iwao Uchiyama, Department of Environmental Engineering (Examiner)
3. Professor Sadahiko Itoh, Department of Environmental Engineering (Examiner)
4. Associate Professor Minoru Yoneda, Department of Urban and Environmental Engineering
5. Dr. Aki Nakayama, Department of Urban and Environmental Engineering
6. Dr. Yuko Kawamoto, Department of Urban and Environmental Engineering
7. Dr. Miki Sugimoto, Department of Animal Science, Graduate School of Agriculture
8. Satoshi Imanishi and Hanako Nishizawa, Department of Animal Science, Graduate School of Agriculture
9. Yoko Kitanaka, Ken Yasukouchi, Tadanao Miura, and Masahiro Tozaki, Department of Urban and Environmental Engineering
10. Yoshito Matsui, Department of Environmental Engineering
11. Friends of the Chair of the Environmental Risk Analysis Laboratory

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ABSTRACT

Generally, all humans are in contact from conception to death, intentionally or unintentionally, with a multitude of chemicals which are both natural and man-made, and are present in the general environment, the home, the workplace, in ambient air, food and drinking water. The use of chemicals in practically every aspect of life has grown very rapidly over the last few decades in order to meet the social and economic goals of the world community. However, many of these chemicals can pose substantial health risks due to their toxicity to living organisms. It is therefore important that their risks are recognized so that they can be eliminated or otherwise controlled and adverse effects prevented.

Endosulfan (6, 7, 8, 9, 10, 10 – hexachloro – 1, 5, 5a, 6, 9, 9a – hexahydro – 6, 9 – methano – 2, 4, 3 – benzodioxathiepin – 3 – oxide), an organochlorine (OC) insecticide of the cyclodiene group, has widespread use in agriculture and forestry to control a wide variety of insect pests and on non – food crops such as cotton and tobacco. It is also used as wood preservative. Endosulfan is used in India, Turkey, Malaysia, Mexico and many other developing countries despite its low persistent characteristic compared to other OC pesticides.

Endosulfan is a mixture of two stereoisomers: α – and β – endosulfan in the ratio of 70:30. The metabolites of endosulfan include endosulfan sulfate, diol, hydroxyl – ether, ether, and lactone but most of its metabolites are polar substances which have not yet been identified. Residues of endosulfan are found in low levels in the environment such as sediment, soil, water and air; in animal tissues as well as in humans. The accumulation of endosulfan has also been reported in various food

crops such as vegetables and fish, thus raising a concern that this pesticide may cause health problems to humans.

Studies on the acute and subacute toxicity in rats, mice, rabbits and other species, its regional distribution in the brain and neurotoxic effects in rats have been documented. Repeated administration of endosulfan increased the liver weight and produced biochemical effects in the liver. Reduction in spermatid count in testis and sperm count in cauda epididymis as well as decrease in the weights of testis, epididymis and seminal vesicle were recorded when rats were given repeated administration of endosulfan. Adverse reproductive effects of endosulfan include testicular impairment *in vivo*, daily sperm production along with increased sperm abnormalities and altered activities of testicular marker enzymes in both mature and immature rats as well as reduction in serum testosterone levels. Data based on these studies indicate that the liver, kidneys, brain and testes are the main target organs. Although endosulfan has been studied for the past decades, a kinetic profile for rodents is still missing.

Involvement of endosulfan, a neurotoxic agent, in the central nervous system (CNS) has been studied in various studies. A stimulation by long term exposure of endosulfan at a dose of 3 mg / kg body weight for 15 and 30 days respectively increased foot shock – induced fighting behavior in male rats. Induction of hyperactivity, tremors and convulsions was observed in male rats given 40 mg / kg of endosulfan intraperitoneally.

Data concerning human exposures are scarce and limited to a small number of cases, with only sporadic data on the tissue concentrations of endosulfan and its isomers.

Toxicokinetic data in humans are lacking. Cases of acute intoxication or suicidal attempts caused by ingestion of endosulfan have been well documented, all of which developed life – threatening symptoms, resulting in fatalities. Signs of poisoning included vomiting, restlessness, irritability, convulsions, pulmonary edema and cyanosis. The first case of a patient who developed chronic brain syndrome following poisoning by endosulfan was reported elsewhere.

Chapter 1 compares the extent of contamination of endosulfan between the Malaysian and Japanese environment. This chapter also describes the physical and chemical properties of endosulfan followed by the possible routes of exposure, human exposures, effects on the environment and its environmental fate. Chapter 2 describes the general toxic effects of endosulfan in mammals (rats) including the neurological, reproductive and endocrine effects.

In Chapter 3, the toxicokinetics of ^{14}C – Endosulfan following single oral administration of 5 mg / kg body weight was investigated in male Sprague – Dawley rats. Three rats were sacrificed at 30 min, 1, 2, 4 and 8 h after dosing. Radioactivity of ^{14}C – Endosulfan was detected in all tissues at each time point. Urine and feces were collected in a separate experiment for up to 96 h. The total radioactivity recovered in the excreta for four days was 106.8 ± 26.2 % of the total administered dose with fecal elimination as the major elimination route (94.4 ± 21.4 %). The cumulative excretion in the urine for four days was 12.4 ± 4.8 %. The relative amounts of radioactivity found 8 h after administration was the highest in gastrointestinal (GI) tract (20.28 ± 16.35 mg – eq. / L) and the lowest in muscle (0.18 ± 0.06 mg – eq. / L). Toxicokinetic parameters obtained from ^{14}C – Endosulfan –

derived radioactivity in blood were the distribution half – life ($T_{1/2x}$) = 31 min; and the terminal elimination half – life ($T_{1/2y}$) = 193 h. The blood concentration reached its maximum (C_{max}) of 0.36 ± 0.08 mg – eq / L at 2 h after oral dose. Endosulfan was rapidly absorbed through into the GI tract in rats with an absorption rate constant (k_a) of 3.07 h^{-1} .

To date, no physiologically based pharmacokinetic (PBPK) model was located for endosulfan in animal species and humans. The estimation by a mathematical model is essential since information on humans can scarcely be obtained experimentally. In Chapter 4, the PBPK model was constructed based on the pharmacokinetic data of the experiment following single oral administration of ^{14}C – Endosulfan to male Sprague – Dawley rats. The model was parameterized by using reference physiological parameter values and partition coefficients that were determined in the experiment and optimized by manual adjustment until the best visual fit of the simulations with the experimental data was observed. The model was verified by simulating the disposition of ^{14}C – Endosulfan *in vivo* after single and multiple oral dosages and comparing simulated with experimental results. The model was further verified by using experimental data retrieved from the literature.

The present PBPK model could reasonably predict target tissue dosimetries in rats. Simulation with three – time repeated administration of ^{14}C – Endosulfan and experimental data retrieved from the literature by the constructed model fitted fairly well with the experimental results; thus suggesting that the newly – developed PBPK model was developed and verified. Sensitivity analyses were used to determine those input parameters with the greatest influence on endosulfan tissue concentrations.

With regards to the impact of the partition coefficients (PCs), the PCs for all the tissues had similar impact on the endosulfan and endosulfan metabolites concentrations across all time points. The impact of maximum metabolic rate (V_{max}) and Michaelis – Menten constant (K_M) on endosulfan tissue concentration was most evident at 8 h and 24 h. Other parameters had little impact in the blood and tissues concentrations of endosulfan and endosulfan metabolites across all time points, except for the fecal elimination (KE_{GD}) and biliary excretion rate (K_{bD}) rates on endosulfan metabolites concentrations, where the impact was most evident at 24 h.

The PBPK model for endosulfan in rats was extrapolated to humans, without adjusting the previously established model parameters to test the ability of the model to predict the pharmacokinetic behavior of endosulfan in humans. The ability of the model was tested by predicting the daily intake of endosulfan per individual and comparing the residue levels of endosulfan in selected tissues for both the parent isomers (endosulfan) and its metabolites (endosulfan metabolites). From the PBPK model simulations, the estimated dietary intake for the pregnant women from Chiba and Kyushu, Japan were 0.76×10^{-5} mg / kg / day and 9.09×10^{-5} mg / kg / day respectively, whereas the estimated dietary intake for the Malaysian school children was 1.06×10^{-5} mg / kg / day. Generally, reasonable agreement was observed between model predictions and experimental data, indicating that the parameters used in the model were quite well predicted and the model was / could be partially validated since data concerning human exposures are scarce and difficult to obtain experimentally.

In Chapter 5, the effects of α - endosulfan, β - endosulfan and endosulfan sulfate on the tight junctions of the blood – brain barrier (BBB) was examined by investigating the transendothelial electrical resistance (TEER) and permeability effects across cultured monolayers of porcine brain microvascular endothelial cells (PBMECs). Following exposure to a series of concentrations of endosulfan (0.01 μ M to 10 μ M), the BBB permeability, measured as TEER, decreased significantly in a dose – and time – dependent manner when monolayers were treated with α - endosulfan, β - endosulfan and endosulfan sulfate at concentrations of 0.01, 0.1, 1 and 10 μ M. TEER declined significantly and reached the bottom level as concentrations and exposure periods increased. Cytotoxicity tests indicated that the concentrations of 10 μ M and below did not cause cell death for all compounds. The integrity of the brain endothelium was further investigated by measuring the transendothelial permeability to 14 C – Endosulfan. It was observed that the transport of endosulfan through the BBB was reversible, in which endosulfan transported from the blood – brain compartment and from the brain – blood compartment, thus suggesting that residues of endosulfan has little potential to accumulate in the brain, although other possibilities could not be ruled out. The ratio between the outer – to – inner and the inner – to – outer compartments for the transport study of 14 C – Endosulfan in the concentration range of 0.01 – 10 μ M was approximately 1.2 – 2.1.

Taking into account the present concern regarding the environmental impact of endosulfan on public health, Chapter 6 studied the *in vitro* neurotoxic effects of α - endosulfan, β - endosulfan and endosulfan sulfate by comparing the ability of α - endosulfan, β - endosulfan and endosulfan sulfate to cause cell death in glial and neuronal cell cultures from rat and human by using WST – 8 assay. The present study

shows that α - endosulfan, β - endosulfan and endosulfan sulfate cause cytotoxicity in neuronal and glial cell cultures from rat and human in a concentration - dependent manner. α - endosulfan was less cytotoxic in rat neuronal PC12 cell line than in human glial CCF - STTG1 and human neuronal NT2 cell lines. β - endosulfan showed a higher potency in human NT2 cell line than in other cultures. Endosulfan sulfate was more cytotoxic in human CCF - STTG1 cell line than in human NT2, rat glial C6 and rat PC12 cell lines. α - endosulfan produces a manifest selective neurotoxicity with lethal concentration to cause 50 % cell death (LC_{50}) ranging from 11.2 μ M to 48.0 μ M. In contrast, selective neurotoxicity was not so manifested in glial and neuronal cell cultures after exposure to endosulfan sulfate, as LC_{50} values were in the range of 10.4 - 21.6 μ M. Human glial cells were the most sensitive cell type to the cytotoxic effects of α - endosulfan followed by human neuronal, rat glial and rat neuronal cells. Human neuronal cells were the most sensitive cell type to the cytotoxic effects of β - endosulfan followed by rat neuronal, rat glial and human glial cells. Human glial cells were the most sensitive cell type to the cytotoxic effects of endosulfan sulfate followed by human neuronal, rat neuronal and rat glial cells.

Chapter 7 was aimed to present a quantitative risk assessment of endosulfan, which utilizes principles of PBPK modeling as well as *in vitro* experiments of cytotoxicity. The neurotoxic, and reproductive risks were estimated since the brain and testes were among the target organs for toxicity. The neurotoxic risk was estimated by using the cytotoxic data on the percentage of viability cells of glial and neuronal cell cultures from rat and human after exposure to endosulfan as previously reported in Chapter 6. At the no effect level of 12.5 mg / kg for neurotoxicity in rat, the brain concentration was calculated by the PBPK model to be 1.56 mg / L. In humans, the same

concentration would be achieved following exposure to 12.7 ppb (0.0312 μM) endosulfan. Exposure of 0.0312 μM endosulfan to glial and neuronal cell cultures from human did not cause cell death and the estimated percentage of cell viability for all cell lines were above 90 %.

The reproductive risk was estimated by using the cytotoxic data on the percentage of viability of detached cells in rat Sertoli – germ cell cocultures after exposure to endosulfan (from the literature). At the No – Observed – Adverse – Effect – Level (NOAEL) of 6 mg / kg for reproductive toxicity in rat, the testes concentration was calculated by the PBPK model to be 1.07 mg / L. In humans, the same concentration would be achieved following exposure to 6.14 ppb (1.51 μM) endosulfan. It was observed that the estimated percentages of viability of detached cells exposure to 1.51 μM endosulfan for 24 h and 48 h were 71.6 % and 64.0 % respectively. This may suggest that the shedding of germ cells *in vitro* may be a possible reason for low sperm production observed in *in vivo* studies on mature and immature rats and may lead to testicular dysfunction.

Based on these results, the estimated exposure level for neurotoxicity in humans was 1.6 – fold higher and exceeded the acceptable daily intake (ADI) of 0.008 mg / kg (equivalent to 8 ppb). In contrast, the estimated exposure level for reproductive toxicity in humans was 6.14 ppb, which was below the ADI.

The estimated exposure levels for the pregnant women from Chiba and Kyushu as well as the Malaysian school children (previously mentioned in Chapter 4) were below the no effect level of 12.7 ppb for neurotoxicity and 6.14 ppb for reproductive

toxicity in human, thus suggesting that these people were unlikely to develop any serious health problems.

CHAPTER 1

1.0 Research background

Large numbers and large quantities of man – made chemicals have been released into the environment since World War II and chemical industry began to boom in the 1950s. Many of man – made chemicals can disturb development of the endocrine system and of the organs that respond to endocrine signals in organisms indirectly and / or early postnatal life; effects of exposure during development are permanent and irreversible. An endocrine disrupter has been defined as an exogenous agent that interferes with the synthesis, secretion, transport, binding, action, or elimination of natural hormones in the body of an intact organism or its progeny that are responsible for the maintenance of homeostasis, reproduction, development, and / or behavior. Among the chemicals which are known or suspected to exert endocrine – disrupting effects are organochlorine pesticides such as endosulfan, DDT and lindane; phthalate plasticizers such as benzylbutylphthalate (BBP) and di-n-butylphthalate (DBP); industrial chemical such as bisphenol A (BPA) and styrenes. Target organs potentially affected include male and female reproductive systems, the central nervous system, the thyroid, and the immune system.

Since the late 1950s to the present day, many dramatic declines in wildlife populations, caused by reproductive failure and problems with the development of young, have been associated with exposure to environmental pollutants. Yet it was not until 1991, that scientists realized that a common thread could link these problems in wildlife. Many of the observed effects were synonymous with what would be expected from disruption of the body's hormones.

In 1962, Rachel Carson warned of the dangers of man – made chemicals to the environment and to humans in her book, *Silent Spring*, concluding, "*Our fate is connected with the animals*". In 1993, after reviewing both human and wildlife data on endocrine-disrupters, scientists similarly proposed that wildlife could be acting like "sentinels", or mirrors to health effects which could also occur in humans. Presently, it is only hypothesis rather than fact that endocrine-disrupting chemicals are affecting human health, but evidence is mounting. Research suggests that the chemicals may be implicated in the rise of several deleterious reproductive and neurological disorders in humans over the past few decades, including reduced sperm counts and increased breast cancer. They may also be associated with reduced intellectual capacity and behavioral problems such as memory deficits and low IQ. Like the effects in wildlife, many effects in humans appear to be on the next generation, although adults may be also be affected.

There is already evidence which suggests that some endocrine – disrupting chemicals have reached levels in the environment where they could cause adverse effects on development in humans and wildlife. Effects on development are often not gross, but instead represent diminished potential - a loss of health and competency, such as reduced fertility, reduced intellectual capacity and weakened immune systems. These sorts of effects may not obviously threaten the existence of an individual but, considered at a population level, they could change the whole character of human society or destabilise wildlife populations. There is now great concern among many scientists that endocrine - disrupting chemicals could pose a long – term threat to world biodiversity and to human society.

1.1 Objectives of the research

Generally, all humans are in contact from conception to death, intentionally or unintentionally, with a multitude of chemicals which are both natural and man-made, and are present in the general environment, the home, the workplace, in ambient air, food and drinking water. The use of chemicals in practically every aspect of life has grown very rapidly over the last few decades in order to meet the social and economic goals of the world community. However, many of these chemicals can pose substantial health risks due to their toxicity to living organisms. It is therefore important that their risks are recognized so that they can be eliminated or otherwise controlled and adverse effects prevented.

Endosulfan, which is known as an organochlorine (OC) pesticide is still widely used across the globe despite its low persistence compared to other OCs. It is currently one of the major pesticides permitted and used extensively in the Malaysian agriculture sector but its usage is severely restricted in Japan. Despite its low persistence and lipophilic characteristics, residue levels of endosulfan continued to be detected in a wide range of biota and abiotic environment. Brain, testes, liver and kidneys are the main target organs for toxicity after exposure to endosulfan. Hence, there is a research need to develop a risk assessment paradigm.

Briefly, the objectives of the current research are as follow:

- (a) To develop a physiologically based pharmacokinetic (PBPK) model for endosulfan in male rats that could reasonably predict tissue dosimetry after single and repeated oral administration of ^{14}C - Endosulfan.

- (b) To extrapolate the newly – developed PBPK model from rat to human in order to test the ability of the model to predict the pharmacokinetic behavior of endosulfan in humans.
- (c) To evaluate the transendothelial electrical resistance (TEER) and permeability effects of endosulfan on the tight junctions of the blood – brain barrier (BBB) within porcine brain microvascular endothelial cells (PBMECs).
- (d) To study the *in vitro* neurotoxic effects of α – endosulfan, β – endosulfan and endosulfan sulfate by comparing the ability of these compounds to cause cell death in glial and neuronal cell cultures from rat and human by using the WST – 8 assay.
- (e) To use the newly – developed PBPK model coupled with *in vitro* assays experiments as mentioned in (c) and (d) to predict / estimate the neurological and reproductive risks.

1.2 Pesticides: An overview

The United States Environmental Protection Agency (U.S. EPA) defined “pesticides” as any substance or mixture of substances intended for preventing, destroying, repelling or mitigating any pest. Pesticides may also be described as any physical, chemical or biological agent that will kill an undesirable plant or animal pest. The term “pesticide” is a generic name for a variety of agents that are usually more specifically classified on the basis of the pattern of use and organism killed. The

major agricultural classes encompass insecticides, herbicides and fungicides (Ecobichon, 1995).

The widespread use and disposal of pesticides by farmers, institutions and the general public provide many possible sources of pesticides in the environment. Pesticides may possess different fates and behavior when they are released into the environment, namely they may be degraded by the action of sunlight, water or other chemicals or microorganisms (i.e. bacteria). Some pesticides may be resistant to degradation and remained unchanged in the environment for a certain period of time. (EXTOXNET, 1993).

From the mid - 1940s to the mid - 1960s, organochlorine (OC) pesticides were used extensively in all aspects of agriculture and forestry, in building and structural protection, and in human situations to control a wide variety of insect pests (Ecobichon, 1995). Their use was discontinued in many countries in subsequent years following their inclination to bio-accumulate in the lipid component of the biological species and their resistance to degradation (Pandit et al., 2001). In humans, the OC pesticides affect the nervous system but are not cholinesterase inhibitors (Pinkston et al). However, OC pesticides are still used in large quantities in some of the third – world, developing nations for the control of agricultural pests because of their low cost and versatility in industry, agriculture and public health.

Endosulfan is still used in chemical formulations although it is no longer produced in the United States (ATSDR, 2000). Endosulfan is unrestricted and widely used in Turkey (Oktay et al., 2003), Mexico (Castillo et al., 2002), Malaysia (Chan et al.,

2004) and Brazil (Dalsenter et al., 1999). In India endosulfan is used against a variety of agricultural pests with about 81, 000 metric tons of endosulfan being manufactured during the 1999 – 2000 (Saiyed et al., 2003) and the annual consumption of endosulfan is about 4, 200 metric tons (Sinha et al., 2001). Endosulfan is severely restricted in countries such as Japan, Korea and Taiwan (EJF, 2002).

1.3 Pesticides in Malaysia

1.3.1 Pesticide usage

Agriculture is an important sector in the Malaysian economy. In 1997, it accounted for 13.2 % of Gross National Product (GNP), 14.8 % of total export earnings and employed 21.3 % of total work force. Hence, pesticide industry is an essential contribution to agriculture. In 1996, the pesticide market was valued at RM 301.0 million (end – user level). Most pesticides used are herbicides (75 %) followed by insecticides (16 %) and fungicides (5%) (Table 1.1) (MACA, 1997) and they are mainly applied in the plantation industry, vegetable growing, rice cultivation and public health control (Department of Statistics, 1994).

The first few organochlorine (OC) insecticides to appear in the market were DDT, lindane and the cyclodienes (aldrin, dieldrin, endrin). They were well received by farmers because they were cheap, effective, persistent and had a wide spectrum of activity that could give total kill.

Table 1.1. Pesticides market in Malaysia (The Malaysian Agricultural Chemicals Association [MACA], 1997).

Pesticide	RM million						
	1990	1991	1992	1993	1994	1995	1996
Herbicides	261.3	230.0	210.0	200.0	201.0	220.0	227.0
Insecticides	42.8	40.0	41.0	39.0	41.0	43.0	47.0
Fungicides	14.5	13.0	13.0	13.0	14.0	15.0	16.0
Rodenticides	10.5	10.0	12.0	10.0	11.0	11.0	11.0
Total	329.1	293.0	276.0	262.0	267.0	289.0	301.0

Most of the manufacturers of pesticides in Malaysia import the active ingredients and formulate them locally (Table 1.2) (Department of Statistics, 1994). The local production of pesticides is illustrated in Table 1.3 (Department of Statistics, 1994).

Table 1.2. Imports of pesticides in Malaysia, 1992 – 1993
(Department of Statistics, 1994).

Pesticide	RM '000	
	1992	1993
Herbicides	23, 479	23, 290
Insecticides	48, 540	52, 291
Fungicides	19, 832	23, 463

Table 1.3. Local production of pesticides in Malaysia, 1992 – 1993
(Department of Statistics, 1994).

Pesticide	RM '000	
	1992	1993
Herbicides	203, 526	216, 173
Insecticides	69, 347	57, 698
Fungicides	4, 202	3, 623

In the rice growing region of West Malaysia, especially in Province Wellesley and in Krian District of Perak, endosulfan was generally favored by many farmers in both localities. 52 % of the farmers reported that endosulfan was effective against many species of paddy pests. 32 % of the farmers claimed that the chemical was effective against leaf – rollers, 4 % found it to be effective against paddy field crabs and 20 % noted that it acted as a repellant to rats. The farmers in Krian District also favored endosulfan for similar reasons. 52 % of the farmers noted that endosulfan was cheap and fast acting on most insect pests (Yunus and Lim, 1971), thus suggesting that endosulfan is widespread in Malaysia. Endosulfan is currently permitted in Malaysia.

1.3.2 Endosulfan in the Malaysian environment

Due predominantly to its persistent characteristic, residues of endosulfan continue to in water, sediment and biota including foodstuff such as agricultural products and seafood. Tables 1.4 and 1.5 show the extent of contamination of endosulfan in both biotic and abiotic components in the Malaysian environment.

Table 1.4. Endosulfan residues in the Malaysian aquatic environment.

Location	Survey Year	Chemical	Concentration (ng ml ⁻¹ / ng g ⁻¹)			References
			Water	Sediment	Fish	
Selangor (Paddy field)	1983	α - Endosulfan	1 - 30	nil	nil	Lee and Ong, 1983
		β - Endosulfan	1 - 2	nil	nil	
		Endosulfan sulfate	0-2	nil	nil	
Tanjong Karang	1982	α - Endosulfan	nil	nil	5130	Soon and Hock, 1987
		β - Endosulfan	nil	nil	1700	
Penang	1984-1987	α - Endosulfan	nil	nil	3.4	Jothyl et al., 1987
		β - Endosulfan	nil	nil	2.0	
Major rivers in Peninsular Malaysia Selangor River	1989-1990 1992-1993	α - Endosulfan	nd - 0.044	nil	nil	Tan and Vijayaletchumy, 1994
		β - Endosulfan	nd - 0.010	nil	nil	
		α , β -Endosulfan	nil	5.4	nil	
Bernam River Selangor River	1994	α , β -Endosulfan	nil	0.96	nil	Tan and Vijayaletchumy, 1994
		α , β -Endosulfan	nil	5.35	nil	
Straits of Malacca Muda area Non Muda area	1995	α - Endosulfan	nil	< 0.02	nil	Ahmad et al., 1998
		β - Endosulfan	nil	< 0.02	nil	
		Endosulfan sulfate	nil	< 0.02 - 3.42	nil	
		α - Endosulfan	nil	< 0.02	nil	
		β - Endosulfan	nil	< 0.02	nil	
		Endosulfan sulfate	nil	< 0.02	nil	

Table 1.5. Endosulfan residues in marine biota from Malaysian waters and human blood.

Location	Survey Year	Chemical	Concentration (ng/g)	References
Batu Lintang, Kedah Mussels	1990	α -Endosulfan	0.05	Ismail et al., 1992
Various locations in Peninsular Malaysia (human blood)	1999	Endosulfan#	nd - 0.6	Chan et al., 2004

α , β and sulfate

1.4 Endosulfan in Japan

1.4.1 Sales of endosulfan in Japan

Endosulfan is severely restricted in Japan (EJF, 2002). In Japan, endosulfan is produced in the form of liquid, powder, emulsion and granules as well as imported from other countries. Sales of endosulfan in Japan have been declining from year to year (Table 1.6).

Table 1.6. Sales of endosulfan in Japan, 1992 – 2001 (National Institute of Environmental Science, Japan).

Year	1992	1993	1994	1995	1996	1997	1998	1999	2000	2001
Tonne	156	157	128	115	113	100	77	61	59	50

1.4.2 Endosulfan in the Japanese environment and population

No endosulfan residues have been detected in the Japanese environment. The surveillance of the intake from usual diets of several kinds of OC pesticides including endosulfan has been carried out up to the time in the total diet study in Japan in 1982. Samples were collected from nine prefectures (Miyagi, Niigata, Yamanashi, Osaka, Nara, Wakayama, Shimane, Ehime and Fukuoka) across Japan. None of the OC pesticides including endosulfan were detected in the study, hence suggesting that intake of those pesticides from the usual diets in Japan was considered to be negligible (Sekita et al., 1985). Residues of endosulfan were detected in maternal serum, umbilical cord serum and umbilical cord samples of pregnant women around the Chiba or Yamanashi and Kyushu areas (Table 1.7).

Table 1.7. Endosulfan residues in the Japanese population.

Area	Sample	Compound	Concentration (pg / g)	Reference
Chiba or Yamanashi	Maternal serum	Endosulfan #	0 – 8.4	Fukata et al., 2005
	Umbilical cord serum		0	
	Umbilical cord		0 – 7.2	
Kyushu	Maternal blood	α -endosulfan	13 – 21	
		β -endosulfan	2.6 – 6.3	
		E. sulfate *	0.16 – 0.78	
	Cord blood	α -endosulfan	14 – 38	
		β -endosulfan	2.1 – 6.8	
		E. sulfate	0.18 – 0.35	
	Placenta	α -endosulfan	52 – 200	
		β -endosulfan	9.4 – 21	
		E. sulfate	0.94 – 3.7	
	Breast milk	α -endosulfan	15 – 46	
		β -endosulfan	3.1 – 7.3	
		E. sulfate	1.7 – 70	

: Mixture of α – and β - endosulfan

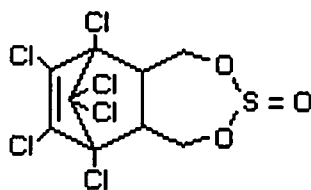
* : Endosulfan sulfate

1.5 Endosulfan – A short review

1.5.1 Introduction

Endosulfan was developed and introduced by Farbwerke Hoechst A. G. in 1954 under the registered trade mark “Thiodan”. It belongs to the cyclodiene group. The other alternative names of endosulfan are Cyclodan, Thimol, Thiofar and Malix. It is chemically known as 6, 7, 8, 9, 10, 10-hexachloro-1, 5, 5a, 6, 9a-hexahydro-6, 9-methano-2, 4, 3-benzodioxathiepine-3-oxide or a, b, 1, 2, 3, 4, 7, 7-hexachloro-bicyclo-(2, 2, 1)-heptene-(2)-bis-hydroxymethylene (5-6) sulfide. The insecticide endosulfan is obtained by the action of thionylchloride on the addition product from hexachlorocyclopentadiene and *cis*-butene-diol-1, 4 (Martin and Worthing, 1977).

Endosulfan is a mixture of two stereoisomers, the alpha of melting point (m. p.) 108 - 110°C, the beta of melting point (m. p.) 208 - 210°C, having a molecular weight of 406.93. It is stable to sunlight but subject to slow hydrolysis by alcohol and sulphur dioxide. It is compatible with most non-alkaline pesticides but incompatible with strongly alkaline materials. The structural formula of endosulfan is given below.



Structural formula of endosulfan

Usually, the technical grade consists of α - and β - isomers in the ratio 70:30. The pure mixture (90 - 95%) of isomers is a brownish crystalline with a terpene like odour [melting point (m. p.) 70 - 100°C, vapor pressure (v. p.) 1×10^{-5} torr at 25°C]. Endosulfan is practically insoluble in water but moderately soluble in most organic solvents. Under normal conditions, it is stable on storage, non-inflammable and be hydrolysed slowly by aqueous alkali and acids with the formation of the diol and sulphur dioxide (Gupta and Gupta, 1979; Briggs).

Endosulfan is a non - systemic contact and stomach insecticide. It is used to control the sucking, chewing and boring insects and mites on a very wide range of crops, such as fruit (including citrus), vines, olives, vegetables, ornamentals, potatoes, cucurbits, cotton, tea, coffee, rice, cereals, maize, sorghum, oilseed crops, hops, hazels, sugar cane, tobacco, lucerne, mushrooms, forestry, glasshouse crops and tsetse flies (Briggs). It is also used as wood preservative and on non - food crops such as tobacco and cotton.

Endosulfan is currently classified as Class II – *moderately hazardous* to human health (WHO, 1984). However, the United States Environmental Protection Agency (US EPA) rates endosulfan as Class Ib – *highly hazardous*.

1.5.2 Summary of the characteristics of endosulfan

1.5.2.1 Chemical identity

Endosulfan is an OC insecticide. Technical endosulfan consists of a mixture of two stereoisomers, α -endosulfan stereochemistry 3 α , 5 $\alpha\beta$, 6 α , 9 α , 9 $\alpha\beta$ -; β -endosulfan stereochemistry 3 α , 5 $\alpha\alpha$, 6 β , 9 β , 9 $\alpha\alpha$ -. Technical grade endosulfan contains at least 94 % of the two pure isomers (Maeir – Bode, 1968). The α – and β – isomers of endosulfan are present in the ratio of 7:3 respectively. Technical grade endosulfan may also contain up to 2 % endosulfan alcohol and 1 % endosulfan ether. Endosulfan sulfate is a reaction product found in technical endosulfan; it is also found in the environment due to photolysis and in organisms as a result of oxidation by biotransformation [Agency for Toxic Substances and Disease Registry (ATSDR), 2000]. The physical and chemical properties are listed in Tables 1.8 – 1.10.

Table 1.8. Chemical identity of endosulfan.

Characteristic	Information
Common name	Endosulfan
IUPAC name	1, 4, 5, 6, 7, 7-hexachloro-8, 9, 10-trinorborn-5-en-2, 3-ylenebismethylene) sulfite
CAS name	6, 7, 8, 9, 10, 10-hexachloro-1, 5, 5a, 6, 9, 9a-hexahydro-6, 9-methano-2, 4, 3-benzodioxathiepin-3-oxide
Empirical formula	C ₉ H ₆ Cl ₆ O ₃ S
Molecular weight	406.9

1.5.2.2 Physical and chemical properties

Table 1.9. Physical and chemical properties of pure active constituent.

Characteristic	Information
Color	Colorless crystalline solid
Odour	Odourless
Physical state	Pure α -isomer-crystalline solid; pure β -isomer-crystalline solid
Melting point	α -: 109.2 °C; β -: 213.3 °C
Vapor pressure	α -: 1.9; β -: 0.09 mPa at 25 °C. α -: 0.96; β -: 0.04 mPa at 20 °C
Specific gravity	1.745 at 20 °C
Solubility in water	α -: 0.33; β -: 0.32 mg/L at 22 °C
Solubility in organic solvents (100g solvent at 20 °C)	Ethyl acetate, Dichloromethane, Toluene (200 g/L), Ethanol (65 g/L), Hexane (24 g/L)

Table 1.10. Physical and chemical properties of technical grade endosulfan

Characteristic	Information
Color	Brown
Odour	Terpene odour
Physical state	Crystalline flakes
Melting point	70 – 100 °C
Vapor pressure	1×10^{-5} mm Hg at 25°C; 1.7 mPa
Specific gravity	1.745 at 20 °C
Solubility in water	60 – 150 μ g/L
Diffusion coefficient in water	4.55×10^{-6} cm ² /sec at 37 °C
Solubility in organic solvents (100g solvent at 20 °C)	Chloroform (50 g); Xylene (45 g); Benzene (37 g); Acetone (33 g); Carbon tetrachloride (29 g); Kerosene (20 g); Methanol (11 g); Ethanol (5 g)

1.5.2.3 Exposure guidelines

Table 1.11. Exposure guidelines (ATSDR, 2000; McGregor, 1998; WHO, 1984).

Criteria	Abbreviation	Exposure guideline
Acceptable daily intake	ADI	0.008 mg / kg / day (WHO)
Lowest effect level	LEL	0.75 mg / kg / day (rat)
No – observed – adverse – effect – level (for reproductive toxicity)	NOAEL	6 mg / kg / day (rat)
No – observed – effect – level (for neurotoxicity)	NOEL	12.5 mg / kg / day (rat)
Reference dose	RfD	0.00005 mg / kg / day (EPA)
Threshold limit value - short term exposure limit	TLV - STEL	0.3 mg / m ³
Threshold limit value - Time weighted average	TLV - TWA	0.1 mg / m ³

1.5.3 Possible routes of exposure

Endosulfan is well absorbed through ingestion, inhalation and skin contact. Food contaminants with endosulfan are the main portal of human exposure. The most likely way for people to be exposed to endosulfan is by eating food contaminated with endosulfan. Endosulfan has been found in some food products such as oils, fats, and fruits and vegetable products, but residues have generally been found to well below the FAO / WHO maximum residue limits (WHO, 1984). People may also be exposed to low levels of endosulfan by skin contact with contaminated soil or by smoking cigarettes made from tobacco that has endosulfan residues on it (Lonsway et al., 1997). Well water and public water supplies are not likely sources of exposure to endosulfan. Workers can breathe in the chemical when spraying the pesticide on

crops. Exposure can occur by breathing the dust or getting the pesticide on the skin if people do not follow all the safety and handling procedures. Accidental spills and releases to the environment at hazardous waste disposal sites are also possible sources of exposure to endosulfan for occupational workers. The most likely exposure to endosulfan for people living near hazardous waste sites is through contact with soils containing it.

Populations that are usually susceptible to endosulfan include the unborn and neonates, the elderly and people with liver, kidney, immunological, haematological or neurological disease.

Table 1.12. Maximum residue limits (MRL) for endosulfan (The Agrochemical Handbook)

Compound	Maximum residue limit (MRL)
Endosulfan	Root vegetables 0.2 ppm; other fruit and vegetables 1ppm; maize 0.2 ppm; other cereals 0.1 ppm; all feedstuffs 0.1 ppm (except maize 0.2 ppm, oilseeds 0.5 ppm, complete feedstuffs for fish 0.005 ppm)

1.5.4 Exposure of endosulfan to humans

In general, characterization of the dose of endosulfan in poisoning cases has been poor. Several cases of accidental and suicidal poisoning have been reported. The lowest reported dose that resulted in death in humans was 35 mg / kg body weight, and deaths have also been reported after ingestions of approximately 295 and 467 mg/kg, with death occurring within 1 hour of administration in some cases. Intensive medical treatment within 1 hour of endosulfan administration was reportedly

successful at doses of 100 and 1000 mg / kg with clinical signs in these patients consistent with those seen in laboratory animals, dominated by tonic-clonic spasms. In a case where the dose was 1000 mg / kg, neurological symptoms requiring anti-epileptic therapy, which resulted from anoxia during treatment, were still required one year after endosulfan exposure (NRA, 1998). Signs of poisoning included vomiting, restlessness, irritability, convulsions, pulmonary edema and cyanosis [Agency for Toxic Substances and Disease Registry (ATSDR), 2000; World Health Organization (WHO), 1984].

A small number of case reports on the tissue concentrations of endosulfan and its metabolites were reported in the literature (Chan et al., 2004; Cooper et al., 2001; Kumar et al., 2000; Saiyed et al., 2003; Sarcinelli et al., 2003; Cerrillo et al., 2005). This pesticide is also implicated in several cases of accidental (Demeter et al., 1977; Oktay et al., 2003), non – accidental (Boereboom et al., 1998) and suicidal deaths (Blanco – Coronado et al., 1992; Eyer et al., 2004; Lo et al., 1995) as well as occupational human poisoning (Aleksandrowicz, 1979; Brandt et al., 2001).

1.5.5 Effects on the environment

Endosulfan is not readily bioaccumulated and it is not very persistent in biological tissues as compared to other OC pesticides. It is hazardous as an acute poison for some aquatic species, particularly fish, even at application rates recommended for wetland areas and can cause massive mortalities. In fish, it caused marked changes in sodium (Na) and potassium (K) concentrations, decrease in blood Ca^{2+} and magnesium (Mg) levels and inhibits Na, K and Mg – dependent ATPase (in brain) (Naqvi and Vaishnavi, 1993). The high toxicity to fish means that fish kills can result

from discharges to waterways. It is moderately toxic to honey bees. It is moderately to highly toxic for birds in a laboratory setting, but no poisonings have been reported under field conditions. Endosulfan contamination is not widespread in the aquatic environment as it is easily degraded with a half – life of 4 days which could be increased at low pH or under anaerobic conditions (Naqvi and Vaishnavi, 1993).

1.5.6 Environmental fate of endosulfan

Endosulfan has low water solubility. It is strongly absorbed onto soil particles and hence immobile in the soil column. Transport of this insecticide is most likely in the form of surface runoff. Large amounts of endosulfan can be found in surface water near areas of application (Farms Chemical Handbook, 1992).

The breakdown of endosulfan in water is more rapid under neutral conditions with the half-life of five weeks that at more acidic condition with the half – life of five months. Under strongly alkaline conditions, the half-life of the insecticide is one day. The two isomers have different degradation times in soil. The half – life for the α – isomer is 35 days and 150 days for the β – isomer under neutral conditions. The half – live on plants is three to seven days for most fruits and vegetables (Martin and Worthing, 1977).

Due to its widespread application, the accumulation of endosulfan has been reported in the environment (Gamón et al., 2003; Bhattacharya et al., 2003; Louie and Sin, 2003; Tan and Vijayaletchumy, 1994).

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CHAPTER 2

TOXICITY TO MAMMALS

2.0 Introduction

Chlorinated hydrocarbon insecticides which are ubiquitous in the environment in nature have become an integral part in the tissues of animals. Recognition of the incorporation of the parent compound and / or its metabolites in lower organisms, in tissues of fishes, birds, wild animals and humans has caused serious concern (Martin, 1964). Endosulfan due to its insecticidal properties has an extensive use in agriculture sector as a potent pesticide in control pests. Due to its wide application, it may enter human or animal systems either directly or as an environmental pollutant. Generally, mammals are not as sensitive to endosulfan as aquatic organisms. The degree of toxicity depends upon the species and sex of animals, the route of exposure and vehicle used (McGregor, 1998). Brain, testes, liver and kidneys are the main target organs for toxicity after exposure to endosulfan (ATSDR, 2000).

2.1 Toxicity to mammals (rats)

2.1.1 General toxic effects of endosulfan

Tables 2.1 and 2.2 show the existing information on health effects of endosulfan in human and animal. The LD₅₀ of endosulfan (isomeric mixture) for rats varies markedly depending upon the route of administration, species, dosing vehicle and the sex of the animal. The LD₅₀ ranges from 47 – 89 mg / kg for male rats and 8 – 49 mg / kg for female rats (Gupta, 1976; Gupta and Gupta, 1977). The inhalation lethal dose (LC₅₀) in male rats was 350 mg / m³ when exposed for 4 hours. The dermal LD₅₀

Table 2.1. Existing information on health effects of endosulfan in human.

Health effect	Route of exposure		
	Inhalation	Oral	Dermal
Death		*	
Systemic (Acute)	*	*	*
Systemic (Intermediate)	*		*
Systemic (Chronic)			
Immunologic		*	*
Neurologic	*	*	*
Reproductive			
Developmental		*	
Genotoxic	*		
Cancer	*		

Table 2.2. Existing information on health effects of endosulfan in animal.

Health effect	Route of exposure		
	Inhalation	Oral	Dermal
Death	*	*	*
Systemic (Acute)	*	*	*
Systemic (Intermediate)	*	*	*
Systemic (Chronic)		*	
Immunologic	*	*	*
Neurologic	*	*	*
Reproductive	*	*	*
Developmental		*	*
Genotoxic		*	
Cancer		*	

Note.

* : Existing studies

varied from 74 – 681 mg / kg depending on the vehicle and sex used. When rats were fed on diets containing 0 %, 3.5 %, 9 %, 26 % or 81 % protein as casein, the LD₅₀ was 5.1, 24.0, 57.0, 102.0 and 98.0 mg / kg, respectively, but the prominent signs and

symptoms of intoxication remained unchanged (Gupta and Gupta, 1977). The prominent signs of intoxication include hypersensitivity, respiratory distress, diarrhea, tremors, hunching and convulsions followed by death. Rats fed two years on a diet containing 30 ppm suffered no ill effect (Martin and Worthing, 1977).

The isomers of endosulfan show acute oral toxicity profiles similar to that of technical grade endosulfan. The acute toxicity of formulations containing endosulfan was dependent upon the concentration of the active ingredient in the end – used products, and was similar to those seen following administration of the active ingredient. The toxicity of the metabolites varies depending upon vehicle and species used. Generally, the toxicities of the metabolites were similar to the parent compound, except for endosulfan diol which has low acute oral toxicity in the mouse [National Registration Authority (NRA), 1998].

More than 90 % of an oral dose of endosulfan was absorbed in rats, with maximum plasma concentrations occurring after 3 - 8 h in males and about 18 h in females. Elimination occurs mainly in the feces and to a lesser extent in the urine, more than 85 % being excreted within 120 h. The highest tissue concentrations were in the kidneys. The metabolites of endosulfan include endosulfan sulfate, diol, hydroxy-ether, ether, and lactone but most of its metabolites are polar substances which have not yet been identified. Endosulfan would not be expected to accumulate significantly in human tissues.

Metabolism studies in rats, after an intraperitoneal injection of 20 mg / kg of technical endosulfan in an oil solution revealed the presence of endosulfan diol and an

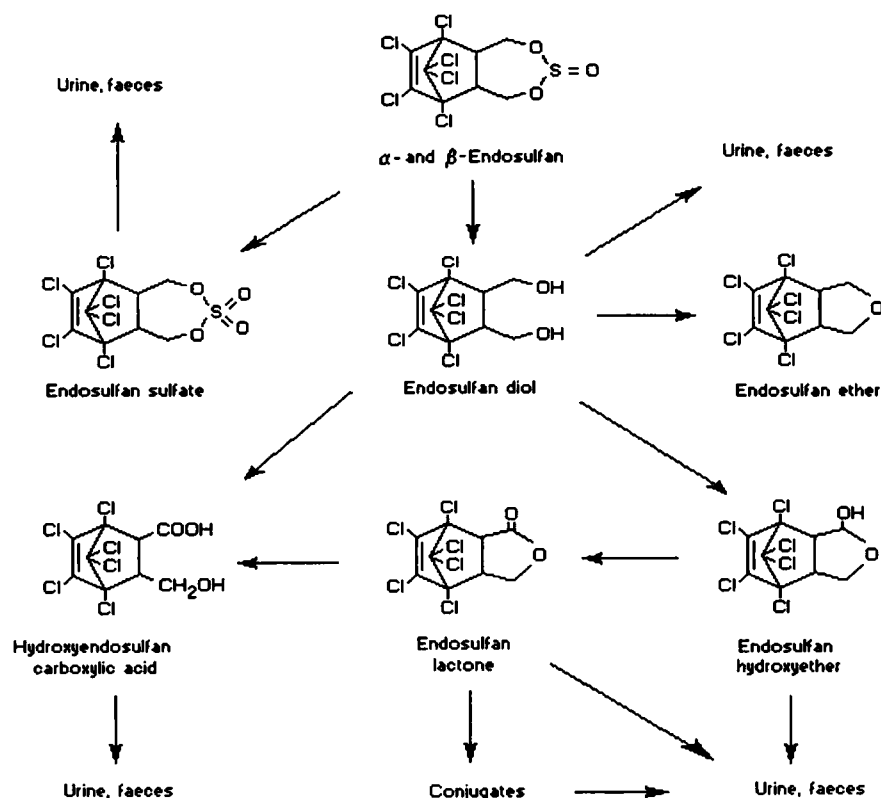
unknown compound in the urine as a water soluble conjugation metabolite. It was also reported that endosulfan was not excreted in rat urine as endosulfan diol, but as endosulfan- α -hydroxy ether. In addition, transient amounts of endosulfan and endosulfan sulfate was also detected in the body fat and liver of mice after the administration of [^{14}C] endosulfan where endosulfan metabolites were detected in feces (Gupta and Gupta, 1979). There is no accumulation in milk, fat or muscle and it is excreted as conjugates of the diol and other highly polar compounds depending on the species (Martin and Worthing, 1977). Dorrough et al. (1978) indicated that the major portion of residues in the excreta and / or tissues consisted of unidentified polar metabolites that could not be extracted from the substrate, whereas the non – polar metabolites, including sulfate, diol, α – hydroxyether, lactone and ether derivatives of endosulfan, represented only minor amounts. The available evidence indicates that endosulfan can be metabolized in animals to other lipophilic compounds, which can rapidly enter tissues, and to more hydrophilic compounds that can be excreted.

The distribution pattern of endosulfan was estimated in the plasma and brain of the rats when they were fed daily doses of endosulfan (5 or 10 mg / kg) for 15 days. The animals were sacrificed 24 hours later. The concentration of α -isomer was in the order of cerebrum (3.76 μg / g) > remaining parts of the brain (2.66 μg / g) > cerebellum (2.04 μg / g). The concentration of the β -isomer was 0.06 μg / g in the cerebrum and 0.02 μg / g in the cerebellum, where as no β -isomer was detected in the “remaining part” of the brain. The plasma concentrations of α - and β - isomers were 2.26 and 0.46 μg / g respectively and endosulfan sulfate was the only metabolite detected in the plasma (Gupta, 1978). Endosulfan is neither carcinogenic (ATSDR,

2000; Hack et al, 1995; McGregor, 1998; Naqvi and Vaishnavi, 1993) nor genotoxic (ATSDR, 2000; McGregor, 1998; Naqvi and Vaishnavi, 1993).

2.1.2 Neurological effects

Involvement of endosulfan, a neurotoxic agent, in the central nervous system (CNS) has been studied in various studies (Anand et al., 1980; Paul et al., 1994; Seth et al., 1986; Subramoniam et al., 1994). Long term inhalation or oral exposure to endosulfan results in neurotoxicity which is a primary effect. Farmers exposed to endosulfan exhibit epilepsy, hyperactivity, irritability, tremors, convulsions and paralysis. However, the CNS effects described for acute exposure are not usually



Note. Most endosulfan metabolites are polar and are yet to be identified.

Figure 2.1. Mammalian metabolism and excretion of endosulfan

(Adapted from McGregor, 1998).

found with chronic oral exposure in experimental animals. A stimulation by long term exposure of endosulfan at a dose of 3 mg / kg body weight for 15 and 30 days respectively increased foot shock – induced fighting behavior in male rats (Agrawal et al., 1983). Induction of hyperactivity, tremors and convulsions was observed in male rats given 40 mg / kg of endosulfan intraperitoneally (Ansari et al., 1987). Dikshith and co – workers (1984) observed signs of CNS stimulation for the first 3 – 4 days only in rats given oral gavage doses of endosulfan for 30 days.

Dermal exposure to endosulfan has not been reported to cause neurological effects in humans. No effects on brain weight or histopathology were observed with dermal application of endosulfan to male rats (62.5 mg / kg / day) and to female rats (32 mg / kg / day) for 30 days (Dikshith et al., 1988).

2.1.3 Reproductive effects

Human studies demonstrating reproductive effects due to inhalation, oral or dermal exposures are unavailable in the literature survey. Reproductive performance in animals is not adversely affected even though adverse effects on the reproductive organs have been observed. Endosulfan induced alteration on spermatogenesis in young growing as well as adult rats have been reported (Sinha et al., 2001). The impairments include decreased sperm count, intratesticular spermatid number, sperm morphology, altered activities of testicular marker enzymes [Pandey et al., 1990; National Cancer Institute (NCI), 1978] as well as reduction in serum testosterone levels (Choudhary and Joshi, 2003; Wilson and LeBlanc, 1998). Dalsenter et al. (1999, 2003) reported reproductive effects of endosulfan on the male offspring of rat exposed during pregnancy and lactation at high dose. The daily sperm production

was permanently decreased at puberty and adulthood. In addition, the percentage of seminiferous tubules showing complete spermatogenesis was significantly decreased at puberty. This finding may explain the decrease in daily sperm production observed in the endosulfan – exposed male rats. Other testicular impairment *in vivo* have been reported elsewhere (Sinha et al., 1995; Sinha et al., 1997; Chitra et al., 1999; Dikshith et al., 1984; Singh and Pandey, 1989).

2.1.4 Endocrine effects

An estrogen is a substance that can induce estrus or a biological response associated with estrus; one such effect is proliferation of cells in the female genital tract. In recent years, naturally occurring and man-made substances in the environment that may be estrogenic have come under increasing scrutiny. Suspicion that endosulfan may have estrogenic properties was stimulated by observation of the reduced sperm counts described above and of testicular atrophy in rats given endosulfan in the diet in long-term studies. Perhaps the first study of estrogenic effects *in vivo* was conducted by Raizada et al. (1991), who treated groups of eight ovariectomized Wistar rats, weighing an average of 100 g, with endosulfan at 1.5 mg / kg bw per day by gavage, estradiol dipropionate intraperitoneally (dose unspecified), or a combination of these treatments for 30 days. Endosulfan did not change the weights of the uterus, cervix, or vagina, whereas estradiol propionate produced large increases (Hiremath and Kaliwal, 2003). The increased weights seen with the combined treatment were similar to those with estradiol propionate alone. Persistent depressed testicular testosterone was seen in male rats after intermediate oral exposures to endosulfan.

Concern that endosulfan might be estrogenic persisted as a result of the findings of an 'E - screen' assay, which was developed to assess the estrogenic effects of environmental chemicals by observing their proliferative effect on a target cell. The numbers of cells present after similar inocula of the human breast cancer cell line, MCF - 7, were compared in the absence of estrogens (negative control), in the presence of estradiol - 17β (positive control), and with a range of concentrations of endosulfan. In this assay, endosulfan was estrogenic at concentrations of 10 - 25 μM , with a proliferative effect about 80 % that of 17β - estradiol at 10 μM ; the relative proliferative potency (i.e. the ratio of the doses of endosulfan and estradiol - 17β required to produce the maximum effect) was 0.0001 %. In addition, endosulfan competed with estradiol - 17β for binding to the estrogen receptor and increased the concentrations of progesterone receptor and pS2 in MCF - 7 cells, as would be expected for a compound that mimics estrogens (Soto et al., 1994, Soto et al., 1995).

No studies were located regarding endocrine disruption in humans after exposure to endosulfan. Overall, *in vitro* evidence in favor of endosulfan estrogenicity indicates relatively weak potency compared to estradiol - 17β . Contradictory results were reported in different studies, indicating that cautions should be used in interpreting the collective *in vitro* results.

Table 2.3. Levels that cause no toxic effect (ATSDR, 2000; McGregor, 1998).

Species	No toxic effect level
Mouse:	3.9 ppm, equal to 0.58 mg / kg bw per day (females in a 78 - week study of toxicity)
Rat:	15 ppm, equal to 0.6 mg / kg bw per day (two - year dietary study of toxicity) 75 ppm, equal to 6 mg / kg bw per day (reproductive toxicity) 0.66 mg / kg bw per day (maternal toxicity in a study of developmental toxicity) 2 mg / kg bw per day (fetotoxicity in a study of developmental toxicity)
Rabbit:	0.7 mg / kg bw per day (maternal toxicity in a study of developmental toxicity)
Dog:	10 ppm, equivalent to 0.57 mg / kg bw per day (one - year study of toxicity)

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CHAPTER 3
TOXICOKINETICS OF ^{14}C – ENDOSULFAN FOLLOWING SINGLE AND
REPEATED ORAL ADMINISTRATION IN THE MALE SPRAGUE –
DAWLEY RATS

3.0 Pharmacokinetics: An overview

Pharmacokinetics define the “science and study of the factors which determine the amount of drugs at sites of biologic effect at various times after application of an agent to a biologic system”. Pharmacokinetic data therefore include all data leading to the ‘effective dose’ of any chemical at the site of effect (Hoang, 1995). Pharmacokinetic models have the potential to estimate time – course concentrations of parent compounds and metabolites for different exposure conditions. Much of the earlier work with pharmacokinetic modeling in toxicology was based on determining the time – course of chemical concentrations in various tissues after different doses (Figure 3.1). These time – course curves were then analyzed with the so – called data – based compartmental pharmacokinetic models to estimate the dose – dependence of kinetic parameter. The compartments in these models do not correspond directly to the anatomy, physiology, or biochemistry of the animal itself (Gibaldi and Perrier, 1982). Despite the relative simplicity of these descriptions, these data – based compartmental pharmacokinetic approaches were instrumental in uncovering dose dependencies of pharmacokinetics and examining the influence of dose – dependent kinetics on dosimetry in target tissues. However, this approach of pharmacokinetic analysis was primarily of use for interpolation between doses or for limited extrapolation in the test animal. Interpolation is not adequate for most risk assessment

needs that extrapolate to very low doses and from animals to humans (Andersen, 2003).

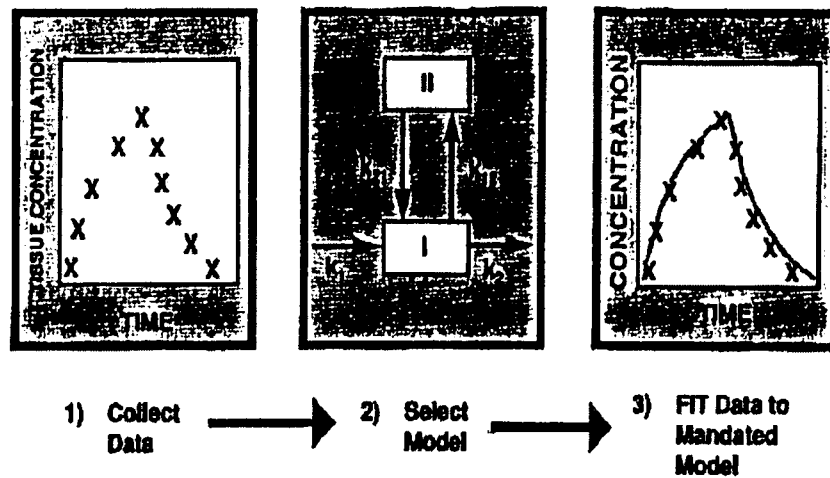


Figure 3.1. Data – based pharmacokinetic modeling: in this common approach to pharmacokinetic modeling, time – course data are collected (left – most panel) and fit to specified models that describe the body as a series of compartments (middle panel). These compartments have no direct physiological correspondence with specific anatomical structures within the body. The best fit of the model to the data provides estimates of the various micro – constants (k_{12} , k_{21} , k_1 , k_2) in the model (Adapted from Andersen, 2003).

Drugs are often administered by various routes. The two most common of these routes being intravenous infusion and oral fixed – dose or fixed – time interval regimens, e.g., a specific dose, taken one, two or three times daily. Administration of a drug by fixed doses rather than by continuous infusion is often more convenient. Orally administered drugs may be absorbed slowly and the plasma concentration of the drug is influenced by both the rate of absorption and the rate of drug elimination (Mycek et al., 2000).

3.1 Background introduction

Organochlorine (OC) pesticides are ubiquitous in the environment and in organisms. These chemicals, still in use in many countries have low volatility and slow rate of biotransformation, are highly lipid soluble, and resistant to degradation. Despite these characteristics that make OC pesticides such effective pesticides also brought to their demise in many countries due to its detrimental and deleterious effects in the environment, bioconcentration and biomagnification in various food chains (Gonzalez – Farias et al., 2002).

Endosulfan (6, 7, 8, 9, 10, 10 – hexachloro – 1, 5, 5a, 6, 9, 9a – hexahydro – 6, 9 – methano – 2, 4, 3 – benzodioxathiepin – 3 – oxide), an OC insecticide of the cyclodiene group, has widespread use in agriculture and forestry to control a wide variety of insect pests and on non – food crops such as cotton and tobacco. It is also used as wood preservative [Agency for Toxic Substances and Disease Registry (ATSDR), 2000]. Endosulfan is still used in chemical formulations although it is no longer produced in the United States (ATSDR, 2000). Endosulfan is unrestricted and widely used in Turkey (Oktay et al., 2003), Mexico (Castillo et al., 2002) and Brazil (Dalsenter et al., 1999). In India endosulfan is used against a variety of agricultural pests with about 81, 000 metric tons of endosulfan being manufactured during the 1999 – 2000 (Saiyed et al., 2003) and the annual consumption of endosulfan is about 4, 200 metric tons (Sinha et al., 2001). Endosulfan is severely restricted in countries such as Japan, Korea and Taiwan (EJF, 2002). Because of its widespread application, the accumulation of endosulfan has been reported in various food crops (Arrebola et al., 1999; Antonious et al., 1998; Novak and Ahmad, 1989; Gunderson, 1995), environment (Gamón et al., 2003; Bhattacharya et al., 2003; Louie and Sin, 2003; Tan

and Vijayaletchumy, 1994) and animal tissues (Gupta, 1978) as well as in humans (Burke et al., 2003; Chan et al., 2004; Younglai et al., 2002).

Endosulfan is a mixture of two stereoisomers: α - and β - endosulfan (Hayes and Laws, 1991) in the ratio of 70:30. Undiluted endosulfan is slowly and incompletely absorbed in the digestive tract of warm – blooded animals but absorption is enhanced in the presence of alcohols, oils and emulsifiers (Maeir – Bode, 1968). When given to rats by various routes, endosulfan is rapidly metabolized and excreted in the urine and feces as oxidation products such as endosulfan sulfate, diol, hydroxyether or ketone resulting from the cleavage of the cyclic sulfite group. The ratio of the different compounds in the excreta differs with routes of exposure and isomers (FAO / WHO, 1969). The LD₅₀ of endosulfan for rats, mice, dogs, guinea pigs and rabbits varies markedly depending upon the route, strain, vehicle and the sex used (McGregor, 1998; WHO, 1984; Gupta 1979).

Studies on the acute and subacute toxicity in rats, mice, rabbits and other species (Gupta, 1979; Gupta and Chandra, 1977; Naqvi and Vaishnavi, 1993; Singh and Pandey, 1989), its regional distribution in the brain (Gupta, 1978) and neurotoxic effects (Agrawal et al., 1983; Castillo et al., 2002; Dikshith et al., 1984) in rats have been documented. Repeated administration of endosulfan increased the liver weight and produced biochemical effects in the liver (Tyagi et al., 1984). Reduction in spermatid count in testis and sperm count in cauda epididymis as well as decrease in the weights of testis, epididymis and seminal vesicle were recorded when rats were given repeated administration of endosulfan (Dalsenter et al., 2003; Sinha et al., 2001).

Data based on these studies indicate that the liver, kidneys, brain and testes are the main target organs (ATSDR, 2000).

A pharmacokinetic profile for female rabbits (Gupta and Ehrnebo, 1979) following intravenous administration of racemic endosulfan showed pronounced differences between α – and β – isomers and these dissimilarities may partly explain reported differences in toxicity between the two isomers. However, it is uncertain that the differences in disposition are in the same direction in rats as in rabbits.

3.2 Objective of the study

The present study was intended to establish the basic toxicokinetic parameters of endosulfan in male Sprague – Dawley rats following oral administration of ^{14}C – Endosulfan at a dose of 5 mg / kg.

3.3 Materials and methods

3.3.1 Chemicals

Radiolabelled endosulfan (2, 3 – ^{14}C – Endosulfan) with a molar activity of 20.88 mCi / mmole; specific activity of 1.895 MBq / mg and a radiochemical purity of > 95% by Radio TLC was obtained from the Institute of Isotopes Co., Ltd. (Budapest, Hungary). All chemicals used in the present study were of reagent grade. Olive oil, 30 % hydrogen peroxide (H_2O_2), 2 – propanol and heparin sodium salt were supplied by Nacalai Tesque, Inc. (Kyoto, Japan). All solvents and chemicals used in the experiment such as ethanol, diethyl ether, sodium chloride and phosphate buffered saline powder were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). The tissue solubilizer (Soluene[®] – 350), Hionic – Fluor[™] and Ultima Gold

used for liquid scintillating counting were obtained from Packard Bioscience B. V. (Groningen, The Netherlands).

3.3.2 Animals and general conditions

Thirty - seven male Sprague Dawley rats, weighing 92 – 110 g were bought from Shimizu Laboratory Supplies (Kyoto, Japan) on post natal day (PND) 21 and housed in three per cage in plastic cages (20 x 25 x 47 cm) containing shredded wood chips as bedding and roofed with stainless wire covers. Animals were fed pelleted rodent chow supplied by MS Oriental Yeast (Tokyo, Japan) and water *ad libitum*, in a room with a cycle of 12 hours light, 12 hours dark, temperature of 23 °C and a relative humidity of 55 %. The animals were allowed to acclimatize to their new surrounding for one week prior the actual treatment started on PND 28. The use of animals in this study was in accordance with the Guideline for Animal Experiments of Kyoto University.

3.3.3 Preparation of endosulfan dosage

Purchased ^{14}C – Endosulfan was dissolved in ethanol at the concentration of 10 mg / mL. A 5 mg ^{14}C – Endosulfan / kg body weight dosing solution was prepared by adding olive oil to the 10 mg / mL stock solution in order to obtain a final concentration of 1 mg / mL ^{14}C – Endosulfan.

3.3.4 Rationale for dose selection

The dose was selected based on the LD₅₀ of 7 - 121 mg / kg depending upon species, sex, formulation tested, vehicle used and nutritional status of the animal established by WHO (1984). At a dose of more than 7 mg / kg body weight; i.e. 7.5 mg / kg

body weight administered for 60 days (Ansari et al., 1984) and 10 mg / kg body weight administered for 15 days (Gupta, 1978; Gupta and Chandra, 1977), mortality was observed. In order to maintain the number of animals in each group and to detect minimal residue levels in various organs or tissues which were believed to be low, administration dose was set at 5 mg / kg body weight.

3.3.5 Experimental design and administration of ^{14}C – Endosulfan

(1) Single administration - Blood time course and distribution of ^{14}C – Endosulfan following single oral administration of 5 mg ^{14}C – Endosulfan / kg body weight

In the single dose experiment, eighteen animals were used. The animals were arbitrarily divided into six groups ($n = 3$); (i) Group I served as the control and was given olive oil only; and (ii) Group II – VI were given ^{14}C – Endosulfan once by oral gavage at a dose of 5 mg / kg body weight and sacrificed at intervals of 30 min, 1, 2, 4 and 8 h following administration. Each animal was weighed prior to treatment and the dosage was adjusted for body weight. Treatments were administered by oral gavage, using an 18 - gauge gavage needle (1 inch length, with a 2.25 mm ball) and a 2.5 mL glass syringe. The compound was administered in 0.5 mL per 100 g body weight.

(2) Excretion analysis following single oral administration of 5 mg ^{14}C – Endosulfan / kg body weight

Three animals ($n = 3$) were given ^{14}C – Endosulfan once by oral gavage at a dose of 5 mg / kg body weight. Immediately following dosing, each rat was housed

individually in metabolic cages to collect for urine and feces. Urine and feces were collected every 24 hour until 96 h.

(3) Repeated administration - Blood time course and distribution of ^{14}C - Endosulfan following three - time repeated oral administration of 5 mg ^{14}C - Endosulfan / kg body weight

In the repeated dose experiment, thirteen animals were used. The animals were randomly assigned to five groups with three animals in each group ($n = 3$) except for Group I, where only one animal ($n = 1$) was used. (i) Group I was given ^{14}C - Endosulfan once by oral gavage at a dose of 5 mg / kg body weight and sacrificed 2 hour following administration; (ii) Group II was given ^{14}C - Endosulfan once and sacrificed 3 hour following administration; (iii) Group III was given ^{14}C - Endosulfan twice at an interval of 3 hour per dose and sacrificed 2 hour following the last administration; (iv) Group IV was given ^{14}C - Endosulfan thrice at an interval of 3 hour per dose and sacrificed 2 hour following the last administration; and (v) Group V was given ^{14}C - Endosulfan thrice at an interval of 3 hour per dose and sacrificed 25 hour following the last administration.

3.3.6 Necropsy

Rats were lightly anesthetized with diethyl ether prior necropsy. At necropsy, tissue samples such as liver, kidneys, fat, gastrointestinal (GI) tract consists of large intestine, small intestine, stomach and cecum, muscle, brain, heart, lung, spleen, testis and thyroid gland were removed and blood was collected at the vena cava immediately at each interval (30 min, 1, 2, 4 and 8 h) for radioactivity analysis for the single dose experiment whereas blood was collected at the intervals of 2 h, 3 h, 5 h, 8

h and 25 h for the three – time repeated dose experiment. The remaining blood samples were collected in a 5 mL test tube and centrifuged at 3000 rpm for 10 minutes to obtain serum. The serum supernatant was aspirated out and stored in new test tubes at – 80 °C until further analysis. The contents of the GI tract were separated from each of its tissues for the single dose experiment.

3.3.7 Measurement of radioactivity

Immediately upon necropsy tissue samples were minced, aliquots of blood, serum, feces and contents from the GI tract were dissolved in Soluene[®] - 350. The solubilized blood, feces and contents samples from the GI tract were mixed with isopropyl alcohol (1 / 1 v / v) and decolorized with 30 % (v / v) H₂O₂ before mixing with Hionic – Fluor[™] where as the solubilized tissues samples were mixed with Hionic – Fluor[™] directly and counted for radioactivity to determine the total concentration with liquid scintillation counter (LSC 6100, Aloka, Tokyo, Japan). Aliquots of urine were mixed with Ultima Gold directly and counted for radioactivity. The quenching correction was made automatically by external standardization. Radioactivity was represented as weight equivalent to Endosulfan {ex. mg Endosulfan (ES) eq.} based on the specific activity of ¹⁴C – Endosulfan.

3.3.8 Toxicokinetic parameters

Toxicokinetic parameters were determined from the individual blood ¹⁴C – Endosulfan – derived radioactivity concentration – time curve from the same dose (5 mg / kg) of orally administered ¹⁴C – Endosulfan. The area under the blood ¹⁴C – Endosulfan – derived radioactivity concentration – time curve (AUC_(0 – 8 h)) was calculated by the linear trapezoidal method.

The blood concentration – time curve for ^{14}C – Endosulfan after oral dosing was fitted to a two – compartment first – order input, first – order elimination model using the nonlinear regression analysis program $^{\circ}\text{SPSS}$ for Windows software package (Release 10.0, SPSS, Inc) to obtain estimates for the absorption rate constant (k_a), distribution and elimination half – lives ($T_{1/2x}$, $T_{1/2y}$), central – peripheral transfer rate (k_{12}), peripheral – central return rate (k_{21}) and elimination rate constant (k_{10}).

The two – compartment model system is illustrated in Figure 3.2. The following equation was used for the two – compartment model system (Takada, 1996):

$$C_t = -H e^{-k_a t} + I e^{-x t} + J e^{-y t} \quad (3-1)$$

where C_t is the concentration of ^{14}C – Endosulfan in the central compartment at time t , H and k_a represent the absorption phase, I and x represent the distribution phase, while J and y represent the terminal phase. H , I and J are the ordinate axis intercepts. H , I , J , k_a , x and y are predicted by the nonlinear regression analysis. The individual rate constants of the model: k_{12} , k_{21} and k_{10} were calculated from H , I , J , x (rapid rate constant of the central compartment) and y (terminal elimination rate constant) parameters as detailed in Section 3.3.9.

The half – lives for the distribution and elimination phases ($T_{1/2x}$ and $T_{1/2y}$) were calculated using the formulas:

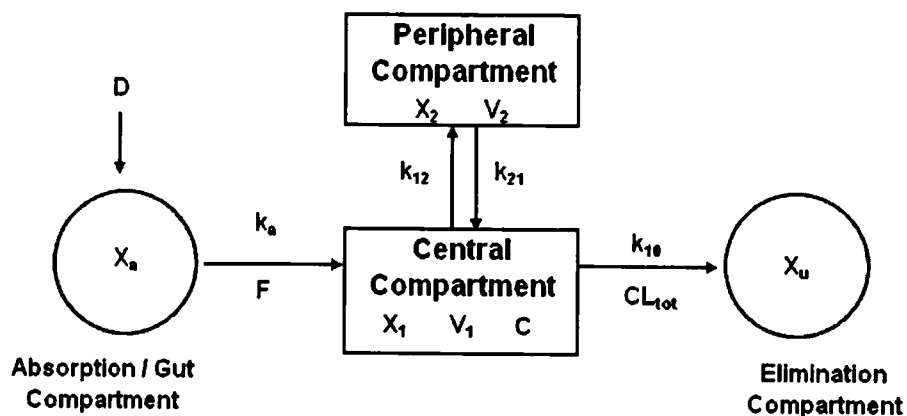
$$T_{1/2x} = \ln 2 / x \quad (3-2)$$

$$T_{1/2y} = \ln 2 / y \quad (3-3)$$

Total clearance (Cl_{tot}) and apparent volume of distribution area (V_d) were determined by the following equations:

$$Cl_{tot} = D / AUC_{blood} \quad (3-4)$$

$$V_d = D / y (AUC_{blood}) \quad (3-5) \quad \text{where } D \text{ is the dose (mg } ^{14}\text{C – Endosulfan / kg)}$$



D : Dose of the chemical (mg)

X_a : Amount of chemical absorbed in the gut (mg)

F : Oral bioavailability (%)

k_a : Absorption rate constant (hr^{-1})

k_{12} : Central – peripheral transfer rate (hr^{-1})

k_{21} : Peripheral – central return rate (hr^{-1})

k_{10} : Elimination rate constant (hr^{-1})

X_1 : Amount of chemical in the central compartment (mg)

X_2 : Amount of chemical in the peripheral compartment (mg)

X_u : Amount of chemical eliminated (mg)

V_1 : Volume of distribution in the central compartment (L)

V_2 : Volume of distribution in the peripheral compartment (L)

C : Concentration of chemical in the blood (mg / L)

CL_{tot} : Total clearance (mL / min)

Figure 3.2. Two – compartment model system.

3.3.9 Calculation of the Laplace Transforms

The differential system connected with this model is:

$$\frac{dX_a}{dt} = -k_a \cdot X_a \quad (3-6)$$

$$\frac{dX_1}{dt} = k_a \cdot X_a - (k_{12} + k_{10}) \cdot X_1 + k_{21} \cdot X_2 \quad (3-7)$$

$$\frac{dX_2}{dt} = k_{12} \cdot X_1 - k_{21} \cdot X_2 \quad (3-8)$$

By applying the Laplace Transforms, the time domain of the rate expression is replaced with the complex domain of the Laplace operator s .

When $X_a = F \cdot D$; $X_1 = X_2 = 0$; then;

$$s \cdot x_a - F \cdot D = -k_a \cdot x_a \quad (3-9)$$

$$s \cdot x_1 = k_a \cdot x_a - (k_{12} + k_{10}) \cdot x_1 + k_{21} \cdot x_2 \quad (3-10)$$

$$s \cdot x_2 = k_{12} \cdot x_1 - k_{21} \cdot x_2 \quad (3-11)$$

$$\text{Rearrange,} \quad (s + k_a) \cdot x_a = F \cdot D \quad (3-12)$$

$$-k_a \cdot x_a + (s + k_{12} + k_{10}) \cdot x_1 - k_{21} \cdot x_2 = 0 \quad (3-13)$$

$$-k_{12} \cdot x_1 + (s + k_{21}) \cdot x_2 = 0 \quad (3-14)$$

With

$$\Delta = \begin{vmatrix} s + k_a & 0 & 0 \\ -k_a & s + k_{12} + k_{10} & -k_{21} \\ 0 & -k_{12} & s + k_{21} \end{vmatrix} \quad (3-15)$$

$$= (s + k_a) \cdot (s + k_{12} + k_{10}) \cdot (s + k_{21}) - k_{12} \cdot k_{21}(s + k_a) \quad (3-16)$$

$$= (s + k_a) \cdot \{s^2 + k_{12} + k_{10} + k_{21}\} \cdot s + k_{10} \cdot k_{21} \quad (3-17)$$

We denote by α and β the two roots of $\Delta = 0$, that is

$$\Delta = (s + k_a) \cdot (s + \alpha) \cdot (s + \beta) \quad (3-18)$$

However, $\alpha + \beta = k_{12} + k_{10} + k_{21}$ (3-19)

$$\alpha \cdot \beta = k_{10} \cdot k_{21} \quad (3-20)$$

By using Cramer's Rule, equations (3-12) to (3-13) will be;

$$x_1 = \frac{\begin{vmatrix} s + k_a & F \cdot D & 0 \\ -k_a & 0 & -k_{21} \\ 0 & 0 & s + k_{21} \end{vmatrix}}{\Delta} \quad (3-21)$$

$$x_1 = \frac{F \cdot D \cdot k_a \cdot (s + k_{21})}{\Delta} \quad (3-22)$$

From Benet's Rule; $x_1 = \frac{P(s)}{Q(s)} = \frac{F \cdot D \cdot k_a \cdot (s + k_{21})}{(s + k_a) \cdot (s + \alpha) \cdot (s + \beta)}$ (3-23)

When $Q(s) = 0$, $-k_a$, $-\alpha$ and $-\beta$;

$$L^{-1} = \frac{P(s)}{Q(s)} = \frac{P_1(-k_a) \cdot \exp(-k_a \cdot t)}{Q_1(-k_a)} + \frac{P_2(-\alpha) \cdot \exp(-\alpha \cdot t)}{Q_2(-\alpha)} + \frac{P_3(-\beta) \cdot \exp(-\beta \cdot t)}{Q_3(-\beta)} \quad (3-24)$$

With, $Q_1(-k_a) = (\alpha - k_a) \cdot (\beta - k_a)$ (3-25)

$$Q_2(-\alpha) = (k_a - \alpha) \cdot (\beta - \alpha) \quad (3-26)$$

$$Q_3(-\beta) = (k_a - \beta) \cdot (\alpha - \beta) \quad (3-27)$$

$$P_1(-k_a) = F \cdot D \cdot k_a \cdot (k_{21} - k_a) \quad (3-28)$$

$$P_2(-\alpha) = F \cdot D \cdot k_a \cdot (k_{21} - \alpha) \quad (3-29)$$

$$P_3(-\beta) = F \cdot D \cdot k_a \cdot (k_{21} - \beta) \quad (3-30)$$

From equations (3-25) to (3-30), substitute into equation (3-23), the analytical expression of concentration in compartment 1 (central compartment) can be obtained;

$$\begin{aligned}
 L^{-1} = \left(\frac{P(s)}{Q(s)} \right) = X_1 = & -\frac{F \cdot D \cdot k_a \cdot (k_a - k_{21})}{(\alpha - k_a) \cdot (\beta - k_a)} \cdot \exp(-k_a \cdot t) \\
 & + \frac{F \cdot D \cdot k_a \cdot (k_{21} - \alpha)}{(k_a - \alpha) \cdot (\beta - \alpha)} \cdot \exp(-\alpha \cdot t) \\
 & + \frac{F \cdot D \cdot k_a \cdot (k_{21} - \beta)}{(k_a - \beta) \cdot (\alpha - \beta)} \cdot \exp(-\beta \cdot t)
 \end{aligned} \tag{3-31}$$

To calculate the concentration of chemical in the blood, C, by using the relationship of $X_1 = C \cdot V_1$;

$$\begin{aligned}
 C = & -\frac{F \cdot D \cdot k_a \cdot (k_a - k_{21})}{V_1(\alpha - k_a) \cdot (\beta - k_a)} \cdot \exp(-k_a \cdot t) \\
 & + \frac{F \cdot D \cdot k_a \cdot (k_{21} - \alpha)}{V_1(k_a - \alpha) \cdot (\beta - \alpha)} \cdot \exp(-\alpha \cdot t) \\
 & + \frac{F \cdot D \cdot k_a \cdot (k_{21} - \beta)}{V_1(k_a - \beta) \cdot (\alpha - \beta)} \cdot \exp(-\beta \cdot t)
 \end{aligned} \tag{3-32}$$

Therefore, in equation (3-1);

$$H = \frac{F \cdot D \cdot k_a \cdot (k_a - k_{21})}{V_1(\alpha - k_a) \cdot (\beta - k_a)} \cdot \exp(-k_a \cdot t) \tag{3-33}$$

$$I = \frac{F \cdot D \cdot k_a \cdot (k_{21} - \alpha)}{V_1(k_a - \alpha) \cdot (\beta - \alpha)} \cdot \exp(-\alpha \cdot t) \tag{3-34}$$

$$J = \frac{F \cdot D \cdot k_a \cdot (k_{21} - \beta)}{V_1(k_a - \beta) \cdot (\alpha - \beta)} \cdot \exp(-\beta \cdot t) \tag{3-35}$$

By integrating equation (3-1);

$$\int_0^{\infty} C dt = -H \cdot \int_0^{\infty} \exp(-k_a \cdot t) dt + I \cdot \int_0^{\infty} \exp(-\alpha \cdot t) + J \cdot \int_0^{\infty} \exp(-\beta \cdot t) \quad (3-36)$$

$$= -\frac{H}{k_a} + \frac{I}{\alpha} + \frac{J}{\beta} \quad (3-37)$$

$$\int_0^{\infty} C dt = \frac{F \cdot D \cdot k_{21}}{V_1 \cdot \alpha \cdot \beta} \quad (3-38)$$

From equation (3-19);

$$\int_0^{\infty} C dt = \frac{F \cdot D \cdot k_{21}}{V_1 \cdot k_{10}} \quad (3-39)$$

By rearranging the form of equation (3-38);

$$\frac{V_1 \cdot k_{10}}{F} = \frac{D}{\int_0^{\infty} C dt} \quad (3-40)$$

By substituting equation (3-37);

$$\frac{V_1 \cdot k_{10}}{F} = \frac{D}{-\frac{H}{k_a} + \frac{I}{\alpha} + \frac{J}{\beta}} \quad (3-41)$$

Let's say $P = -\frac{H}{k_a} = \frac{F \cdot D}{V_1} \left(\frac{k_{21} - k_a}{(\alpha - k_a) \cdot (\beta - k_a)} \right)$ (3-42)

$$Q = \frac{I}{k_a} = \frac{F \cdot D}{V_1} \left(\frac{k_{21} - \alpha}{(k_a - \alpha) \cdot (\beta - \alpha)} \right) \quad (3-43)$$

$$R = \frac{J}{k_a} = \frac{F \cdot D}{V_1} \left(\frac{k_{21} - \beta}{(k_a - \beta) \cdot (\alpha - \beta)} \right) \quad (3-44)$$

and $\frac{F \cdot D}{V_1} = Q \cdot (k_a - \alpha) + R \cdot (k_a - \beta)$ (3-45)

By rearranging the form of equation (3-45);

$$V_1 = \frac{F \cdot D}{Q \cdot (k_a - \alpha) + R \cdot (k_a - \beta)} \quad (3-46)$$

From equation (3-41), substitute $-H$, I and J with $k_a \cdot P$, $k_a \cdot Q$ and $k_a \cdot R$;

$$\frac{V_1 \cdot k_{10}}{F} = \frac{D}{P + \frac{k_a \cdot Q}{\alpha} + \frac{k_a \cdot R}{\beta}} \quad (3-47)$$

By using the equation (3-20); $\frac{1}{k_{10}} = \frac{k_{21}}{\alpha \cdot \beta}$

$$k_{21} = \frac{\beta \cdot k_a \cdot Q + \alpha \cdot k_a \cdot R + \alpha \cdot \beta \cdot P}{Q \cdot (k_a - \alpha) + R \cdot (k_a - \beta)} \quad (3-48)$$

After obtaining k_{21} , k_{10} and k_{12} can be calculated.

$$k_{10} = \frac{\alpha \cdot \beta}{k_{21}} \quad (3-49)$$

$$k_{12} = \alpha + \beta - (k_{21} + k_{10}) \quad (3-50)$$

3.4 Results

3.4.1 General condition of animals

None of the rats showed any sign of toxicity through the period of observation following single and three – time repeated oral administration of 5 mg / kg ^{14}C – Endosulfan.

3.4.2 Disposition of ^{14}C – Endosulfan (5 mg / kg): Excretion routes

Figure 3.3 show the cumulative percentage of urinary and fecal elimination after single oral administration of 5 mg / kg ^{14}C – Endosulfan. Following single oral administration of 5 mg / kg ^{14}C – Endosulfan to male rats, the urinary excretion of the radioactivity during 24 h was 9.6 ± 5.0 % of the total administered dose. The cumulative excretion in the urine for four days was 12.4 ± 4.8 %. Fecal elimination of ^{14}C – Endosulfan – derived radioactivity represented the major elimination route in male rats. The fecal excretion of the radioactivity during four days was 94.4 ± 21.4 %. The total radioactivity recovered in the excreta for four days was 106.8 ± 26.2 %. Over four days, rats excreted more radioactivity in feces than in urine. Generally the standard deviation (SD) for urinary elimination was below 10 % for each time point, which was considered excellent. The SD for fecal elimination at each time point was above 20 %; however it was consistent at each time point. The total elimination of endosulfan recovered in the excreta, which exceeded 100 % could be attributed to experimental error.

3.4.3 ^{14}C – Endosulfan residues in tissues and contents of the GI tract

Table 3.1 shows the residue levels of ^{14}C – Endosulfan in various tissues of rats after single oral administration of 5 mg / kg ^{14}C – Endosulfan. The relative amounts of

radioactivity found 8 h after administration were in the following sequence (mg ES eq. / L): GI Tract (20.28) > liver (5.52) > fat (3.61) > thyroid gland (2.50) > kidneys (1.83) > lung (1.31) > serum (0.75) > heart (0.42) > spleen (0.37) > blood (0.28) > brain (0.27) > testes (0.25) > muscle (0.18).

The relative amounts of radioactivity found 8 h after administration for the contents of each tissue from the GI tract were (mg ES eq. / L): Cecum (25.84) > large intestine (6.76) > small intestine (6.45) > stomach (3.57).

Table 3.2 shows the residue levels of ^{14}C – Endosulfan in various tissues of rats after three – time repeated oral administration of 5 mg / kg ^{14}C – Endosulfan. It was observed that the radioactivity levels in all tissues on the 25th hour decreased after administration was terminated.

3.4.4 ^{14}C – Endosulfan toxicokinetics in blood

The log concentration – time curve of ^{14}C – Endosulfan – derived blood radioactivity concentration over 8 h oral dose at 5 mg ^{14}C – Endosulfan / kg is shown in Figure 3.4. Toxicokinetic parameters estimated from the blood – time course data for oral administration are summarized in Table 3.3. Following single oral administration of 5 mg ^{14}C – Endosulfan / kg to rats, the ^{14}C – Endosulfan was rapidly absorbed through GI tract with absorption rate constant (k_a) of 3.07 h^{-1} and peak blood concentration (C_{max}) of $0.36 \pm 0.08\text{ mg – eq. / L}$ with time to C_{max} (T_{max}) of 2 h. The terminal elimination half life ($T_{1/2\gamma}$) of the radioactivity in blood was 193 h.

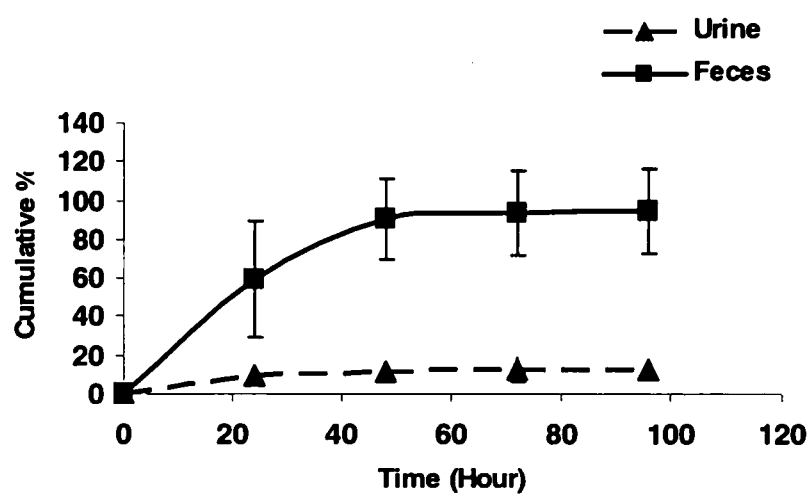


Figure 3.3. Cumulative percentage of urinary and fecal elimination of ^{14}C – Endosulfan in male rats after single oral administration of 5 mg / kg ^{14}C – Endosulfan (Means $\pm$ SD; for $n = 3$ at each time point).

Table 3.1. The concentrations of ^{14}C – Endosulfan – derived radioactivity in various tissues of male rats following single oral administration of ^{14}C – Endosulfan at 5 mg / kg (Means $\pm$ SD; for $n = 3$ for each time point).

Organ	Radioactivity concentration (mg - Endosulfan - eq. / L)				
	Post dosing time				
	30 min	1 h	2 h	4 h	8 h
Blood	0.09 $\pm$ 0.07	0.33 $\pm$ 0.12	0.36 $\pm$ 0.08	0.30 $\pm$ 0.09	0.28 $\pm$ 0.09
Serum	0.22 $\pm$ 0.04	0.43 $\pm$ 0.09	1.36 $\pm$ 0.31	1.46 $\pm$ 0.30	0.75 $\pm$ 0.34
Liver	3.94 $\pm$ 0.71	4.56 $\pm$ 0.74	10.79 $\pm$ 0.53	13.69 $\pm$ 2.69	5.52 $\pm$ 4.75
Kidneys	0.23 $\pm$ 0.05	2.35 $\pm$ 2.85	1.61 $\pm$ 0.41	8.00 $\pm$ 3.06	1.83 $\pm$ 1.78
Stomach ^a	12.67 $\pm$ 12.13	16.91 $\pm$ 18.34	5.81 $\pm$ 1.15	7.29 $\pm$ 5.28	3.04 $\pm$ 0.49
Large intestine ^a	1.35 $\pm$ 0.90	2.04 $\pm$ 2.96	1.14 $\pm$ 0.62	3.03 $\pm$ 1.24	2.27 $\pm$ 1.02
Small intestine ^a	5.46 $\pm$ 2.48	10.00 $\pm$ 2.11	12.03 $\pm$ 1.43	12.12 $\pm$ 5.39	4.74 $\pm$ 3.38
Cecum ^a	0.83 $\pm$ 0.27	0.50 $\pm$ 0.33	0.82 $\pm$ 0.39	1.30 $\pm$ 0.31	10.23 $\pm$ 11.46
GI Tract ^b	20.31 $\pm$ 15.78	29.45 $\pm$ 23.74	19.79 $\pm$ 3.59	23.73 $\pm$ 12.21	20.28 $\pm$ 16.35
Spleen	4.48 $\pm$ 5.30	0.47 $\pm$ 0.62	0.57 $\pm$ 0.16	0.47 $\pm$ 0.07	0.37 $\pm$ 0.11
Heart	0.10 $\pm$ 0.05	0.19 $\pm$ 0.03	0.58 $\pm$ 0.06	0.66 $\pm$ 0.15	0.42 $\pm$ 0.14
Lung	0.14 $\pm$ 0.05	1.08 $\pm$ 0.22	0.93 $\pm$ 0.93	0.88 $\pm$ 0.14	1.31 $\pm$ 0.25

Continue.....

Organ	Radioactivity concentration (mg - Endosulfan - eq. / L)				
	Post dosing time				
	30 min	1 h	2 h	4 h	8 h
Brain	0.11 ± 0.05	0.14 ± 0.01	0.61 ± 0.06	0.41 ± 0.16	0.27 ± 0.10
Thyroid gland	6.50 ± 10.60	0.30 ± 0.07	2.62 ± 0.94	1.53 ± 0.66	2.50 ± 1.15
Muscle	0.04 ± 0.02	0.05 ± 0.01	0.26 ± 0.01	0.29 ± 0.10	0.18 ± 0.06
Testes	0.03 ± 0.01	0.06 ± 0.02	0.95 ± 0.16	0.33 ± 0.04	0.25 ± 0.07
Fat	2.17 ± 1.36	1.43 ± 0.42	4.44 ± 1.66	4.23 ± 0.93	3.61 ± 1.56
Contents of each tissue from GI tract					
Stomach	6.95 ± 2.01	25.71 ± 20.06	11.86 ± 2.31	10.37 ± 2.75	3.57 ± 3.48
Large intestine	4.71 ± 2.61	3.99 ± 5.37	5.10 ± 4.42	28.28 ± 17.73	6.76 ± 0.81
Small intestine	3.85 ± 2.01	3.03 ± 3.73	4.19 ± 3.62	24.55 ± 12.83	6.45 ± 0.33
Cecum	0.02 ± 0.02	0.03 ± 0.04	7.53 ± 13.02	1.77 ± 1.81	25.84 ± 26.04

Note.

a : The radioactivities reported for the stomach, large intestine, small intestine and cecum were counted without contents.

b : Sum of radioactivities in the stomach, large intestine, small intestine and cecum without contents.

Table 3.2. The concentrations of ^{14}C – Endosulfan – derived radioactivity in various tissues of male rats following three – time repeated oral administration of ^{14}C – Endosulfan at 5 mg / kg (Means $\pm$ SD; for $n = 3$ for each time point except for the first time point [2 h] where $n = 1$).

Organ	Radioactivity concentrtrion (mg - Endosulfan - eq. / L)				
	Post dosing time				
	2 h	3 h	5 h	8 h	25 h
Blood	0.22	0.53 ± 0.32	1.10 ± 0.75	0.93 ± 0.14	0.56 ± 0.25
Serum	1.08	1.55 ± 0.71	2.60 ± 0.25	3.48 ± 0.71	2.57 ± 0.35
Liver	6.07	7.01 ± 1.54	14.19 ± 0.97	18.81 ± 2.98	10.29 ± 0.66
Kidney	2.16	3.28 ± 0.17	5.77 ± 0.33	12.23 ± 3.97	8.33 ± 0.62
Brain	0.30	0.56 ± 0.13	1.26 ± 0.03	1.58 ± 0.28	0.37 ± 0.04
Thyroid gland	0.39	0.77 ± 0.29	2.37 ± 1.73	1.88 ± 0.41	1.12 ± 0.15
Muscle	0.10	0.15 ± 0.03	0.26 ± 0.02	0.42 ± 0.02	0.35 ± 0.04
Testes	0.57	0.66 ± 0.54	1.13 ± 0.77	2.04 ± 0.46	1.56 ± 0.37
Fat	1.31	1.76 ± 0.78	4.86 ± 2.28	5.75 ± 0.88	0.62 ± 0.18

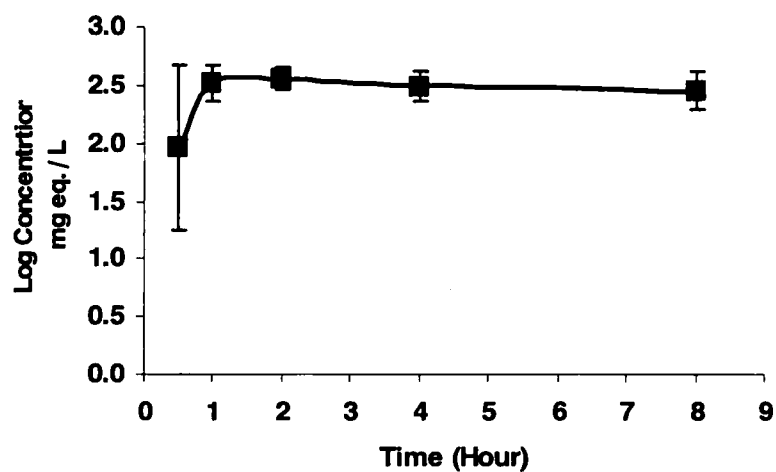


Figure 3.4. Plot of the log concentration – time course of ^{14}C – Endosulfan in blood of male rats following single oral administration of 5 mg / kg ^{14}C – Endosulfan (Means $\pm$ SD; for $n = 3$ at each time point).

Table 3.3. Toxicokinetic parameters of ^{14}C – Endosulfan – derived radioactivity in blood of male rats following single oral administration of ^{14}C – Endosulfan at 5 mg / kg.

Definitions	Parameters	Dose route (Oral)	Source
Time to $C_{\max}$	$T_{\max}$ (h)	2.0	Experiment
Peak blood concentrations	$C_{\max}$ (mg – eq. / L)	0.36 ± 0.08	Experiment
Half - life in the distribution phase	$T_{1/2\alpha}$ (h)	0.52	Calculated
Half - life in the elimination phase	$T_{1/2\beta}$ (h)	193	Calculated
Absorption rate constant	k_a (h^{-1})	3.07	Predicted
Central – peripheral transfer rate	k_{12} (h^{-1})	0.40	Calculated
Peripheral – central return rate	k_{21} (h^{-1})	0.94	Calculated
Elimination rate constant	k_{10} (h^{-1})	0.005	Calculated
Apparent volume of distribution area	V_d (L / kg)	605	Calculated
Total body clearance	Cl_{tot} (L / h / kg)	2.18	Calculated
Area under curve	$AUC_{(0-8\text{ h})}$ (mg – eq. h / L)	2.30	Calculated
Area under the moment curve	$AUMC_{(0-8\text{ h})}$ (mg – eq. h^2 / L)	27.72	Calculated
Mean residence time	MRT (h)	12.07	Calculated

3.5 Discussion

Following oral administration of 2 mg / kg of ^{14}C - Endosulfan to male Wistar rats, 90 % of the oral dose was eliminated in the urine and feces within seven days; elimination was essentially complete within the 1 - 2 days (McGregor, 1998). Female rats treated with a single dose (2 mg / kg) of α - or β - [^{14}C] - Endosulfan eliminated 88 % of the α - endosulfan and 87 % of the β - endosulfan within 5 days of oral administration (Dorough et al., 1978). They also suggested that the biliary metabolites of endosulfan would not enter the enterohepatic cycle and hence were voided in the feces. In metabolic studies using BALB / c mice fed with single dose of 0.3 mg ^{14}C - Endosulfan, approximately 65 % of ^{14}C - Endosulfan was recovered in the excreta and tissues of mice 24 h later after ingesting endosulfan in their diet (Deema et al., 1966). These observations were consistent with our results that most of the radiocarbons were excreted in the urine (12.4 ± 4.8 %) and feces (94.4 ± 21.4 %) after 96 h. Elimination was essentially complete after 48 h (101.6 ± 25.1 %). Our data also suggested that endosulfan would not enter enterohepatic recirculation as shown by the radioactivity concentrations in the contents of each tissue from the GI tract (Table 3.1). The present study of disposition of endosulfan in rats showed that the principal excretion route was the feces in rats.

The tissue concentrations of residues after 8 h as observed in the present study were generally highest in the liver and kidneys and lower in other tissues such as brain, testes and muscle; thus suggesting that these tissues do not appear to bioaccumulate. High radioactivity which was found in the liver may indicate that liver is the site of high metabolic activity (Khanna et al., 1979).

The distribution pattern of α - and β - endosulfan in rats after administration of a mixture varies depending on the tissues (Ansari et al., 1984; Nath et al., 1978). The regional differences in the quantity of fat content may also partly account for the differences in the distribution of endosulfan in various organs (Ansari et al., 1984). Ansari and co – workers (1984) indicated a differential ability to accumulate the two isomers of endosulfan which may help to explain the difference in the toxic potential of α - and β - isomers. Accumulation of endosulfan was greater in kidney than liver as kidney is one of the target organs for toxicity (FAO / WHO, 1999). A rapid biodegradation in the liver may be one of the causes of low concentration in this organ since it is the main site of detoxification, however the possibility of other factors cannot be ruled out as suggested by Ansari and co – workers (1984).

The tissue concentration of residues in all tissues on the 25th hour decreased after the administration was terminated as observed in the present study. This suggests that endosulfan does not appear to bioaccumulate and rats will recover once administration is terminated. When technical endosulfan (mixture of α - and β - isomers) was fed orally to male rats at 5 mg / kg for 15 days, maximum residues of endosulfan, which occurred in the kidney and liver, were 1.46 $\mu\text{g} / \text{g}$ and 0.09 $\mu\text{g} / \text{g}$ respectively while only 0.05 $\mu\text{g} / \text{g}$ was detected in the liver 15 days after the last administration (Chan and Mustafa, 2004). Similar distribution pattern was also observed when technical endosulfan was administered orally to male rats at 10 mg / kg for 15 days. 2.26 $\mu\text{g} / \text{g}$ and 0.09 $\mu\text{g} / \text{g}$ of endosulfan residues were detected in the kidney and liver respectively, whereas only 0.06 $\mu\text{g} / \text{g}$ was detected in the liver 15 days after the last administration (Chan and Mustafa, 2004). Deema and co – workers (1966) observed that accumulation in liver (2883 cpm) was higher than kidney (1390

cpm) when BALB / c mice were fed with a single dose of [^{14}C] – Endosulfan after 24 h. Dorrough and co – workers (1978) reported that tissue concentrations of residues were generally highest in the kidneys (3 ppm) and liver (1 ppm) and lower in other tissues (< 1 ppm) when rats were fed endosulfan in the diet at a concentration of 5 ppm for 14 days. At the end of the 14 – day recovery period, residues were confined to the kidneys (0.9 ppm) and to the lesser extent, the liver (0.1 ppm).

The log – concentration – time curve (Figure 3.4) for blood endosulfan after oral administration demonstrates kinetics which can be described by a two – compartment model. It consists of a central compartment and a peripheral compartment. Elimination was allocated into the central compartment. All processes were assumed to underline first – order kinetics. The model delivers an excellent fit to the data with a goodness of fit (R^2) value of 0.999 (Figure 3.4).

The blood concentration reached its maximum (C_{max}) of 0.36 ± 0.08 mg – eq / L at 2 h (T_{max}) after oral dose (Table 3.1). Our observation was comparable when male Wistar rats were administered 2 mg / kg ^{14}C – Endosulfan orally, the blood concentration reached its C_{max} of 0.25 ± 0.06 mg / L at T_{max} of 3 – 8 h (McGregor, 1998). The concentration – time curve is approximated by two exponentials: the first term with a half – life ($T_{1/2\alpha}$) of 31 min describes the decline after reaching the maximum concentration. Then, the slope flattens over time approaching a terminal phase with a half – life ($T_{1/2\beta}$) of 193 h. Both phases are governed by the distribution together with the elimination process. However, McGregor (1998) reported that the half – life in the distribution phase ($T_{1/2\alpha}$) for male Wistar rats was 8 h and the terminal half – life ($T_{1/2\beta}$) was 110 h.

The present model yields half – life of 31 min for the uptake into the central compartment and an elimination rate constant of 0.3 min from this compartment. The obtained results also show that the absorption of endosulfan into the GI tract in rats is rapid with a k_a of 3.07 h^{-1} . The absorption of endosulfan in the present study was estimated to be 46 % on a basis of a comparison of area under the curve (*AUC*) after oral administration of ^{14}C – Endosulfan as compared to 60 – 70 % in male Wistar rats (McGregor, 1998). The rapid distribution of endosulfan to lipid tissues may be responsible for its slow elimination half – life (Abdel – Rahman et al., 2002). Intravenous administration of 2 mg / kg endosulfan (7:3 ratio of α – and β – isomers) in rabbits produced much longer half – life of the terminal slope of the α – isomer (235 h) than for β – endosulfan (5.97 h) (Gupta and Ehrnebo, 1979). Excretion of the two isomers occurred primarily via the urine (29 %) with much less excreted via the feces (2 %). It had been shown that marked differences occurred in the pharmacokinetics, metabolism and excretion between α – and β – isomers in rabbits (Gupta and Ehrnebo, 1979). However, it was unclear whether the differences in disposition were similar between rats and rabbits as the level of radiocarbon was insufficient to attempt identification of separate isomers in the present study.

The variations in the excretion and absorption patterns may be attributable to the differences in exposure routes, species differences or to both. In a draft presented by McGregor (1998), the percentage of elimination of endosulfan in the excreta, absorption efficiency and accumulation in various tissues differ with route of exposure, sex, strain and species (McGregor, 1998).

3.6 Conclusions

The present study indicates that ^{14}C – Endosulfan was rapidly excreted in the feces and urine after 96 h following single oral administration with fecal elimination as the major elimination route in male rats. Elimination was essentially complete within a few days. The present study also indicates that differences in sex, strain, route of exposure may influence the pharmacokinetic parameters for endosulfan when compared to other studies. Further study is necessary to investigate the toxicokinetics and biotransformation of ^{14}C – Endosulfan in rats after oral and intravenous administration. A more reliable and sensitive analytical method would also be incorporated into our future experiments to enhance the separation and identification of the parent compounds and metabolites present in different matrices.

Note.

Some sections in this chapter will be published and available in Environmental Toxicology, Volume 20, Issue 5; October 2005.

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CHAPTER 4

DEVELOPMENT OF A PHYSIOLOGICALLY BASED PHARMACOKINETIC MODEL FOR ENDOSULFAN IN THE MALE SPRAGUE – DAWLEY RATS

4.0 Physiologically based pharmacokinetic (PBPK) model: An overview

The structure of a physiologically based pharmacokinetic model is based to as large an extent as practicable on the actual physiological and biochemical structure of the animal being described. PBPK modeling is generally well – suited to calculation of tissue doses of chemicals and their metabolites over a wide range of exposure conditions in different species (Andersen, 2003).

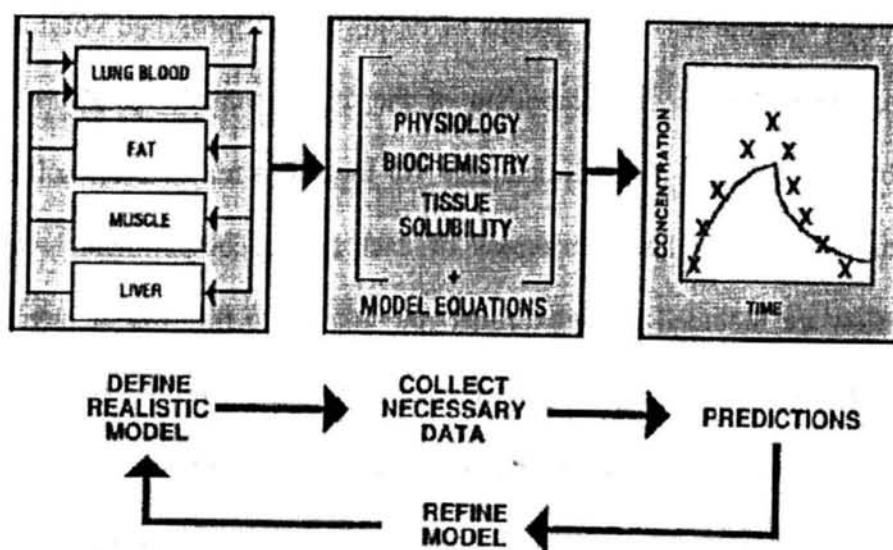


Figure 4.1. Idealized approach of PBPK modeling.

In PBPK modeling, the compartments in the model are developed based on an understanding of the anatomy and physiology of the test animals (left panel). The complexity of any model for a specific compound depends on inclusion of routes of absorption, organs that serve as sites of storage, metabolism and excretion, and

tissues that are the target organs for chemical toxicity. The parameters for metabolism, excretion, tissue partitioning, blood and air flow rates are introduced as constants into the model and kinetic behavior in various compartments – blood, tissues, excreta, etc. Middle panel is predicted by computer simulation. Predictions made from the PBPK models can be tested by comparison with the observed time – course results (right panel). When discrepancies are noted between experiment and model predictions (as often happen), the model can be refined in a hypothesis generation loop and tested against available data (Adapted from Andersen, 2003).

In PBPK models (Figure 4.1), the biological system is envisaged as comprising of a small number of physiologically relevant compartments and each compartment corresponds to discrete tissues or groups of tissues with appropriate volumes, blood flow rates, and pathways of metabolism of the test chemical. In PBPK models, pertinent biochemical and physical constants for metabolism and tissue solubility are incorporated directly in the descriptions of each tissue compartment. Routes of administration are included in their proper relationship to the overall physiological structure and exposure scenario differences are accounted for in the time sequence of the dose input terms. Each compartment (i. e., tissue) in the model is described by a mass – balance differential equation, and the set of equations is solved by numerical integration to predict tissue time – course concentrations of the test chemical and its metabolites (Andersen, 2003).

The steps involved in developing a PBPK model are (Aarons et al., 1999):

- Specification of the model based on the anatomical / physiological structure of the species of interest and the determinants of disposition and biotransformation of the substance concerned;
- Development of a mathematical description of the biological processes involved, including physiological, physicochemical and biochemical constants;
- Design of new experiments to test the robustness of the model description;
- Collection of the necessary experimental data to confirm or refute the model structure;
- Adjustments to refine the model.

PBPK models offer a number of advantages over the compartmental models used previously in classical pharmacokinetic analyses. A physiologically based model can be interpreted in biological terms, and lead to an understanding of the actual pharmacokinetic processes governing chemical disposition in the body. Lack of fit of a particular model may suggest alternative hypotheses about pharmacokinetic processes involved in the distribution and metabolism of chemicals. They can be used to estimate dose – effect data over a wide range of exposure conditions. If they are validated, they can even be used to predict the outcomes of exposure conditions which have not been experimentally tested. Hence, PBPK models can provide a means of extrapolating from the high – dose situation commonly used in laboratory studies to the low – dose condition relevant to environmental exposure. Similarly, it may conceivably be used for extrapolating from acute to repeated exposure scenarios. PBPK models may also offer a powerful tool for predicting target tissue dose from

one route of exposure to another and between species. A PBPK model established on the basis of studies in non – human mammalian species can be applied to humans after substituting the appropriate allometric, biochemical, and pharmacokinetic parameters for humans. Finally, by providing a means of estimating the dose of reactive metabolites reaching target tissues, PBPK models may lead to more accurate predictions of risk than can be obtained using environmental exposure levels, particularly when saturation effects lead to a non – linear relationship between environmental exposures and tissue doses (Leung and Paustenbach, 1995; Moolgavkar et al., 1999).

4.1 Background introduction

Endosulfan (6, 7, 8, 9, 10, 10 – hexachloro – 1, 5, 5a, 6, 9, 9a – hexahydro – 6, 9 – methano – 2, 4, 3 – benzodioxathiepin – 3 – oxide), an organochlorine (OC) insecticide of the cyclodiene group, has widespread use in agriculture and forestry to control a wide variety of insect pests and on non – food crops such as cotton and tobacco. It is also used as wood preservative [Agency for Toxic Substances and Disease Registry (ATSDR), 2000]. Endosulfan is used in India (Saiyed et al., 2003), Turkey (Oktay et al., 2003), Malaysia (Chan et al., 2004), Mexico (Castillo et al., 2002; González – Farias et al., 2004) and many other developing countries.

Residues of endosulfan are found in low levels in the environment such as sediment (González – Farias et al., 2004; Bhattacharya et al., 2003), soil (Gamón et al., 2003), water (Tan and Vijayaletchumy, 1994) and air (Louie and Sin, 2003); in animal tissues (Ansari et al., 1984; Chan and Mustafa, 2005; Dikshith et al., 1984; Nath et al., 1978; National Cancer Institute, 1978) as well as in humans (Aleksandrowicz, 1979;

Blanco – Coronado et al., 1992; Boereboom et al., 1998; Brandt et al., 2001; Chan et al., 2004; Coutselinis et al., 1978; Demeter et al., 1977; Eyer et al., 2004; Lo et al., 1995). The accumulation of endosulfan has also been reported in various food crops such as vegetables (Antonious et al., 1998) and fish (Novak and Ahmad, 1989).

Endosulfan is a mixture of two stereoisomers: α - and β - endosulfan (Hayes and Laws, 1991) in the ratio of 70:30. When given to rats by various routes, endosulfan is rapidly metabolized and excreted in the urine and feces as oxidation products such as endosulfan sulfate, diol, hydroxyether or ketone resulting from the cleavage of the cyclic sulfite group (McGregor, 1998). The ratio of the different compounds in the excreta differs with routes of exposure and isomers (FAO / WHO, 1969).

Endosulfan induced alteration on spermatogenesis in young growing and adult rats. The impairments included decreased sperm count, intratesticular spermatid number, sperm morphology and altered activities of testicular marker enzymes (Chitra et al., 1999; Choudhary and Joshi, 2003; Sinha et al., 1995; Sinha et al., 1997; Sinha et al., 2001) as well as reduction in serum testosterone levels (Choudhary and Joshi, 2003; Wilson and LeBlanc, 1998). In addition, reduction in the weights of testes and sex accessory organs was observed (Choudhary and Joshi, 2003). In an *in vitro* experiment, cytotoxicity in Sertoli – germ cell coculture was observed following exposure to endosulfan, which might disturb the normal interaction between Sertoli and germ cells, thus leading to testicular dysfunction (Sinha et al., 1999).

Involvement of endosulfan, a neurotoxic agent, in the central nervous system (CNS) has been studied in various studies (Anand et al., 1980; Paul et al., 1994; Seth et al.,

1986; Subramoniam et al., 1994). A stimulation by long term exposure of endosulfan at a dose of 3 mg / kg body weight for 15 and 30 days respectively increased foot shock – induced fighting behavior in male rats (Agrawal et al., 1983). Induction of hyperactivity, tremors and convulsions was observed in male rats given 40 mg / kg of endosulfan intraperitoneally (Ansari et al., 1987). Data based on these studies indicate that the liver, kidneys, brain and testes are the main target organs (ATSDR, 2000).

According to ATSDR (2000) and the PubMed Database (2005), no calibration or validation of PBPK model predictions was located for endosulfan.

4.2 Objective of the study

The present study was aimed to develop a physiologically based pharmacokinetic (PBPK) model for endosulfan in male rats that could reasonably predict tissues dosimetries after single oral administration of ^{14}C – Endosulfan. The model was verified and given a trial by simulating independent derived data on endosulfan disposition after multiple oral administrations of 5 mg / kg body weight ^{14}C – Endosulfan. The model was further verified by using experimental data retrieved from the literature.

The newly – developed model was extrapolated and scaled up to predict the behavior of endosulfan in humans, and model predictions were compared with the data from human volunteers in Japan and Malaysia.

4.3 Materials and methods

The materials and experimental details in this chapter were similar to those described in Chapter 3, Section 3.3.

4.3.1 PBPK model development

The schematic diagram of the PBPK model for endosulfan in male Sprague – Dawley rats is presented in Figure 4.2. The basic structure was adapted from Ramsey and Anderson (1984) to describe the tissue dosimetry of endosulfan. The model consists of nine compartments which include: (1) gastrointestinal (GI) tract; (2) liver, serving as the metabolizing organ; (3) brain; (4) kidneys; (5) fat; (6) testes; (7) well perfused tissues; (8) poorly perfused tissues; and (9) lung. In this model, the rate of transport of chemical into the tissue is limited by blood flow to that particular tissue while diffusion is assumed instantaneous. The brain was added as a separate tissue compartment since the central nervous system (CNS) is considered the primary target site for the expression of endosulfan neurotoxicity. A testes compartment was separated from the well perfused tissues, because the testes are one of the target organs for endosulfan male reproductive toxicity. Two lumped compartments: (7) well perfused tissues was included and consists of lung, heart and thyroid gland; and (8) poorly perfused tissues was included and consists mainly of skin and muscle tissue. All tissues were described by flow – limited conditions.

The constructed PBPK model assumes that all metabolisms take place in the liver where the rate of metabolism is described by the Michaelis – Menten equation. The parent compounds which include α – and β – endosulfan were lumped (represented as

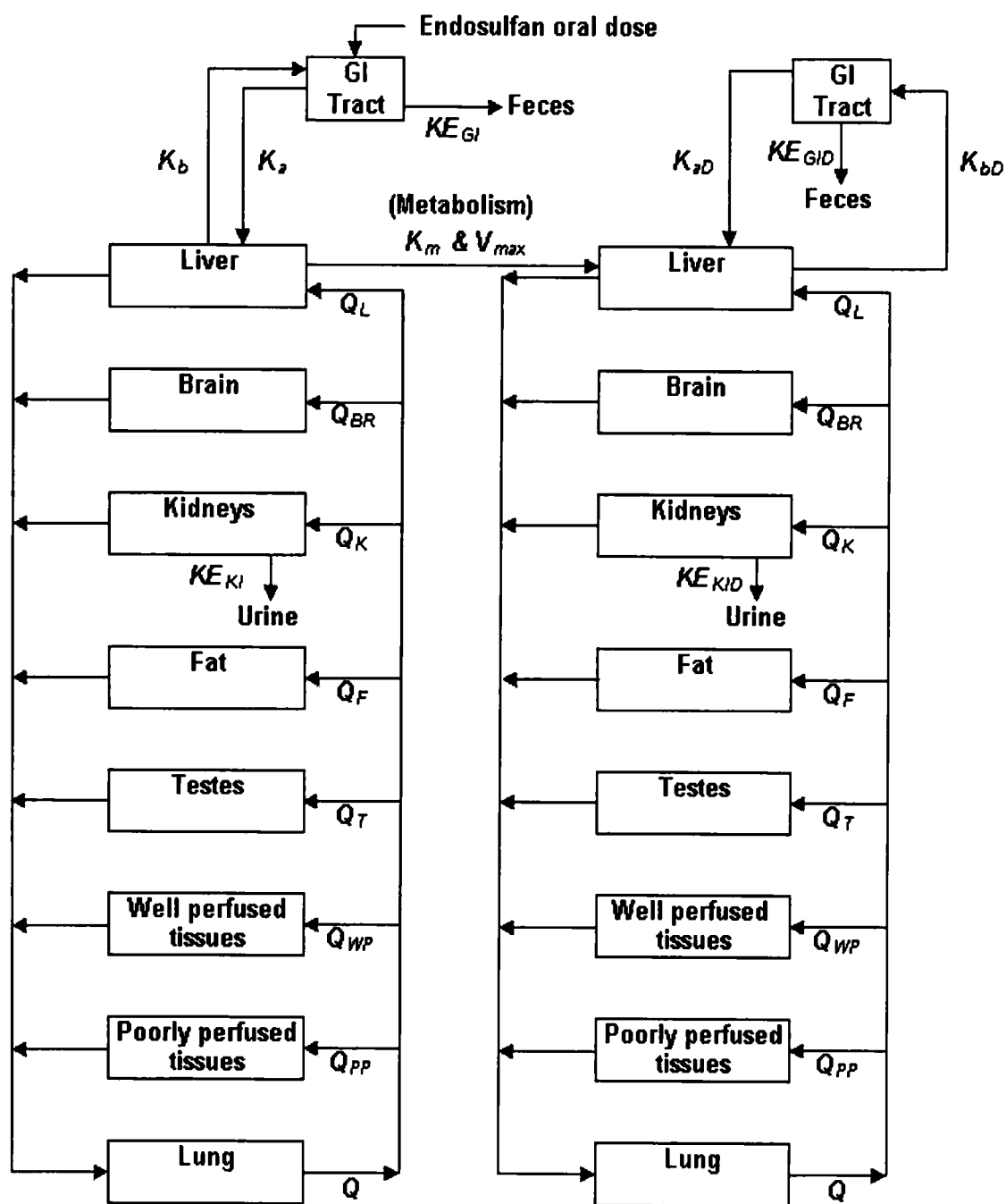


Figure 4.2. The schematic diagram of the PBPK model for endosulfan in male rats.

endosulfan) and the metabolites were represented as endosulfan metabolites in the description of the model. In summary, orally administered endosulfan was taken up by the liver and metabolized.

The model equations for endosulfan are described as follows:

$$\begin{aligned}
 V_{KI} \frac{dC_{KI}}{dt} &= Q_{KI} \left(C_a - \frac{C_{KI}}{P_{KI}} \right) - KE_{KI} C_{KI} V_{KI} && \dots \text{Kidneys} \\
 V_{WP} \frac{dC_{WP}}{dt} &= Q_{WP} \left(C_a - \frac{C_{WP}}{P_{WP}} \right) && \dots \text{Well perfused tissues} \\
 V_{PP} \frac{dC_{PP}}{dt} &= Q_{PP} \left(C_a - \frac{C_{PP}}{P_{PP}} \right) && \dots \text{Poorly perfused tissues} \\
 V_{BR} \frac{dC_{BR}}{dt} &= Q_{BR} \left(C_a - \frac{C_{BR}}{P_{BR}} \right) && \dots \text{Brain} \\
 V_F \frac{dC_F}{dt} &= Q_F \left(C_a - \frac{C_F}{P_F} \right) && \dots \text{Fat} \\
 V_T \frac{dC_T}{dt} &= Q_T \left(C_a - \frac{C_T}{P_T} \right) && \dots \text{Testes} \\
 V_{LI} \frac{dC_{LI}}{dt} &= Q_{LI} \left(C_a - \frac{C_{LI}}{P_{LI}} \right) - \frac{dMET}{dt} V_{LI} + K_a A_{GI} - K_b C_{LI} V_{LI} && \dots \text{Liver} \\
 V_{GI} \frac{dC_{GI}}{dt} &= DOSE - K_a A_{GI} + K_b C_{LI} V_{LI} - KE_{GI} A_{GI} && \dots \text{GI Tract} \\
 V_{BL} \frac{dC_{BL}}{dt} &= \sum Q_{ti} \left(\frac{C_{ti}}{P_{ti}} - C_a \right) && \dots \text{Arterial blood}
 \end{aligned}$$

where V_{ti} is the volume of a tissue (L); C_{ti} is the endosulfan concentration in a tissue (mg / L); Q_{ti} is the blood flow rate to a tissue (L / h); C_a is the arterial endosulfan concentration (mg / L); P_{ti} is the tissue : blood partition coefficient of endosulfan; and $A_{ti} = C_{ti} \cdot V_{ti}$ is the endosulfan amount in an organ. The abbreviations of other parameters in the equations are defined in Tables 4.1 – 4.4.

Table 4.1. Physiologic parameters for the male rats.

Parameter	Abbreviation	Value	Source
Body weight (kg)	BW	0.1	Experiment
Tissue volumes (% BW)			
Kidneys	V_{KI}	0.7	Brown et al., 1997
Well perfused tissues	V_{WP}	1.4	Brown et al., 1997
Poorly perfused tissues	V_{PP}	79.1	Brown et al., 1997
Liver	V_{LI}	3.7	Brown et al., 1997
Brain	V_{BR}	0.6	Brown et al., 1997
Fat	V_F	10.0	Brown et al., 1997
Testes	V_T	1.2	Plowchalk, 2002
Gastrointestinal (GI) Tract	V_{GI}	2.7	Brown et al., 1997
Blood flows (% Q)			
Cardiac output (L / h / kg)	Q	$15 (BW)^{0.74}$	Brown et al., 1997
Kidneys	Q_{KI}	14.1	Brown et al., 1997
Well perfused tissues	Q_{WP}	7.3	Brown et al., 1997
Poorly perfused tissues	Q_{PP}	45.8	Brown et al., 1997
Liver	Q_{LI}	17.4	Brown et al., 1997
Brain	Q_{BR}	2.0	Brown et al., 1997
Fat	Q_F	7.0	Brown et al., 1997
Testes	Q_T	0.78	Plowchalk, 2002

The model equations for endosulfan metabolites are presented below (equations which are similar to endosulfan are not shown):

$$V_{LI} \frac{dC_{LI}^d}{dt} = Q_{LI} \left(C_a^d - \frac{C_{LI}^d}{PD_{LI}} \right) + \frac{dMET}{dt} V_{LI} + K_{aD} A_{GI}^d - K_{bD} C_{LI}^d V_{LI} \quad \dots \text{Liver}$$

where C_{ii}^d is the endosulfan metabolites concentration in a tissue (mg / L); C_a^d is the arterial endosulfan metabolites concentration (mg / L); PD_{ii} is the tissue : blood partition coefficient of endosulfan metabolites; and $A_{ii}^d = C_{ii}^d \cdot V_{ii}$ is the endosulfan

metabolites amount in an organ. The abbreviations of other parameters in the equations are defined in Tables 4.1 – 4.4.

Table 4.2. The tissue : blood partition coefficient values for endosulfan in male rats.

Tissue	Measured ^a	Adjusted
Kidneys	1.22	18.39
Well perfused tissues	1.04	1.43
Poorly perfused tissues	0.58	0.68
Liver	2.98	30.72
Brain	0.58	1.45
Fat	0.87	1.16
Testes	1.06	2.10

Note.

a = Measured in vitro (Jepson et al., 1994)

Table 4.3. The tissue : blood partition coefficient values for endosulfan metabolites in male rats.

Tissue	Estimated ^b	Adjusted
Kidneys	0.44	0.30
Well perfused tissues	0.38	0.27
Poorly perfused tissues	0.21	0.39
Liver	1.08	67.60
Brain	0.21	0.13
Fat	1.26	0.22
Testes	0.38	0.22

Note.

b = Estimated by the algorithm of Poulin and Krishnan (1993)

Table 4.4. Biochemical parameters of the PBPK model for endosulfan and endosulfan metabolites in male rats.

Model parameter	Abbreviation	Estimated value
<i>Endosulfan</i>		
Absorption rate (1 / h)	K_a	150
Excretion rate constant from kidneys (1 / h)	KE_{KI}	0.1
Excretion rate constant from GI Tract (1 / h)	KE_{GI}	0.08
Biliary excretion rate constant (1 / h)	K_b	0.1
<i>Endosulfan metabolites</i>		
Absorption rate (1 / h)	K_{aD}	0.1
Excretion rate constant from kidneys (1 / h)	KE_{KID}	787.5
Excretion rate constant from GI Tract (1 / h)	KE_{GID}	0.057
Biliary excretion rate constant (1 / h)	K_{bD}	8.59
<i>Metabolic parameters</i>		
Maximum metabolic rate (mg / h)	V_{max}	546.5
Michaelis – Menten constant (mg / L)	K_M	5.0

The metabolism of endosulfan into its metabolites is given by:

$$\frac{dMET}{dt} = \frac{V_{max} \cdot C_L / P_L}{K_M + C_L / P_L}$$

where V_{max} is the maximum metabolic rate (mg / h); K_M is the Michaelis – Menten constant (mg / L); C_L is the endosulfan concentration in liver (mg / L); and P_L is the liver : blood partition coefficient of endosulfan.

4.3.2 Parameterization

4.3.2.1 Physiological parameters

The rat physiological parameters, including tissue blood volumes, were taken from Brown et al. (1997) with the exception of the testes blood – flow rate and testes blood – flow volume (Plowchalk and Teeguarden, 2002). The body weight (BW) used in the

model was determined by our experiment. Parameter values are summarized in Table 4.1.

4.3.2.2 Physicochemical parameters

1) Measured partition coefficients

In the partition coefficient (PC) determination experiment, three rats ($n = 3$) were used. PC values for ^{14}C – Endosulfan were determined experimentally in male Sprague – Dawley rats using the centrifugation method for nonvolatile chemicals described by Jepson et al. (1994). Several unexposed rats were lightly anesthetized with diethyl ether and tissues such as liver, kidneys, fat, muscle, brain, heart, lung, spleen, testis, thyroid gland and blood were collected. The blood and minced tissues were weighed into 5 mL scintillation vials capped with Teflon / rubber septa to prevent the absorption of the chemical. Vials were prepared using 1 μg / mL of ^{14}C – Endosulfan in PBS. Typically, 0.25 g of blood or tissue and 5 mL of chemical in saline were added to each vial. Vials were incubated and vortexed at a medium speed setting for 18 h at 37 °C. The supernatant was centrifuged for 10 min at 3000 rpm. The resulting supernatant was filtered through a prewashed Centrifree® Micropartition Device with YMT membrane (Millipore Corp., Bedford, MA) by centrifugation for 3 min at 3000 rpm. 100 μL of the filtrate was removed from the filter cup and 5 mL of Ultima Gold was mixed and counted for radioactivity. The tissue : saline partition coefficients were calculated as in Jepson et al. (1994) and adjusted until agreement was reached between model – predicted and experimental data for the 5 mg / kg single dose group. The physicochemical parameters are summarized in Table 4.2.

The tissue : saline partition coefficient ($P_{T/S}$) was calculated as:

$$P_{T/S} = \frac{C_T}{C_S} = \frac{AMT_T/V_T}{C_S} = \frac{(C_R V_R - C_S V_S)/V_T}{C_S}$$

$$C_S = C_{S,F} \left(\frac{C_{R,U}}{C_{R,F}} \right)$$

where C_T = chemical concentration ($\mu\text{g} / \text{mL}$) in tissue;

C_S = chemical concentration ($\mu\text{g} / \text{mL}$) in saline fraction;

AMT_T = amount (μg) of chemical in the tissue;

C_R = chemical concentration ($\mu\text{g} / \text{mL}$) in reference solution (chemical solution without tissue);

V_R = volume (mL) of the reference solution;

V_S = volume (mL) of the sample solution;

V_T = volume (mL) of tissue or liquid;

$C_{S,F}$ = chemical concentration ($\mu\text{g} / \text{mL}$) in the saline filtrate;

$C_{R,U}$ = chemical concentration ($\mu\text{g} / \text{mL}$) in the unfiltered reference solution;

$C_{R,F}$ = chemical concentration ($\mu\text{g} / \text{mL}$) in the filtered reference solution

The tissue : blood PC can then be determined by dividing the tissue : saline PC by the blood : saline PC.

2) Estimated partition coefficients

The tissue : blood PCs represent an important group of input parameters for the PBPK models. These PCs represent the relative distribution of a chemical between tissues and blood at equilibrium within the organism. In the biologically based algorithm for predicting rat tissue : blood PCs from K_{ow} , Poulin and Krishnan (1995) took into

account the chemical partitioning into phospholipids in tissues and blood. Partitioning into erythrocytes and plasma was also considered separately. Accordingly, the tissue : blood PCs were computed as the ration of the partitioning of chemicals in tissues to the sum total of their partitioning in erythrocytes and plasma.

The partitioning into tissues was described as an additive function of partitioning of a chemical into tissue neutral lipids, phospholipids, and tissue water. The partitioning of chemicals into tissue neutral lipids was assumed to correspond to the $K_{o/w}$, whereas the partitioning of chemicals into tissue water fraction was considered to be equal to 1. To calculate the partitioning of a chemical into a tissue, the fraction of neutral lipids was multiplied with $K_{o/w}$, and the fraction of water in the tissue with 1. The partitioning of chemicals into tissue phospholipids was calculated as a fractional additive function of their partitioning into neutral lipids (0.3) and water (0.7). The following equation representing this approach was used to calculate the tissue partitioning of chemicals (P_t):

$$P_t = (K_{o/w} \times F_{nt}) + (1 \times F_{wt}) + (K_{o/w} \times 0.3 \times F_{pt}) + (1 \times 0.7 \times F_{pt})$$

where F represents the fraction of tissue weight as a particular component (subscripts nt = neutral lipids in tissue; wt = water in tissue; and pt = phospholipids in tissue).

Similarly, the partitioning of chemicals into rat erythrocytes (P_e) and plasma (P_p) was calculated as follows:

$$P_e = (K_{o/w} \times F_{ne}) + (1 \times F_{we}) + (K_{o/w} \times 0.3 \times F_{pe}) + (1 \times 0.7 \times F_{pe})$$

$$P_p = (K_{o/w} \times F_{np}) + (1 \times F_{wp}) + (K_{o/w} \times 0.3 \times F_{pp}) + (1 \times 0.7 \times F_{pp})$$

where the subscripts ne = neutral lipids in erythrocytes; np = neutral lipids in plasma; we = water in erythrocytes; wp = water in plasma; pe = phospholipids in erythrocytes; and pp = phospholipids in plasma.

Based on the data on the fraction of erythrocytes and plasma constituting rat blood, the relative contribution of each of these to the partitioning in whole blood was considered (37 % erythrocytes, 63 % plasma). The tissue : blood PCs of chemicals (P_{tb}) were predicted by dividing their partitioning into tissue by the sum total of their partitioning into erythrocytes ($\times 0.37$) and plasma ($\times 0.63$). The following algorithm represents the approach outlined above for predicting the P_{tb} from $K_{o/w}$ data and tissue / blood composition:

$$P_{tb} = \frac{P_t}{(0.37 \times P_e) + (0.63 \times P_p)}$$

Hence, the PCs of all the tissues for endosulfan metabolites were estimated from the n – octanol : water partition coefficient ($K_{o/w}$) by the algorithm of Poulin and Krishnan (1995), which was outlined above, with some adjustment by fitting. The fitting was obtained by adjusting each value until agreement was reached between model – predicted and experimental data for the 5 mg / kg single dose group. It was assumed that the ratio of PC between the parent isomers and metabolites was similar for all tissues / organs. The physicochemical parameters are summarized in Table 4.3.

4.3.2.3 Biochemical parameters

According to the literature database (PubMed Database, 2005), since *in vitro* derived parameters for the maximum metabolic rate (V_{max}) and Michaelis – Menten constant (K_M), absorption rate (K_a), urinary (KE_{KI}) and fecal excretion (KE_{GI}) rates, and biliary excretion rate (K_b) for endosulfan as well as K_{aD} , KE_{KID} , KE_{GID} and K_{aD} rates for

endosulfan metabolites were unavailable, these parameters were optimized by manual adjustment until the best visual fit of the simulations with the experimental data was observed. The values of the biochemical parameters are summarized in Table 4.4.

4.3.2.4 Model calibration

The PBPK model was numerically solved using the Euler Method. The model simulation was carried out using Microsoft Visual Basic 6.3 (Microsoft Corporation). The simulation of the parameterized model was compared with the experimental data in male Sprague – Dawley rats after single oral administration of 5 mg / kg ^{14}C – Endosulfan as described previously in Chapter 3 (Section 3.4.3, Table 3.1).

4.3.2.5 Model verification and model simulations of endosulfan disposition in other studies

The calibrated PBPK model was applied to the repeated dosing condition {three – time repeated administration of 5 mg / kg as previously described in the Experimental design and administration of ^{14}C – Endosulfan (3)}. The model simulation was compared with the experimental data on time courses of total concentrations following three – time repeated oral administration of endosulfan to male rats as described previously in Chapter 3 (Section 3.4.3, Table 3.2). The model's ability to predict the endosulfan disposition in various tissues was further verified by using experimental data retrieved from the literature (Ansari et al., 1984; Chan and Mustafa, 2005; Dikshith et., 1984; Nath et al., 1978) and the experimental data are specified in Table 4.5. Since the average body weights were unavailable in these studies, simulations were done by using the range of body weights (minimum – maximum) as reported in the literature.

4.3.3 Sensitivity analyses

Sensitivity analyses were implemented to evaluate the relative importance of model parameters on model output at various times. The impacts of the physicochemical and biochemical parameters were examined when each parameter was increased above and decreased below its original value by 1 %. The changes in the predicted blood and tissue concentrations of endosulfan and endosulfan metabolites were examined and compared with the unadjusted model prediction. The sensitivity for each parameter was then calculated by using the following equation:

$$\text{Sensitivity} = \frac{\delta \ln C}{\delta \ln P} = \frac{(D - E) / E}{(F - G) / G}$$

where C = endosulfan concentration; P = parameter; D = endosulfan concentration at altered parameter value; E = original endosulfan concentration; F = altered parameter value; and G = original parameter value.

4.3.4 Extrapolation from rat model to human model

4.3.4.1 Physiological parameters

Since the purpose of this section was to investigate the validity of animal scale – up procedures to investigate with a PBPK model, the parameters predicting the behavior of endosulfan in humans were obtained solely by calculation from the parameters that successfully simulated endosulfan behavior in rats.

The volumes of the tissue compartments in the human model were assumed proportional to body weight, and were therefore scaled up in direct proportion to the ratio of body weight (i. e., 60 kg / 0.1 kg = 600). 60 kg represents the average body weight for a Japanese man whereas 0.1 kg represents the average body weight of a rat obtained by the experiments in this study. The human physiological parameters,

including tissue blood volumes, were taken from Brown et al. (1997) with the exception of the testes blood – flow rate and testes blood – flow volume (Plowchalk and Teeguarden, 2002). The body weight (*BW*) used in the human model was 60 kg, which was an average weight of a Japanese man. Parameter values are summarized in Table 4.6. Physiological parameter generally considered to be more nearly proportional to body surface area than to body weight (and hence to a fractional power of the body weight ratio) is cardiac output. The 0.74 power of the body weight ratios was chosen to scale up these parameters to the human model, i. e., $600^{0.74} = 113.7$ (Ramsey and Andersen, 1984). *BW* of 49 kg was used for the data on the Malaysian school children (Chan et al., 2004). *BW* of 70 kg, which was an average *BW* for a Western man, was used for other data retrieved from the literature.

4.3.4.2 Physicochemical parameters

PCs used for the rat model were unchanged for the human model. PCs are summarized in Tables 4.2 and 4.3.

Table 4.5. Experimental data reported on endosulfan disposition in rats.

No	Dose regimen	Dose (mg / kg)	Carrier	Strain; sex and body weight (kg)	Observed time points	Animals per time point	Residue, µg / g; mg / L; ppm (Mean ± Standard Deviation)	References
1	Multiple oral (for 15 days)	5	Tween - 80	SD ^a ; male; 0.04 - 0.12	16 th day	4 - 7	Liver - Endosulfan sulfate (0.09 ± 0.04); Kidneys - Endosulfan (1.46 ± 0.38)	Chan and Mustafa, 2005
		5	Tween - 80	SD; male; 0.04 - 0.12	30 th day	4 - 7	Liver - Endosulfan sulfate (0.05 ± 0.01); Kidneys - Endosulfan (ND)	
	Multiple oral (for 15 days)	10	Tween - 80	SD; male; 0.04 - 0.12	16 th day	4 - 7	Liver - Endosulfan sulfate (0.09 ± 0.03); Kidneys - Endosulfan (2.26 ± 0.80)	
		10	Tween - 80	SD; male; 0.04 - 0.12	30 th day	4 - 7	Liver - Endosulfan sulfate (0.06 ± 0.01); Kidneys - Endosulfan (ND)	
2	Multiple oral (for 60 days)	2.5	Groundnut oil : alcohol (19 : 1)	NA; male; 0.06 - 0.08	61 st day	3 - 9	Endosulfan Liver (22.77 ± 10.12); Kidneys (587.23 ± 178.6); Brain (23.37 ± 4.40); Testes (37.46 ± 12.59)	Ansari et al., 1984
		7.5	Groundnut oil : alcohol (19 : 1)	NA; male; 0.06 - 0.08	61 st day	3 - 9	Endosulfan Liver (15.62 ± 3.58); Kidneys (1675.89 ± 467.10); Brain (110.54 ± 69.14); Testes (128.70 ± 38.10)	
	Multiple oral (for 60 days)							
3	Multiple oral (for 30 days)	11	Peanut oil	NA; male; 0.15 - 0.175	31 st day	5	Endosulfan Liver (4.68 ± 0.07); Kidneys (6.47 ± 0.69); Brain (3.64 ± 0.01); Testes (4.35 ± 0.19)	Nath et al., 1978
4	Multiple oral (for 30 days)	5	Peanut oil	Wistar; male; 0.087	31 st day	6	Endosulfan Liver (0.14 ± 0.02); Kidneys (0.15 ± 0.03); Brain (ND); Blood serum (0.03 ± 0.0008)	Dikshith et al., 1984
		1.5	Peanut oil	Wistar; female; 0.083	31 st day	6	Endosulfan Liver (0.11 ± 0.02); Kidneys (0.06 ± 0.01); Brain (ND); Blood serum (0.02 ± 0.002)	

Note.

NA = Not available; ND = Not detectable (Below detection limit); ^aSD = Sprague - Dawley

4.3.4.3 Biochemical parameters

Specific metabolic constants are not usually regarded to vary so consistently with body weight, although basal metabolic rate does appear to follow the fractional power rule with an exponent of 0.74. The maximum rate of the enzymatic reaction, V_{max} was assumed approximately proportional to basal metabolic rate (Gehring et al., 1948; Ramsey and Andersen, 1984), and was therefore scaled in the same manner as cardiac output. K_M was also scaled in the same manner as cardiac output. The absorption rate (K_a), urinary (KE_{KI}) and fecal excretion (KE_{GI}) rates, and biliary excretion rate (K_b) for endosulfan and the absorption rate (K_{aD}), urinary (KE_{KID}) and fecal excretion (KE_{GID}) rates, and biliary excretion rate (K_{bD}) for endosulfan metabolites remained unchanged for the human model. The values of the biochemical parameters are summarized in Table 4.7.

4.3.4.4 Model simulation

The model simulation was carried out using Microsoft Visual Basic 6.3 (Microsoft Corporation). Once the model parameters were set, only the concentration / dose of endosulfan in the diet was changed to simulate the concentration of endosulfan in the desired tissues. Each volunteer was assumed to consume meals three times per day (breakfast, lunch and dinner). Each volunteer was expected to have their meals at an average time of 0700, 1300 and 1900 daily.

Table 4.6. Physiologic parameters for humans.

Parameter	Abbreviation	Value	Source
Body weight (kg)	BW	60	
Tissue volumes (% BW)			
Kidneys	V_{KI}	0.4	Brown et al., 1997
Well perfused tissues	V_{WP}	1.7	Brown et al., 1997
Poorly perfused tissues	V_{PP}	58.0	Brown et al., 1997
Liver	V_{LI}	2.6	Brown et al., 1997
Brain	V_{BR}	2.0	Brown et al., 1997
Fat	V_F	21.4	Brown et al., 1997
Testes	V_T	0.4	Plowchalk, 2002
Gastrointestinal (GI) Tract	V_{GI}	1.7	Brown et al., 1997
Blood flows (% Q)			
Cardiac output (L / h / kg)	Q	$15 (BW)^{0.74}$	Brown et al., 1997
Kidneys	Q_{KI}	19.0	Brown et al., 1997
Well perfused tissues	Q_{WP}	8.3	Brown et al., 1997
Poorly perfused tissues	Q_{PP}	27.0	Brown et al., 1997
Liver	Q_{LI}	25.0	Brown et al., 1997
Brain	Q_{BR}	12.0	Brown et al., 1997
Fat	Q_F	5.0	Brown et al., 1997
Testes	Q_T	0.12	Plowchalk, 2002

Table 4.7. Biochemical parameters of the PBPK model for endosulfan and endosulfan metabolites in humans.

Model parameter	Abbreviation	Estimated value
<i>Endosulfan</i>		
Absorption rate (1 / h)	K_a	150
Excretion rate constant from kidneys (1 / h)	KE_{KI}	0.1
Excretion rate constant from GI Tract (1 / h)	KE_{GI}	0.08
Biliary excretion rate constant (1 / h)	K_b	0.1
<i>Endosulfan metabolites</i>		
Absorption rate (1 / h)	K_{aD}	0.1
Excretion rate constant from kidneys (1 / h)	KE_{KID}	787.5
Excretion rate constant from GI Tract (1 / h)	KE_{GID}	0.057
Biliary excretion rate constant (1 / h)	K_{bD}	8.59
<i>Metabolic parameters</i>		
Maximum metabolic rate (mg / h)	V_{max}	62147.3
Michaelis – Menten constant (mg / L)	K_M	568.6

4.3.4.5 Model verification and model simulations of endosulfan disposition in other studies

The simulation of the parameterized model was compared with the data from human volunteers in Japan (Fukata et al., 2005; Kyushu University) and Malaysia (Chan et al., 2004). The dietary intake of endosulfan for each data was estimated based on the model simulation. The model was simulated for five days with three ingested dose per day (average 3 meals per day) as endosulfan was completely eliminated within four days after exposure (Chapter 3; Section 3.4.2).

The model's ability to predict the endosulfan disposition in various tissues was further verified by using experimental data retrieved from the literature (Blanco – Coronado et al., 1992; Coutselinis et al., 1978). Biochemical parameters were scaled in direct

proportion to the ratio of body weight as described in Section 4.3.4.1. For acute intoxication or suicidal deaths, the model was simulated for 24 h with single ingested dose of endosulfan. The ingested dose was predicted for data with unknown amount of ingested endosulfan.

4.4 Results

4.4.1 Parameterization and calibration

No overt toxicity was observed in any animal post – dosing. The data used for the model simulations in this chapter were retrieved from Chapter 3 (Section 3.4.3, Tables 3.1 and 3.2). The time course for cumulative amount of total endosulfan excreted in urine and feces and model predictions are presented in Figure 4.3. Cumulative amounts of total endosulfan excreted in urine and feces and model predictions were also compared well with the experimental data. Following single oral administration of 5 mg / kg ^{14}C – Endosulfan to male rats, the urinary excretion of the radioactivity during 24 h was $9.6 \pm 21.4 \%$ where as the fecal elimination was $59.0 \pm 29.7 \%$. The total radioactivity recovered in the excreta for four days was $106.8 \pm 26.2 \%$ ($12.4 \pm 4.8 \%$ in urine and $94.4 \pm 21.4 \%$ in feces). The results were consistent with other studies that most of the radiocarbons were excreted in the urine and feces after 96 h (Deema et al., 1966; Dorough et al., 1978; McGregor, 1998).

Biochemical parameters that resulted in the best fit of the data are given in Table 4.4. The parameterized model was compared with the experimental pharmacokinetic data in male Sprague – Dawley rats after single oral administration of 5 mg / kg ^{14}C – Endosulfan. The resulting model simulations for blood and target tissues (liver, kidneys, brain and testes) are shown in Figure 4.4. The model simulations correctly

captured the shape of the experimental data, indicating that the model predictions of total endosulfan time course profiles in all tissues were in general agreement with the experimental data. Good consistency was observed for total endosulfan concentrations in liver, indicating that metabolic kinetic parameters were successfully predicted.

4.4.2 Model verification and model simulations of endosulfan disposition in other studies

Following model calibration, the calibrated model was applied to the repeated dosing experiment (three – time repeated oral administration of 5 mg / kg). Generally, reasonable agreement was observed between model predictions and experimental data for all tissues. The time course profiles in target tissues and model predictions are shown in Figure 4.5. However, simulation of total endosulfan disposition from kidneys and liver after multiple oral administration of 5 mg / kg to male rats resulted in slight overestimation of concentrations at all time points where as simulation of total endosulfan disposition from testes resulted in slight underestimation at the last time - point, but was generally considered to be acceptable (Figure 4.5).

Various experimental data retrieved from the literature on endosulfan disposition *in vivo* after multiple oral dosage were simulated without adjusting the previously established model parameters. Simulated concentrations of endosulfan in the liver and kidneys after multiple oral dosage of 5 and 10 mg / kg body weight per day for a period of 15 days and rested for another 15 days before sacrifice were generally in agreement with the experimental results (Figure 4.6) (Chan and Mustafa, 2005). Another study by Ansari et al. (1984) also indicated good agreement between

experimental and simulated concentrations of endosulfan in the liver, brain and kidneys after multiple oral dosage of 2.5 or 7.5 mg / kg body weight per day for a period of 60 days to male rats (Figures 4.7 and 4.8).

Simulated concentrations of endosulfan in the kidneys and liver after multiple oral dosage of 11 mg / kg per day for a period of 30 days to male rats (Figure 4.9) compared well with experimental data (Nath et al., 1978). However, the simulated concentrations in the brain and testes were underestimated compared to the experimental results (Figure 4.9). The experimental results of endosulfan levels in the kidneys, liver and blood of male and female rats after multiple oral dosage of 5 mg / kg per day for 60 days (for male rats) and 1.5 mg / kg per day for 60 days (for female rats) were generally compared well with the simulations (Figures 4.10 and 4.11) (Dikshith et al., 1984).

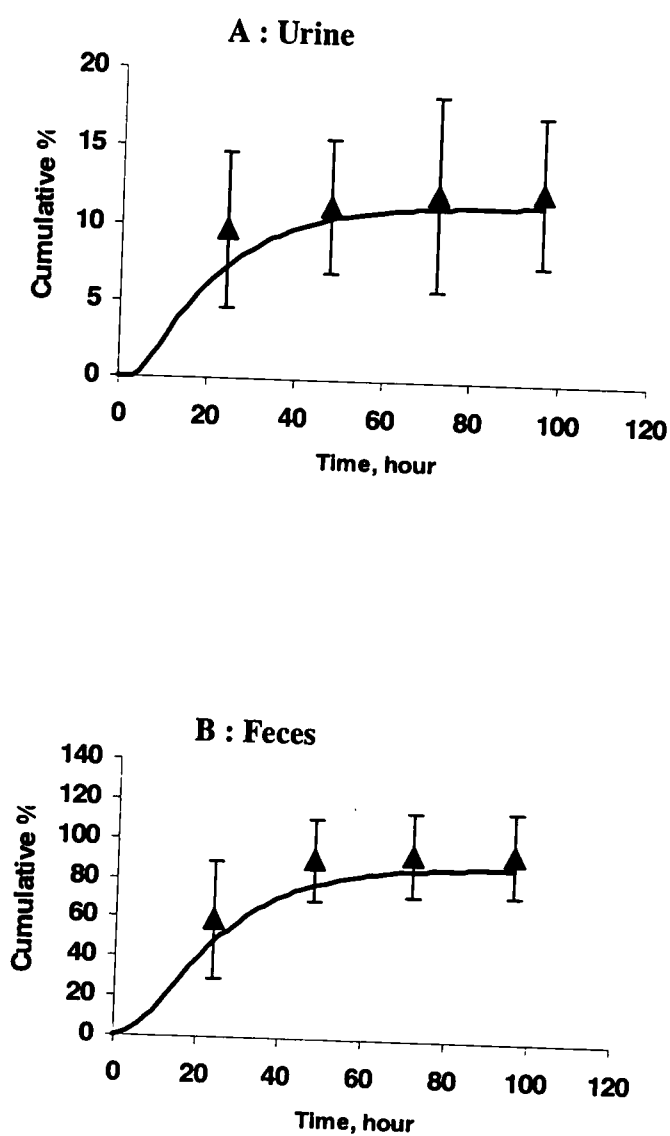
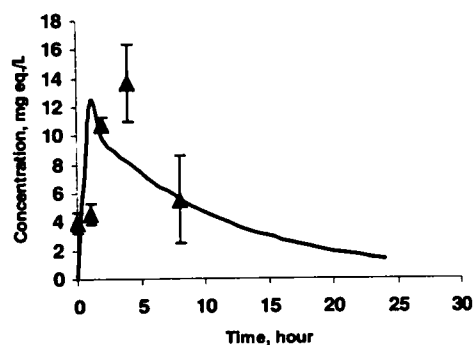
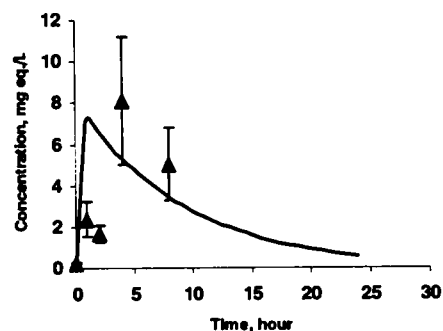


Figure 4.3. Comparison of PBPK model prediction (lines) and experimental cumulative percentage (▲) of urinary (A) and fecal elimination (B) of total endosulfan after single oral administration of 5 mg / kg ^{14}C – Endosulfan to male rats (Values are means $\pm$ SD; for $n = 3$ at each time point).

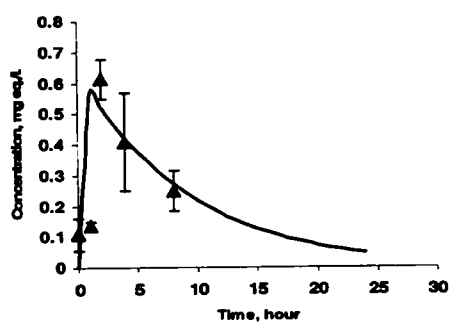
A



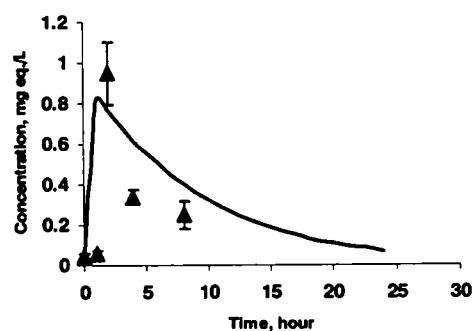
B



C



D



E

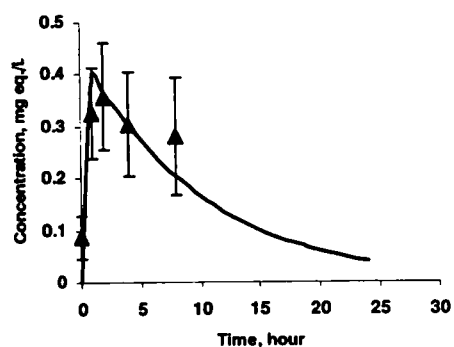


Figure 4.4. Comparison of PBPK model prediction (lines) and experimental concentrations (▲) of total endosulfan in liver (A), kidneys (B), brain (C), testes (D) and blood (E) following single oral administration of ^{14}C - Endosulfan to male rats (Values are means $\pm$ SD; for $n = 3$ at each time point).

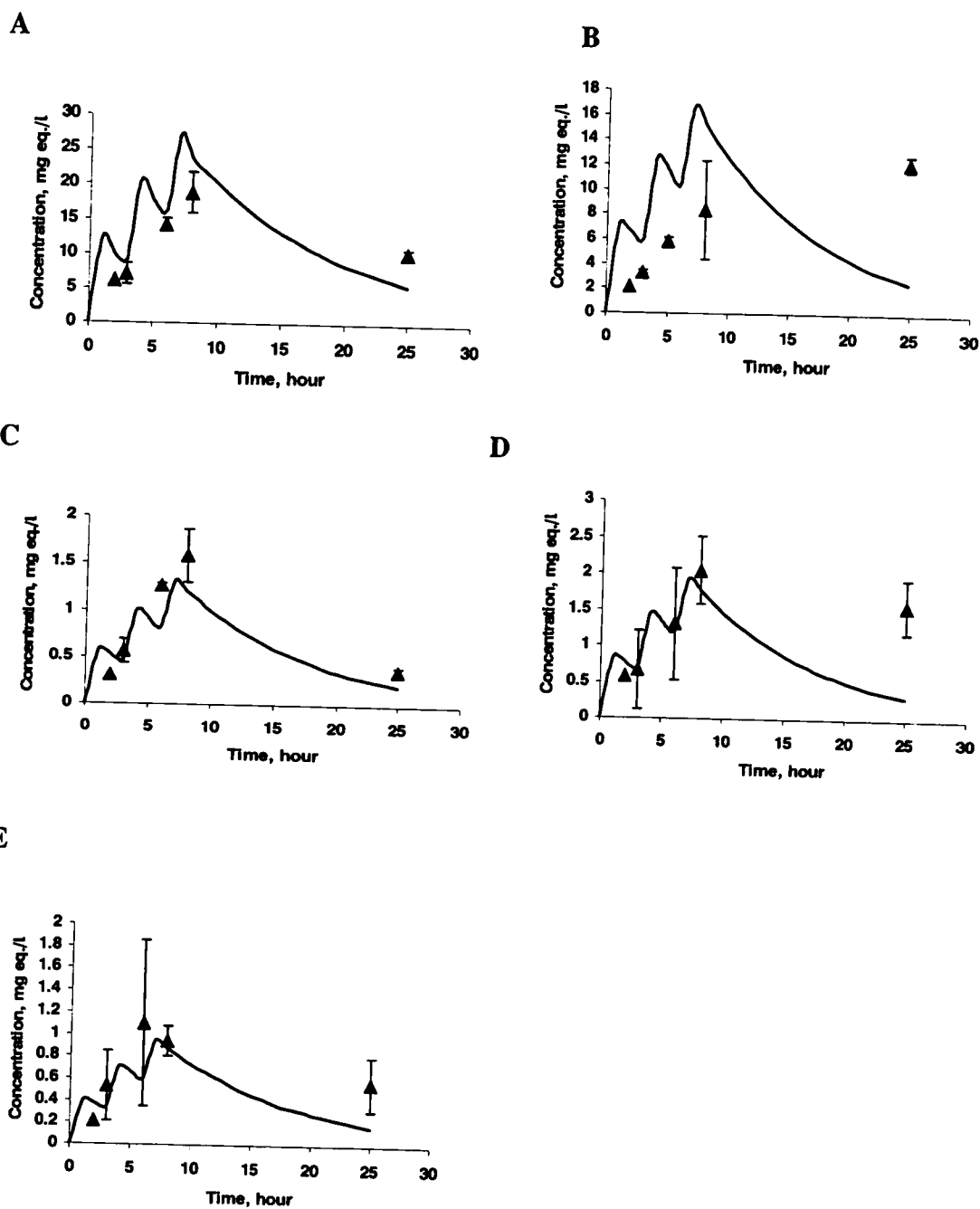
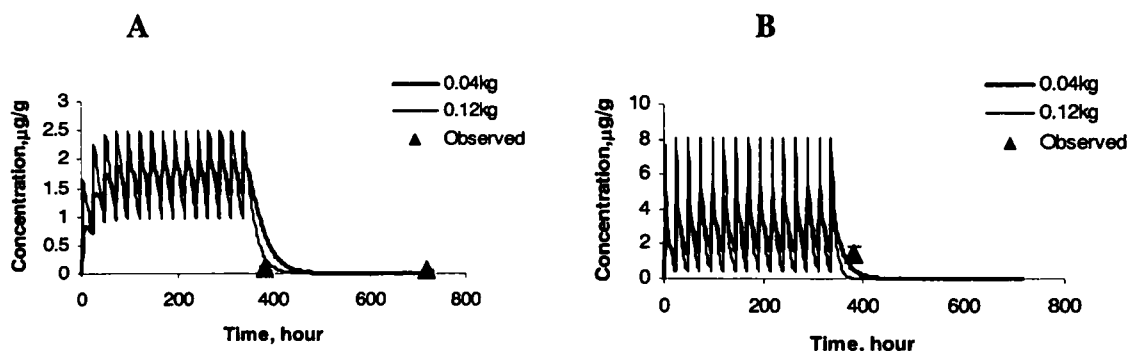


Figure 4.5. Comparison of PBPK model prediction (lines) and experimental concentrations (▲) of total endosulfan in liver (A), kidneys (B), brain (C), testes (D) and blood (E) following three – time repeated oral administration of ^{14}C – Endosulfan to male rats (Values are means $\pm$ SD; for $n = 3$ at each time point except for the first time point where $n = 1$).

Note. Rats were administered 5 mg / kg body weight per day of endosulfan for 15 days and rested for another 15 days before sacrifice.



Note. Rats were administered 10 mg / kg body weight per day of endosulfan for 15 days and rested for another 15 days before sacrifice.

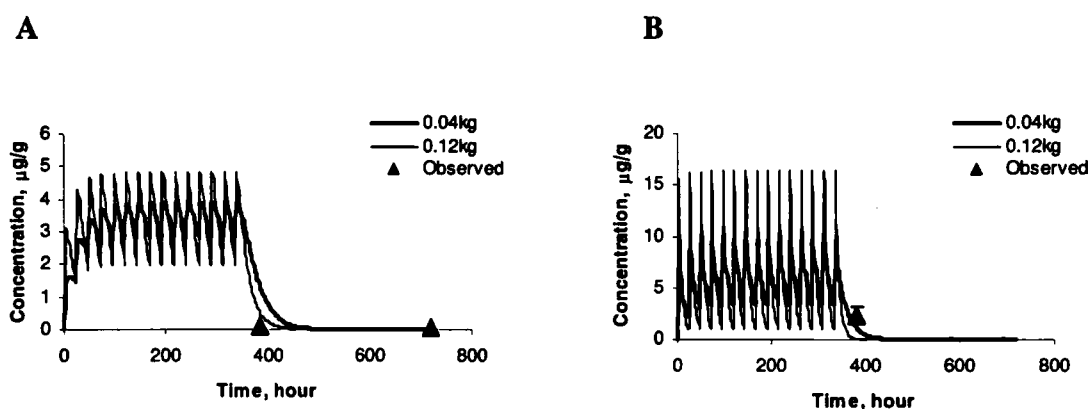


Figure 4.6. Observed (▲) and simulated concentrations (lines) of endosulfan metabolites in liver (A) and endosulfan in kidneys (B) after multiple oral administration of 5 and 10 mg / kg body weight per day of technical grade endosulfan in male Sprague – Dawley rats for 15 days and rested for another 15 days before sacrifice. Body weight was 0.04 – 0.12 kg (Chan and Mustafa, 2005).

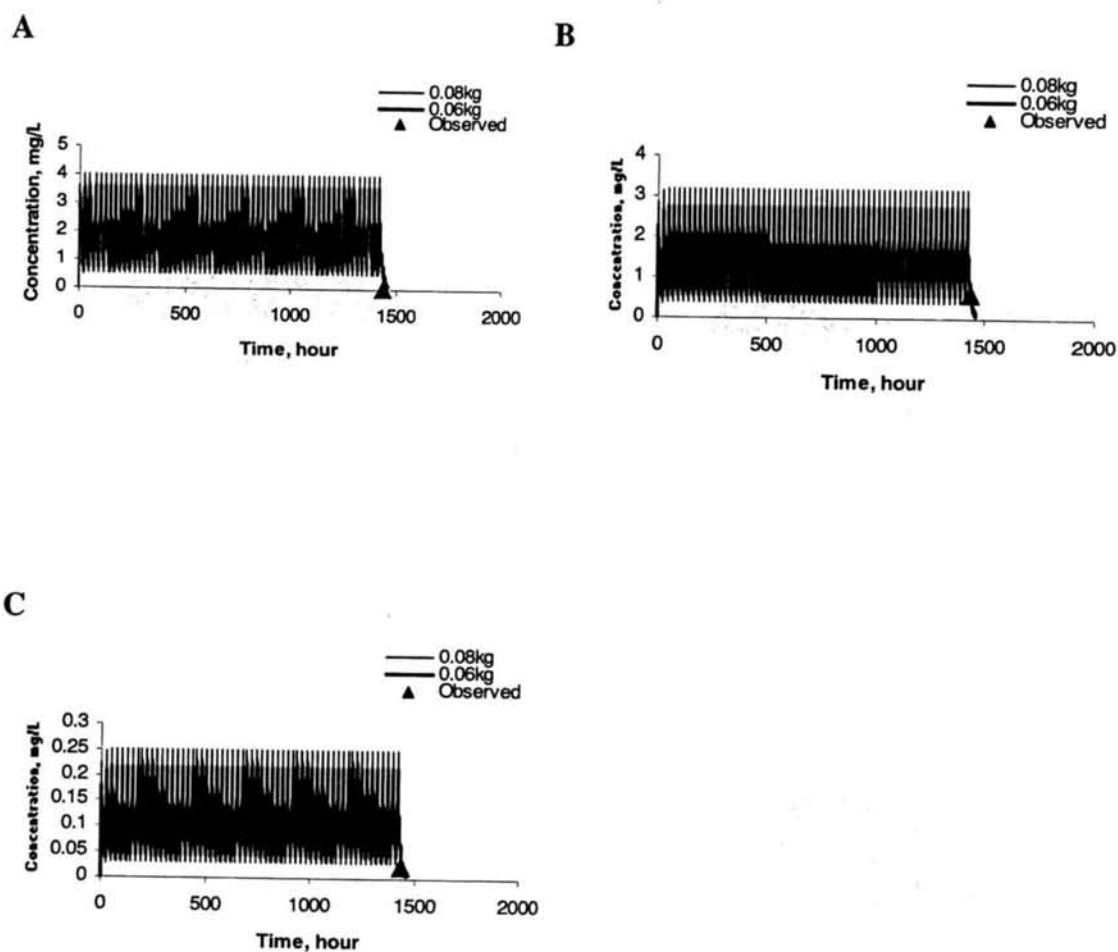


Figure 4.7. Observed (▲) and simulated concentrations (lines) of endosulfan in liver (A), kidneys (B) and brain (C) after multiple oral administration of 2.5 mg / kg body weight per day of endosulfan in male rats for 60 days. Body weight was 0.06 – 0.08 kg (Ansari et al., 1984).

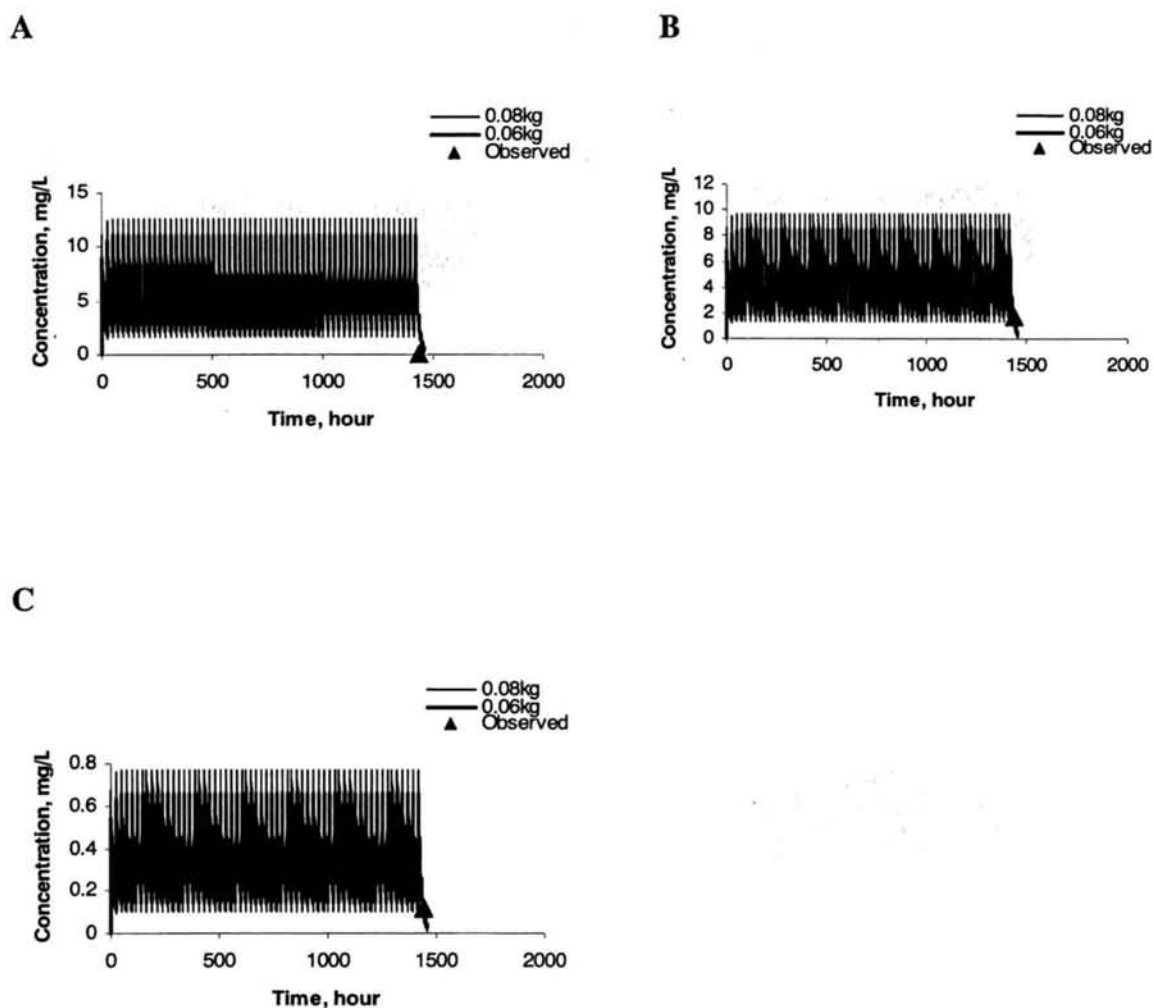


Figure 4.8. Observed (▲) and simulated concentrations (lines) of endosulfan in liver (A), kidneys (B) and brain (C) after multiple oral administration of 7.5 mg / kg body weight per day of endosulfan in male rats for 60 days. Body weight was 0.06 – 0.08 kg (Ansari et al., 1984).

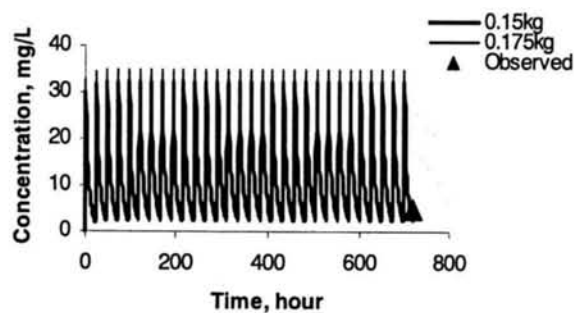
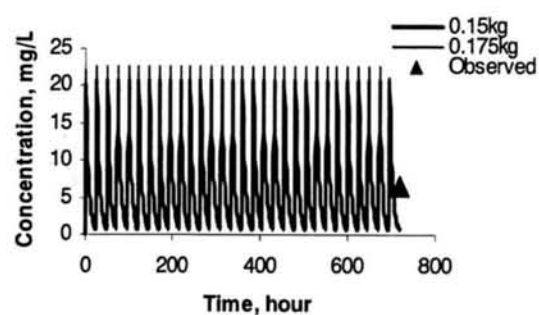
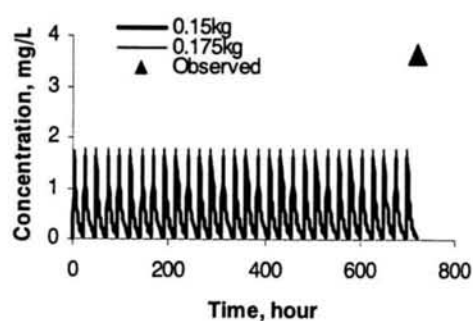
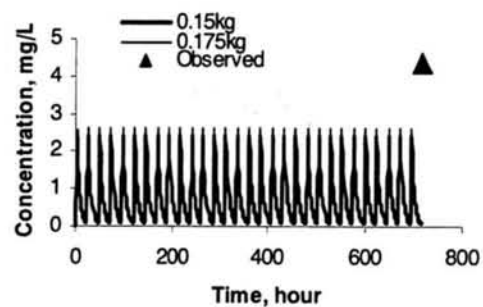
A**B****C****D**

Figure 4.9. Observed (▲) and simulated concentrations (lines) of endosulfan in liver (A), kidneys (B), brain (C) and testes (D) after multiple oral administration of 11 mg / kg body weight per day of endosulfan in male rats for 60 days. Body weight was 0.150 – 0.175 kg (Nath et al., 1978).

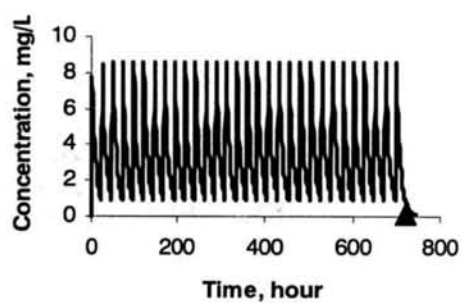
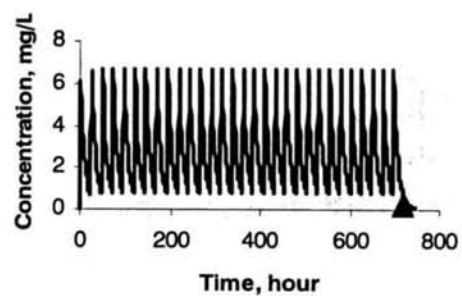
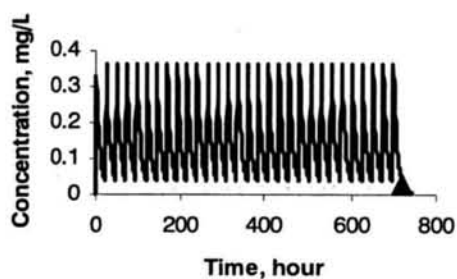
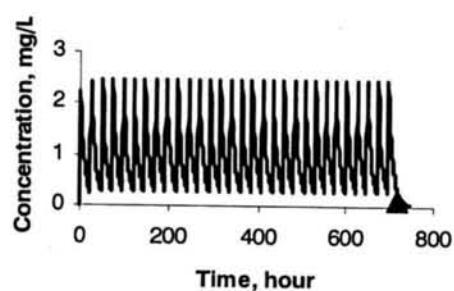
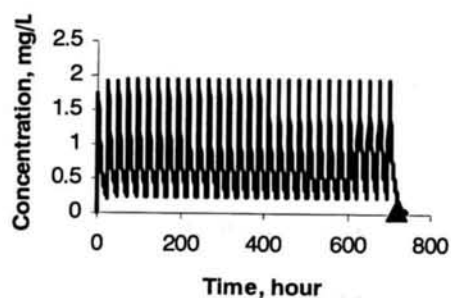
A**B****C**

Figure 4.10. Observed (▲) and simulated concentrations (lines) of endosulfan in liver (A), kidneys (B), and blood serum (C) after multiple oral administration of 5 mg / kg body weight per day of endosulfan in male Wistar rats for 60 days. Body weight was 0.087 kg (Dikshith et al., 1984).

A



B



C

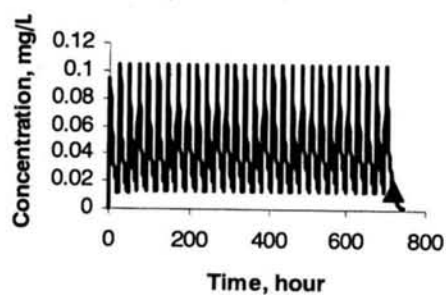


Figure 4.11. Observed (▲) and simulated concentrations (lines) of endosulfan in liver (A), kidneys (B), and blood serum (C) after multiple oral administration of 1.5 mg / kg body weight per day of endosulfan in female Wistar rats for 60 days. Body weight was 0.083 kg (Dikshith et al., 1984).

4.4.3 Sensitivity analyses

4.4.3.1 Endosulfan

The results of the sensitivity analyses at each time point (1 h, 2 h, 4 h, 8 h and 24 h) that tissue endosulfan concentrations were calculated are shown in Table 4.8. With regards to the impact of the PCs, the PCs for all the tissues had similar impact on the endosulfan concentrations across all time points.

The sensitivity for maximum metabolic rate (V_{max}) and urinary excretion rate (KE_{KI}) were consistently negative across tissue time, and external exposure concentration where as the sensitivity for Michaelis – Menten constant (K_M) was consistently positive across tissue time, and external exposure concentration. The impact of V_{max} and K_M on endosulfan tissue concentration was most evident at 8 h and 24 h. Other parameters such as the absorption rate (K_a), biliary (K_b) and fecal excretion (KE_{GI}) rates for endosulfan had no impact in the blood and tissue concentrations of endosulfan (Data not shown).

4.4.3.2 Endosulfan metabolites

The results of the sensitivity analyses at each time point (1 h, 2 h, 4 h, 8 h and 24 h) that tissue endosulfan concentrations were calculated are shown in Table 4.9. With regards to the impact of the PCs, the PCs for all the tissues had similar or little impact on the endosulfan metabolites concentrations across all time points.

The sensitivity for maximum metabolic rate (V_{max}) was consistently positive across tissue time, and external exposure concentration except for the last time point at 24 h where the sensitivity was negative. The sensitivity for Michaelis – Menten constant

(K_M) was consistently negative across tissue time, and external exposure concentration except for the last time at 24 h where the sensitivity for K_M were positive. Other parameters such as the absorption rate (K_{ad}) and urinary excretion (KE_{KID}) rate had little impact in the blood and tissues concentrations of endosulfan metabolites across all time points. The impact of fecal (KE_{GID}) and biliary excretion (K_{ad}) rates on endosulfan metabolites concentrations was most evident at 24 h.

Table 4.8. Sensitivity analyses for the concentration of endosulfan in liver, kidneys, brain, testes and blood.

Endosulfan (mg / L)	Time				
	1 h	2 h	4 h	8 h	24 h
Liver					
Liver PC	1.38	0.99	0.99	1.03	1.17
V_{max}	-0.29	-0.49	-0.72	-1.20	-3.12
K_M	0.27	0.46	0.68	1.14	3.04
KE_{KI}	< -0.01	< -0.01	< -0.01	< -0.01	-0.03
Kidneys					
Kidneys PC	1.01	1.01	1.01	1.01	1.01
V_{max}	-0.05	-0.15	-0.38	-0.84	-2.76
K_M	0.05	0.14	0.35	0.80	2.66
KE_{KI}	< -0.01	< -0.01	< -0.01	-0.01	-0.03
Brain					
Brain PC	1.45	1.45	1.45	1.45	1.45
V_{max}	-0.05	-0.15	-0.38	-0.84	-2.75
K_M	0.05	0.15	0.36	0.80	2.67
KE_{KI}	< -0.01	< -0.01	< -0.01	< -0.01	-0.03
Testes					
Testes PC	1.04	1.07	1.07	1.07	1.05
V_{max}	-0.04	-0.14	-0.37	-0.83	-2.74
K_M	0.04	0.13	0.35	0.79	2.65
KE_{KI}	< -0.01	< -0.01	< -0.01	< -0.01	-0.03
Blood					
V_{max}	-0.05	-0.16	-0.38	-0.84	-2.75
K_M	0.05	0.15	0.36	0.80	2.67
KE_{KI}	< -0.01	< -0.01	< -0.01	< -0.01	-0.03

Note. Sensitivity with an absolute value of 0.50 or greater is in bold. Sensitivity with an absolute value of less than 0.01 was not shown.

Table 4.9. Sensitivity analyses for the concentration of endosulfan metabolites in liver, kidneys, brain, testes and blood.

Endosulfan metabolites (mg/L)	Time				
	1 h	2 h	4 h	8 h	24 h
Liver					
Liver PC	0.15	0.13	0.10	0.08	0.10
V_{max}	0.97	0.90	0.73	0.44	-0.33
K_M	-0.90	-0.83	-0.69	-0.43	0.31
K_{ad}	0.04	0.11	0.23	0.41	0.75
KE_{GID}	-0.01	-0.04	-0.10	-0.23	-0.90
K_{bd}	-0.77	-0.82	-0.85	-0.87	-0.87
KE_{KID}	< -0.01	< -0.01	-0.01	-0.03	-0.07
Kidneys					
Kidneys PC	0.68	0.65	0.60	0.55	0.49
V_{max}	0.99	0.95	0.83	0.56	-0.26
K_M	-0.93	-0.88	-0.77	-0.54	0.24
K_{ad}	0.02	0.06	0.16	0.34	0.73
KE_{GID}	-0.01	-0.02	-0.06	-0.17	-0.82
K_{bd}	-0.68	-0.77	-0.83	-0.87	-0.87
KE_{KID}	-0.32	-0.35	-0.40	-0.46	-0.52
Brain					
Brain PC	1.25	1.25	1.25	1.25	1.25
V_{max}	0.99	0.94	0.83	0.56	-0.26
K_M	-0.93	-0.85	-0.77	-0.54	0.24
K_{ad}	0.02	0.06	0.16	0.34	0.73
KE_{GID}	-0.01	-0.02	-0.06	-0.14	-0.82
K_{bd}	-0.68	-0.77	-0.83	-0.87	-0.87
KE_{KID}	-0.02	-0.05	-0.10	-0.15	-0.22
Testes					
Testes PC	1.03	1.04	1.05	1.05	1.05
V_{max}	0.99	0.94	0.83	0.56	-0.26
K_M	-0.93	-0.88	-0.77	-0.54	0.24
K_{ad}	0.02	0.06	0.16	0.34	0.73
KE_{GID}	-0.01	-0.02	-0.06	-0.17	-0.82
K_{bd}	-0.67	-0.77	-0.83	-0.87	-0.87
KE_{KID}	-0.02	-0.05	-0.10	-0.15	-0.22
Blood					
V_{max}	0.99	0.95	0.83	0.56	-0.26
K_M	-0.93	-0.88	-0.77	-0.54	0.24
K_{ad}	0.02	0.06	0.16	0.34	0.73
KE_{GID}	-0.01	-0.02	-0.06	-0.17	-0.82
K_{bd}	-0.68	-0.77	-0.83	-0.87	-0.87
KE_{KID}	-0.02	-0.05	-0.10	-0.15	-0.22

Note. Sensitivity with an absolute value of 0.50 or greater is in bold. Sensitivity with an absolute value of less than 0.01 was not shown.

4.4.4 Extrapolation from rat model to human model

4.4.4.1 Model verification and model simulations of endosulfan disposition in other studies

The following section of this chapter presents a test of the ability of the PBPK model for endosulfan in rats to predict the pharmacokinetic behavior of endosulfan in humans. The parameters for the human PBPK model (Table 4.6) were obtained solely by scale up of the parameters for the rat as described in Section 4.3.4.1. All parameters remained unchanged except for the V_{max} and K_M , where these parameters were scaled up.

Figures 4.12 and 4.13 show the time course profiles in maternal serum and model predictions of pregnant women from Japan. The estimated dietary intake was simulated until reasonable agreement was observed between model simulations and experimental data. From the survey of thirty – two pregnant women who lived in the cities (Chiba or Yamanashi) near Tokyo, Japan, in 2002 and 2003 (Fukata et al., 2005), endosulfan was detected in the maternal serum of these subjects. The concentration was in the range of nd (non – detectable) – 8.4 pg / g. The estimated dietary intake for the subjects was 0.76×10^{-5} mg / kg / day.

From the data of eleven pregnant women living around the Kyushu area, Japan obtained from Kyushu University, endosulfan (α - and β - endosulfan) with a concentration range of 14.6 – 27.3 pg / g, where as endosulfan sulfate, with a concentration range of nd – 0.78 pg / g were detected in the maternal serum. The estimated dietary intake for the subjects was 9.09×10^{-5} mg / kg / day.

Figure 4.14 shows the time course profiles in blood and model predictions of pregnant school children from Malaysia. The exposure of school children to pesticide residues was investigated in a cross – sectional study involving 577 school children from 60 schools in Peninsular Malaysia. Only three subjects had detectable residue levels of endosulfan in the concentration range of nd – 0.6 ng /g (Chan et al., 2004). The estimated dietary intake for the subjects was 1.06×10^{-5} mg / kg / day.

Generally, reasonable agreement was observed between model predictions and experimental data for blood. The time course profiles in blood and model predictions are shown in Figures 4.12 – 4.14.

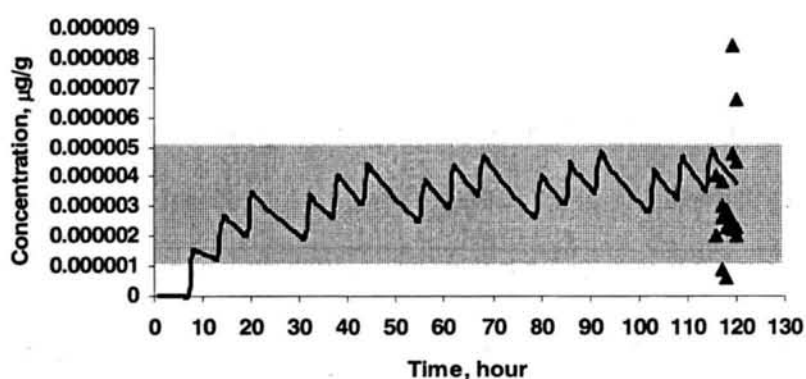
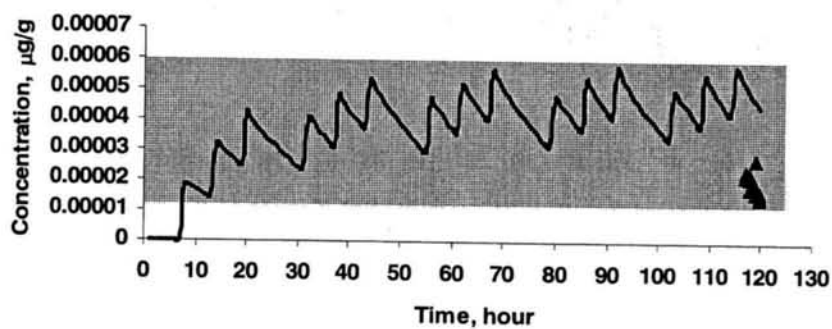


Figure 4.12. Observed (▲) and simulated concentrations (lines) of endosulfan in maternal serum of pregnant women from Chiba, Japan following three – time ingested dose of endosulfan. Each volunteer was assumed to consume meals three times per day (breakfast, lunch and dinner). Each volunteer was expected to have their meals at an average time of 0700, 1300 and 1900 daily (Fukata et al., 2005). The estimated dietary intake was 0.76×10^{-5} mg / kg / day.

A : Endosulfan



B : Endosulfan metabolites

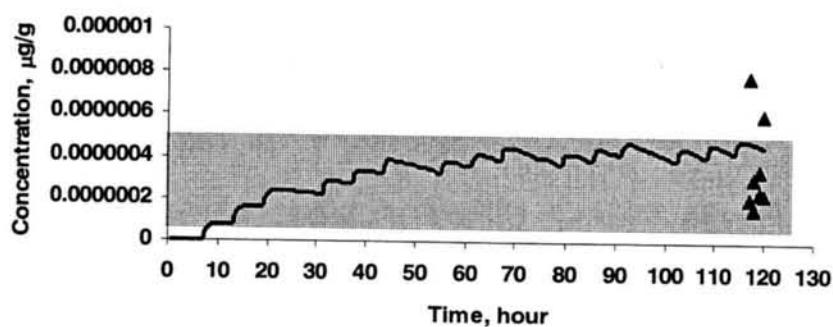


Figure 4.13. Observed (▲) and simulated concentrations (lines) of endosulfan (A) and endosulfan metabolites (B) in maternal serum of pregnant women from Kyushu, Japan following three – time ingested dose of endosulfan. Each volunteer was assumed to consume meals three times per day (breakfast, lunch and dinner). Each volunteer was expected to have their meals at an average time of 0700, 1300 and 1900 daily (Kyushu University). The estimated dietary intake was 9.09×10^{-5} mg / kg / day.

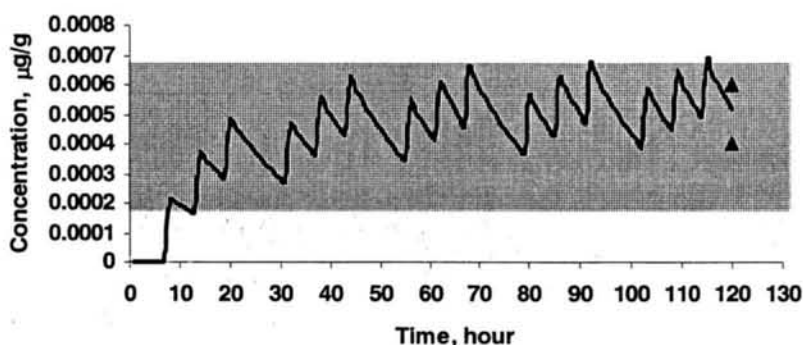


Figure 4.14. Observed (▲) and simulated concentrations (lines) of endosulfan in blood of Malaysian school children following three – time ingested dose of endosulfan. Each volunteer was assumed to consume meals three times per day (breakfast, lunch and dinner). Each volunteer was expected to have their meals at an average time of 0700, 1300 and 1900 daily (Chan et al., 2004). The estimated dietary intake was 1.06×10^{-5} mg / kg / day.

Various experimental data retrieved from the literature on endosulfan disposition in human were simulated without adjusting the previously established model parameters. The concentrations of endosulfan in the biological materials generally fell within the range of predicted concentrations (Coutselinis et al., 1978; Blanco – Coronada et al., 1992) (Table 4.10).

Table 4.10. Concentrations of endosulfan (α – and β – endosulfan) and predicted range of concentrations in biological materials from human.

Specimen	Coutselinis et al., 1978 Case series (n = 3)	Blanco - Coronado et al., 1992 Case series (n = 6)
Ingested dose	Unknown (Predicted = 0.2 mg)	Unknown (Predicted = 1.5)
Blood level - peak	0.63 mg/L ^a (0.12 - 0.03 mg/L)	0.87 mg/L ^a (0.25 - 0.92 mg/L)
Brain	0.028 μ g/g (0.18 - 0.05 μ g/g)	
Liver	1.00 μ g/g (4.33 - 1.00 μ g/g)	
Kidney	2.00 μ g/g (2.24 - 0.61 μ g/g)	

Note.

Values in parentheses are predicted range of concentrations from the simulation.

a : Averaged blood level – peak.

4.5 Discussion

PBPK models are used frequently in toxicology and risk assessment for extrapolation across dose, route of exposures, and species (Simmons et al., 2002). In early days, a typical PBPK model consists of liver, poorly perfused tissues, well perfused tissues, and fat (Krishnan and Andersen, 1994). Increasingly, detailed models with more compartments are being developed to enhance the description of the pharmacokinetics of specific compounds. The advantages of PBPK model compared with classical pharmacokinetic model are: (1) these models can provide the time course of

distribution of xenobiotic to any organ or tissue; (2) they allow estimation of the effects of changing physiological parameters of tissue concentrations (as done by sensitivity analyses in the present study); (3) they can predict the toxicokinetics of chemicals across species by allometric scaling; (4) complex dosing regimes are easily accommodated and more important, the resultant PBPK model have the potential for extrapolations from observed data to predicted situations; while one of the major disadvantages is that physiological input parameters are often ill – defined for various strains and species (Medinsky and Klaassen, 1996).

Given that no calibration or validation of PBPK model predictions of concentrations of endosulfan in various tissues exist (ATSDR, 2000; PubMed Database, 2005), the present study is the first attempt to do so and compares rat PBPK model predictions of endosulfan to measured concentrations following single and multiple oral administrations. It should be emphasized that the parent isomers were lumped together in the current study as an initial step to developing the rat PBPK model. Generally, results show reasonable concordance between model predictions and measured values in all target tissues following oral administration. Brain and testes dosimetries of total endosulfan were reasonably predicted after oral exposures to ^{14}C – Endosulfan with the adjustment of the brain : blood and testes : blood partition coefficients.

For the present study, *in vitro* derived parameters for absorption and metabolism of endosulfan were unavailable. Hence, these parameters were obtained by model calibration to a single set of *in vivo* experimental data after rats were administered single oral dose of 5 mg / kg ^{14}C – Endosulfan. Subsequently, the validity of the

model was tested by simulation with the three – time repeated dose experimental data and other *in vivo* data on endosulfan disposition from the literature, which are independent of the calibration data. An attempt to identify separate isomers in the current study was impossible due to low specific activity of radiocarbon.

The simulation of endosulfan concentrations in the target tissues after multiple oral dosages shows the ability of the model to extrapolate from single oral dosage (Figure 4.4) to multiple oral dosages (Figure 4.5). The shape of the kidneys and liver concentration – time curves were not well predicted after multiple oral dosages. This may be attributable to alternative plausible mechanisms such as diffusion – limitation or enterohepatic recirculation or both is occurring in the kidneys and liver (Keys et al., 1999). However, enterohepatic recirculation was unlikely to occur for endosulfan (Dorough et al., 1978; McGregor, 1998). Dorough and co – workers (1978) suggested that the biliary metabolites of endosulfan would not enter the enterohepatic cycle and were voided in the feces. Another possible reason may be due to the short intervals between administration and residue was not sufficiently excreted before the next administration. Hence, caution should be exercised when applying the model to predict concentrations of endosulfan in kidneys and liver after multiple oral dosages. This also suggests the need for further research.

The simulations of those studies from the literature generally agree with experimental data (Figures 4.6 – 4.11). However, experimental data reported on endosulfan disposition in brain and testes of male rats (Nath et al., 1978) were underestimated (Figure 4.9). Readjustment of the partition coefficients for the respective tissues resulted in an adequate description of endosulfan disposition reported in this study

(Data not shown). When evaluating these simulations, it has to be realized that these studies sometimes differ from the calibration study in strain (Ansari et al., 1984; Dikshith et al., 1984; Nath et al., 1978) or sex of the animals (Dikshith et al., 1984) and also the volume and the type of vehicle used in which endosulfan was applied (Chan and Mustafa, 2005).

Sensitivity analyses allow for a quantitative assessment of input parameters on the model simulations of tissue concentrations (Simmons et al., 2002). The present results indicate that the influence of PBPK model input parameters on total endosulfan tissue dosimetry varies, as expected, across parameter. Comparison of the absolute magnitude of the sensitivity parameters may help guide decisions regarding the usefulness of data collection efforts to further refine / define specific parameters or may help to improve on the designs of future experiments.

It was observed that the estimated dietary intake for the pregnant women from Kyushu area was 12 – fold higher than the estimated dietary intake for the pregnant women from Chiba or Yamanashi area with 9.09×10^{-5} mg / kg / day and 0.76×10^{-5} mg / kg / day respectively. Residue levels of endosulfan from these women living in the Kyushu area were higher [α – and β – endosulfan (14.6 – 27.3 pg / g) and endosulfan sulfate (nd – 0.18 pg / g)] than those living in the urban areas in Chiba or Yamanashi [endosulfan (nd – 8.4 pg / g)]. This could be attributed that perhaps some parts of the Kyushu area, where the samples were collected were of agricultural areas and farming activities were carried out intensively in those areas. In addition, these subjects may also be exposed to endosulfan through consumption of food contaminated with endosulfan. Recent studies regarding predictors of organochlorine

levels indicate that exposure among the general population occurs mainly through the diet (Devoto et al., 1998; Laden et al., 1999).

Endosulfan is permitted for agricultural use in Japan although its usage is restricted (EJF, 2002). Residues of endosulfan were detected in the umbilical cord serum from the newborn babies [α – and β – endosulfan (16.1 – 44.8 pg / g) and endosulfan sulfate (nd – 0.78 pg / g)]. Residues of endosulfan were also found in high levels in the breast milk from the pregnant women [α – and β – endosulfan (19.0 – 52.9 pg / g) and endosulfan sulfate (nd – 70.0 pg / g)], indicating that these subjects were exposed to high levels of endosulfan.

In a survey conducted by Fukata and co – workers (2005), residues of endosulfan were detected in the umbilical cord (nd – 7.2 pg / g) besides maternal serum. These surveys indicated that endosulfan was transferred from mother to fetus and breastfeeding was the highest exposure source as it was the main method for excretion, thus raising a cause for concern since maternal toxic chemicals during the critical development phase may give rise to fetal neurotoxicity (Sarcinelli et al., 2003; Tilson, 1998).

Generally, reasonable agreement was observed between model predictions and experimental data for blood. In Figures 4.12 and 4.14, the observed range of experimental data for the pregnant women from Chiba fell within the simulated range, thus indicating that the newly – developed model has the capacity to reproduce the observed range of endosulfan concentration in humans. In Figure 4.13, the observed range of experimental data for the pregnant women from Kyushu fell within the

simulated range for endosulfan and endosulfan metabolites. By comparing the experimental data with the simulation results for endosulfan, the experimental data for endosulfan metabolites fell within the simulated range, thus indicating that the model can be partially validated. This also demonstrates that the parameters used in the current model were quite well predicted and the model can be applied to estimate the concentrations of endosulfan in human tissues. The estimated dietary intakes for the pregnant women from Chiba and Kyushu as well as school children from Malaysia were below the acceptable daily intake (ADI), in which ADI for endosulfan in human was 0.008 mg / kg / day (WHO, 1984). This suggests that the low estimated dietary intakes for these groups of people will not cause serious health effects. However, other possibilities cannot be ruled out.

At present, the human PBPK model cannot strictly be validated since data concerning human exposures are scarce and limited to a small number of cases, with only sporadic data on the tissue concentrations of endosulfan and its isomers. Toxicokinetic data in humans are lacking. All of the cases reported were of acute intoxication or suicidal attempts caused by ingestion of endosulfan. Another problem for model validation may be due to the large differences among individual concentration data. These differences in concentration may be partially explained by differences in body weight, age, gastrointestinal absorption and food consumption habits.

4.6 Conclusions

In summary, it is concluded that the first and new PBPK model for endosulfan in male rats has been developed in the present study and can predict tissue dosimetries following single and multiple oral dosages of endosulfan. The model was verified with experimental data retrieved from the literature. This model provides the foundation for development of a more complex physiologically based dynamic model that would provide a basis for the design of further experimental investigations. A more reliable and sensitive analytical method would also be incorporated into our future experiments to enhance the separation and identification of the parent compounds and metabolites present in different matrices. Further research is also necessary to expand its current model for endosulfan to include the two isomers of the parent compounds and its major oxidation metabolites.

Generally, reasonable agreement was observed between model predictions and experimental data for the extrapolated PBPK model from rat to human. This suggests that the parameters used in the model were quite well predicted and the model has the capacity to be applied to estimate the concentrations of endosulfan in human tissues. However, more studies on human exposures to endosulfan are necessary in order to validate the model for future health risk assessments..

Extension of the model with *in vitro* experimental data on the toxicodynamics of endosulfan in the CNS would facilitate the interpretation of endosulfan's neurotoxicity *in vivo*. Such studies are currently carried out in our laboratory. The extended model would also provide the basis for more accurate and reliable risk assessments of the effects of endosulfan on neurotoxicity and male reproductive

toxicity since the CNS and testes are among the target organs for the respective toxic effects. It is hoped that the newly developed model could be utilized in the human health risk assessment in near future, particularly in the human reproductive and neurotoxic risks.

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CHAPTER 5

DEVELOPMENT OF AN *IN VITRO* BLOOD – BRAIN BARRIER MODEL TO STUDY THE PERMEABILITY EFFECTS OF ENDOSULFAN ON THE TIGHT JUNCTIONS

5.0 Introduction

As has been discussed in Chapter 2, endosulfan, a neurotoxic insecticide is involved in the neurotoxicity of its central nervous system (CNS). The primary site of action of endosulfan is believed to be the CNS. Involvement of CNS in the neurotoxicity of endosulfan has been shown by several *in vivo* experiments (Anand et al., 1980; Paul et al., 1995; Seth et al., 1986; Subramoniam et al., 1994; Zaidi et al., 1985). However, the precise mechanism of its toxicity and whether endosulfan causes a direct effect on tight junctions at the blood – brain barrier (BBB) remained unidentified.

5.0.1 Blood – brain barrier (BBB)

BBB is formed by complex tight junctions of the brain capillary endothelial cells (ECs) and expresses various transport systems. ECs are joined by tight intercellular junctions that provide a biological barrier to maintain the homeostasis of the brain microenvironment. The distinct property of this barrier can be attributed, in part, to the presence of a continuous ring of tight junctions between neighboring cells. BBB protects the brain from the blood milieu, and delivery of ions and solutes from blood to CNS is limited by the selectivity of BBB. (Cucullo et al., 2004; Igarashi et al., 1999; Jean Harry et al., 1997; Lauer et al., 2004; Lee et al., 2004; Lu et al., 2005; Ohtsuki, 2004; Rubin and Staddon, 1999). Figure 5.1 shows the essential features of the BBB.

From a toxicological viewpoint, three aspects of the BBB are of interest: (a) the BBB regulates uptake and release of endogenous substances and also xenobiotics, (b) toxic substances may interfere with the structural and functional properties of the BBB, and (c) certain parts of the CNS (e. g., areas in the hypothalamus and the choroids plexa), have poorly developed BBB functions. The latter is also true for all parts of the embryonic and juvenile brains [European Center for Validation of Alternative Methods (ECVAM)].

5.0.2 Endpoints for acute toxic effects

For acute toxic effects, there are two endpoints for toxic insult to the BBB: (a) partial or complete breakdown of the barrier function (i. e., effects on the ability of the BBB to exclude endogenous and exogenous substances) and (b) changes in the specific transport capacity of the BBB. There is a need to measure the ability of the normal BBB to transport toxicants into or out of the brain (ECVAM).

The literature varies considerably concerning the different BBB *in vitro* models set up during the past decades, using primary endothelial cells and astrocytes or glial cells, cultured under different experimental conditions and using different cell sources. Several *in vivo* and *in vitro* models for estimation of brain permeation have been proposed in the literature (Pardridge, 1995). Most commonly, bovine, rat or porcine endothelial cells are used for *in vitro* models.

5.0.3 Transendothelial electrical resistance (TEER)

The analysis of transendothelial electrical resistance (TEER) is a simple method to quantify the functionality of the tight junctions of the *in vitro* BBB. TEER represents

the permeability of small ions through the tight junctions between brain capillary endothelial cells. The absolute value of TEER is believed to be mainly dependent on the amount and complexity of tight junctions between the cells (Madara, 1998). Drug transport studies with an *in vitro* BBB model allow the simultaneous determination of the permeability status of the BBB by TEER and permeability coefficient of drugs (Gaillard and de Boer, 2000).

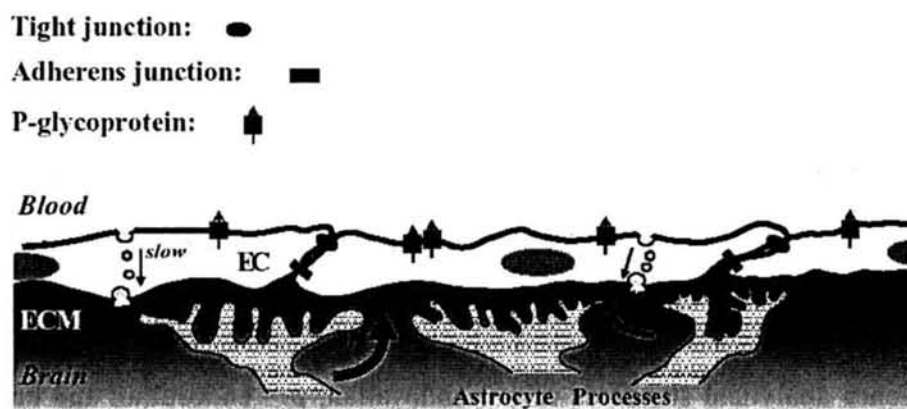


Figure 5.1. Essential features of the blood – brain barrier (BBB). Brain capillary endothelial cells are coupled by adherens and tight junctions, the latter limiting paracellular flux. P – glycoprotein (P-gl) is expressed in the apical membrane of the ECs and actively ejects undesired substances from the CNS. Transcytosis across brain ECs occurs slowly, minimizing transcellular movement into the CNS. Astrocyte processes ensheath the ECs, although an extracellular matrix (ECM) is interposed and may release molecules that influence their phenotype (Adapted from Rubin and Staddon, 1999).

5.1 Objective of the study

The present study was aimed to evaluate the transendothelial electrical resistance (TEER) and permeability effects of endosulfan on the tight junctions of the BBB within porcine brain microvascular endothelial cells (PBMECs). The porcine BBB model was used as the current *in vitro* BBB model because porcine brain physiology is closely related to human (Tewes et al., 1997).

5.2 Materials and methods

5.2.1 Chemicals

Radiolabelled endosulfan (2, 3 – ^{14}C - Endosulfan) with a molar activity of 772.56 MBq; specific activity of 1.895 MBq / mg and a radiochemical purity of > 95% by Radio TLC was obtained from the Institute of Isotopes Co. Ltd. (Budapest, Hungary). All reference analytical standards of α - endosulfan, β - endosulfan and endosulfan sulfate (> 99 % purity), phosphate buffered saline (PBS) powder and dimethyl sulfoxide (DMSO) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Trypsin - EDTA were purchased from Gibco (Grand Island, NY, USA). Hionic – FluorTM used for liquid scintillating counting were obtained from Packard Bioscience B. V. (Groningen, The Netherlands). CS – C Complete Medium Kit was purchased from Cell Systems Corporation (Kirkland, WA, USA). Cell Viability Kit (CCK – 8) was obtained from Dojindo Laboratories (Kumamoto, Japan). All chemicals used in the present study were of the highest grade available.

5.2.2 Materials

Millicell – PCF culture plate inserts (30 mm diameter; 0.4 μm pore size) were purchased from Millipore Corporation (Bedford, MA, USA). Falcon Multiwell™ cell culture plates were obtained from Becton – Dickinson (NJ, USA). Iwaki 96 – well micro plates, tissue culture flasks and scintillation vials were purchased from Wakenyaku Co. Ltd. (Kyoto, Japan).

5.2.3 Preparations of chemicals

α – endosulfan, β – endosulfan and endosulfan sulfate were dissolved in DMSO individually to obtain concentrations of 1 nM, 0.01 μM , 0.1 μM , 1 μM , 10 μM , 100 μM , 1 mM, 10 mM. These standard solutions will be used for transendothelial electrical resistance (TEER) and cytotoxicity studies of porcine brain microvascular endothelial cells (PBMECs). The final concentrations that will be used for these studies were 0.001 nM, 0.01 nM, 0.1 nM, 1 nM, 0.01 μM , 0.1 μM , 1 μM and 10 μM .

Purchased ^{14}C – Endosulfan was dissolved in ethanol at the concentration of 10 mg / mL. The 10 mg / mL stock solution of ^{14}C – Endosulfan was further diluted in DMSO to obtain concentrations of 10 μM , 100 μM , 1 mM and 10 mM. These solutions will be used for transendothelial transport study of PBMECs. The final concentrations that will be used were 0.01 μM , 0.1 μM , 1 μM and 10 μM .

5.2.4 Rationale for dose selection

The concentrations of ^{14}C – Endosulfan in the brain following single administration of 5 mg / kg ^{14}C – Endosulfan to male Sprague rats were 0.11 mg / L (equivalent to 0.27 μM) after 30 min and 0.27 mg / L (equivalent to 0.66 μM) after 8 h as detailed and

shown in Chapter 3 (Section 3.4.3, Table 3.1). The concentration of ^{14}C – Endosulfan in the brain following three – time repeated administration of 5 mg / kg ^{14}C – Endosulfan to male Sprague rats was in the range of 0.74 μM to 3.88 μM as discussed in Chapter 3 (Section 3.4.3, Table 3.2). Since the concentration levels in the brain were low in the experiments in Chapter 3 and in order to maintain maximum survival of the cells, the dose range of 0.001 nM to 10 μM was chosen for all the experiments in Chapter 5.

5.2.5 Cell culture for transendothelial electrical resistance (TEER) study

Porcine brain microvascular endothelial cells (PBMECs) were obtained from Dainippon Pharmaceutical Co. Ltd. (Osaka, Japan). PBMECs were grown to confluence in 75 cm^2 collagen – coated tissue culture flasks in CS – C Complete Medium Kit (CSCCMK) and maintained in 95 % air and 5 % CO_2 at 37 $^\circ\text{C}$. Upon reaching confluence, PBMECs were briefly trypsinized to remove them from the tissue culture flasks and plated at a density of 5×10^5 cells / mL in 2 mL CSCCMK culture medium on the upper side of 6 – well PCF culture plate inserts and maintained in 95 % air and 5 % CO_2 at 37 $^\circ\text{C}$. 3 mL of the CSCCMK medium without PBMECs were added to the lower compartments of the 6 – well plates. Under these experimental conditions, PBMECs reached confluence three days after seeding. Figure 5.2 shows the schematic representation of the *in vitro* BBB model used in the current study.

C_I : Concentration of tracer chemical in the inner chamber
 V_I : Volume of the inner chamber
 C_O : Concentration of tracer chemical in the outer chamber
 V_O : Volume the outer chamber

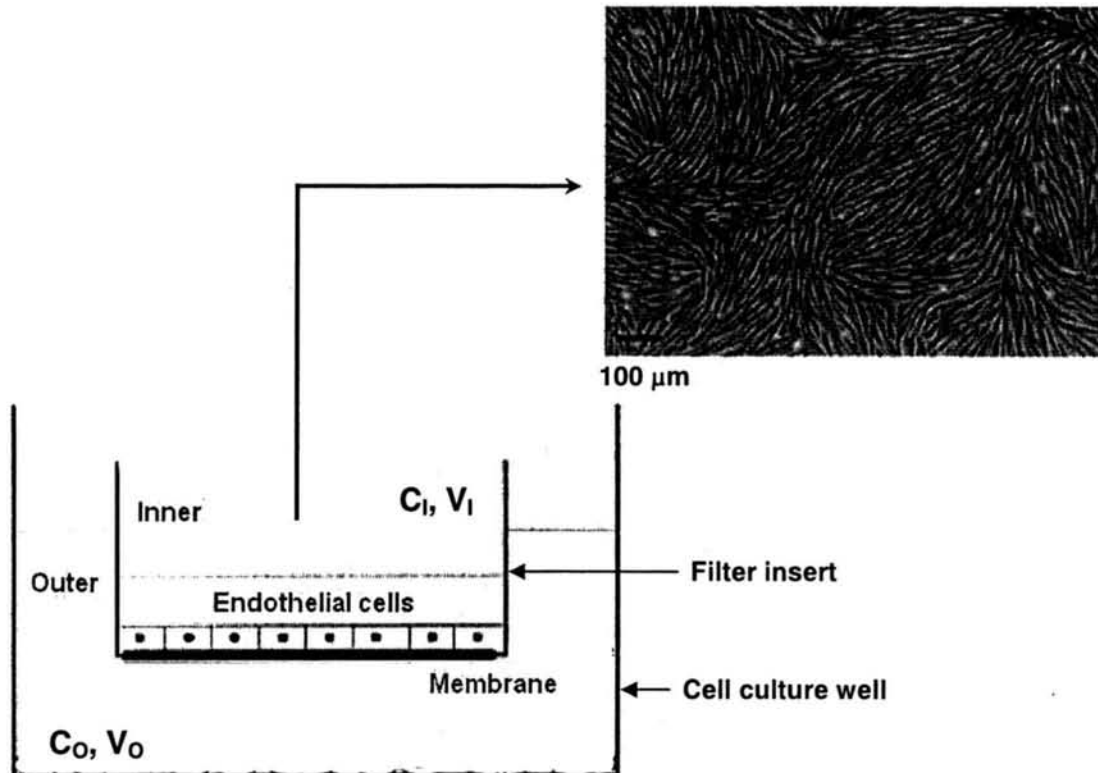


Figure 5.2. Schematic representation of the *in vitro* model of the blood – brain barrier (BBB). (A) Phase contrast micrograph of a confluent brain capillary endothelial cell monolayer. The inner compartment represents the blood compartment while the outer compartment represents the brain compartment. (Modified from Cecchelli et al., 1999).

5.2.6 Measurements of TEER

The TEER measurements were conducted with the Millipore Millicell® - ERS (Electrical Resistance System) (Bedford, MA, USA). The Millipore Millicell® - ERS reliably measures membrane potential and resistance of epithelial cells in culture. This device qualitatively measures cell monolayer health and quantitatively measures cell confluence. Figure 5.3 shows how measurement is taken by using the Millipore Millicell® - ERS.

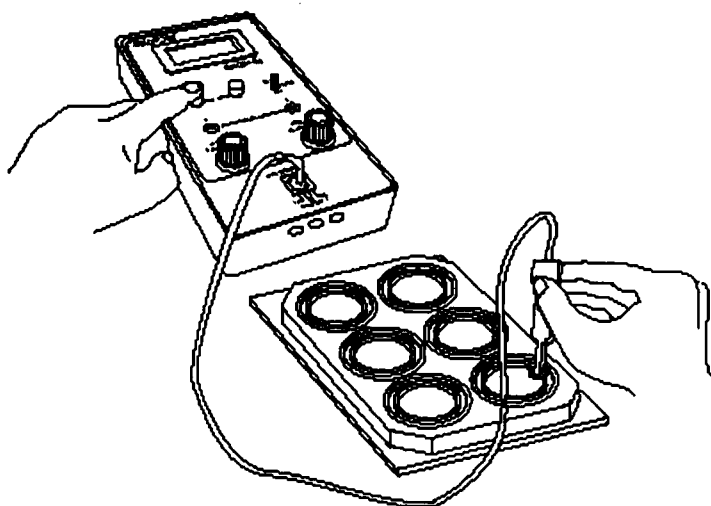


Figure 5.3. Measurement is taken by using the Millipore Millicell® - ERS.

In each experiment, the TEER of the PCF culture plate inserts without cells (control) was measured and subtracted from the inserts with cells and a correction for membrane surface area. TEER values were expressed as $\text{Ohm} \cdot \text{cm}^2$. The insert membrane surface area was 4.2 cm^2 for all calculations. The resistances of the monolayers were monitored every day until they reached a steady state. Once stable resistances were obtained ($> 120 \text{ Ohm} \cdot \text{cm}^2$), the cells were treated with α - endosulfan, β - endosulfan and endosulfan sulfate with final concentrations of 0.01

μM , $0.1 \mu\text{M}$, $1 \mu\text{M}$, $10 \mu\text{M}$ respectively. TEER was measured just before adding the respective endosulfan and at every hour for 6 hours. Measurements were taken in quadruplicate samples ($n = 4$) from three independent experiments (Mean $\pm$ SD).

5.2.6.1 Calculation of the resistance of the cell monolayer

- (1) The resistance of the blank (Millicell – PCF insert without cells) was measured and the blank resistance was denoted $R_{\text{blank}} = A \text{ Ohm}$
- (2) The resistance of the sample – well (Millicell – PCF insert with cells) was measured and the sample – well resistance was denoted $R_{\text{sample}} = B \text{ Ohm}$
- (3) The blank resistance was subtracted from the sample – well resistance measurement. Suppose a typical sample reading was $C \text{ Ohm}$. Then:

$$R_{\text{sample}} - R_{\text{blank}} = R_{\text{monolayer}}$$

$$B \text{ Ohm} - A \text{ Ohm} = C \text{ Ohm}$$

- (4) The resistance of the cell monolayer in this example was $C \text{ Ohm}$. However, a correction for the area covered by the cell monolayer was needed. Therefore, the resistance of the cell monolayer was multiplied by the area of the effective membrane diameter on the Millicell – PCF culture plate insert. In this experiment, the area of effective membrane diameter used was 4.2 cm^2 .

Then,
$$R_{\text{monolayer}} \times 4.2 \text{ cm}^2 = C \text{ Ohm} \times 4.2 \text{ cm}^2 = D \text{ Ohm} \cdot \text{cm}^2$$

5.2.7 Cytotoxicity study of PBMECs after treatment with α - endosulfan, β - endosulfan and endosulfan sulfate

Viability of PBMECs after treatment of α - endosulfan, β - endosulfan and endosulfan sulfate was assessed using a commercially available Cell Viability Kit (CCK – 8). The WST – 8 assay used here is more sensitive than the MTT [3 – (4, 5 –

dimethylthiazol – 2 – yl) – 2, 5 – diphenyltetrazolium bromide] assay, which is widely used in the measurement of cell viability. It measures the activity of mitochondrial dehydrogenase catalyzing the conversion of [2 – (2 – methoxy – 4 – nitrophenyl) – 3 – (4 – nitrophenyl) – 5 – (2, 4 – disulfophenyl) – 2H – tetrazolium monosodium salt] to water – soluble formazan (Dojindo Laboratories, Kumamoto, Japan). Briefly, PBMECs were plated onto the 96 – well microplate in CSCCMK culture medium at a density of 2×10^5 cells / mL together with α – endosulfan, β – endosulfan and endosulfan sulfate at different final concentrations of 0.001 nM, 0.01 nM, 0.1 nM, 1 nM, 0.01 μ M, 0.1 μ M, 1 μ M, 10 μ M respectively and incubated for 96 h. CCK – 8 solution was added to each well of the plate and incubated for another 4 h prior to measurements. The absorbance was measured at wavelength of 450 nm using Bio – Rad Model 550 microplate reader (Bio – Rad Laboratories, Inc., CA, USA). Each value was calculated from 6 to 8 culture wells ($n = 6 - 8$) from three independent experiments. Viability was expressed as a percentage of control (Mean $\pm$ SD).

5.2.8 Transendothelial transport (inner – to – outer) study with ^{14}C – Endosulfan

Transport studies with ^{14}C – Endosulfan were performed at final concentrations of 0.01 μ M, 0.1 μ M, 1 μ M, 10 μ M. 3 mL of the CSCCMK culture medium without PBMECs were added to the outer compartments of the 6 – well plates while 2 mL of the CSCCMK culture medium containing PBMECs were added to the inner compartments. 2 μ L of ^{14}C – Endosulfan were added to the culture plate inserts at various concentrations. Aliquots of 50 μ L were collected from the outer compartments at 20, 30, 45 min, 1, 2, 3, 4, 5, and 6 h for radioactivity analysis. For radioactivity analysis, 5 mL of Hionic – FluorTM was added to each 50 μ L sample and

the radioactivity was measured by means of a liquid scintillation counter (LSC 6100, Aloka, Tokyo, Japan).

5.2.8.1 Data analysis

To obtain a concentration – dependent transport parameter, clearance principle was used (Siflinger – Birnboim et al., 1987). The increment in cleared volumes between successive sampling points was calculated by dividing the amount of solute transport during the interval by the donor chamber concentrations. The volume of ¹⁴C – Endosulfan cleared from the inner chamber to the outer chamber (V) was calculated from:

$$V(\mu L) = \frac{C_o \times V_o}{C_i} \quad (5-1)$$

where C_i is the initial inner tracer concentration, C_o is the outer tracer concentration and V_o is the volume of the outer chamber. During the 6 h experiment, the clearance volume increased linearly with time. The clearance rate (dV / dt) is the slope of the clearance volume over time obtained by linear regression analysis, and given in $\mu L / \text{min}$ (or $10^{-3} \text{ cm}^3 / \text{min}$).

The transendothelial permeability of the filter grown endothelial cell monolayer to ¹⁴C – Endosulfan (Pe) was calculated from the clearance rate (dV / dt) and the surface area (S), and given in $\mu L / \text{cm}^2 / \text{min}$ (or $10^{-3} \text{ cm} / \text{min}$):

$$Pe = \frac{dV / dt}{S} \quad (5-2)$$

In this system, the movement of the tracer molecule occurs by diffusion since there are no hydrostatic and oncotic gradients.

5.2.9 PBMECs reversible transport (outer – to – inner) study with ^{14}C – Endosulfan

To assess the reversible effect of endosulfan on outer – inner compartment, PBMECs were grown on the culture plate inserts as previously described. 3 μL of ^{14}C – Endosulfan were added to the outer compartments of the 6 – well plates at various concentrations. Aliquots of 50 μL were collected from the inner compartments at 20, 30, 45 min, 1, 2, 3, 4, 5, and 6 h for radioactivity analysis. To obtain a concentration – dependent transport parameter, clearance principle was used as described in Section 5.2.8.1.

5.2.10 Statistical analysis

Results were expressed as Mean $\pm$ SD of three independent experiments. Any significant differences between test groups were assessed with one – or two – way ANOVA followed by Tukey's multiple comparison post test at significance level, $P < 0.05$ ($^{\circ}$ SPSS for Windows software package, Release 10.0, SPSS, Inc).

5.3 Results

5.3.1 Measurements of transendothelial electrical resistance (TEER)

The effects of α – endosulfan, β – endosulfan and endosulfan sulfate on TEER were measured in PBMECs monolayers for 6 h (Tables 5.1 – 5.3, Figures 5.4 – 5.6). Blank PCF inserts were used as an indicator of background effects on TEER and showed consistency at $144 \pm 6 \text{ Ohm} \cdot \text{cm}^2$. Controls showed no significant change in TEER over 6 h. TEER decreased significantly ($P < 0.05$) and reached the bottom level after 6 h of exposure to α – endosulfan, β – endosulfan and endosulfan sulfate. TEER values were the lowest at 10 μM as compared to other concentration levels for all

compounds. Generally, TEER declined as concentrations and exposure periods increased for α -endosulfan, β – endosulfan and endosulfan sulfate.

Table 5.1. Time – dependent transendothelial electrical resistance (TEER) values of PBMECs for α -endosulfan. Data are measured for quadruplicate samples ($n = 4$) from three independent experiments (Mean $\pm$ SD).

Time (Hour)	Transendothelial electrical resistance (TEER) values (Ohm $\cdot$ cm ²)				
	Control	0.01 μ M	0.1 μ M	1 μ M	10 μ M
0	125 $\pm$ 4	141 $\pm$ 5	125 $\pm$ 6	128 $\pm$ 2	122 $\pm$ 9
1	124 $\pm$ 13	64 $\pm$ 10	47 $\pm$ 2	39 $\pm$ 1	4 $\pm$ 1
2	116 $\pm$ 3	46 $\pm$ 2	49 $\pm$ 3	31 $\pm$ 4	(-)1 $\pm$ 1
3	107 $\pm$ 3	40 $\pm$ 5	40 $\pm$ 4	21 $\pm$ 1	5 $\pm$ 1
4	92 $\pm$ 2	50 $\pm$ 8	22 $\pm$ 2	10 $\pm$ 1	(-)11 $\pm$ 6
5	88 $\pm$ 1	41 $\pm$ 1	21 $\pm$ 6	4 $\pm$ 3	(-)11 $\pm$ 2
6	109 $\pm$ 9	28 $\pm$ 7	5 $\pm$ 2	(-)12 $\pm$ 3	(-)15 $\pm$ 2

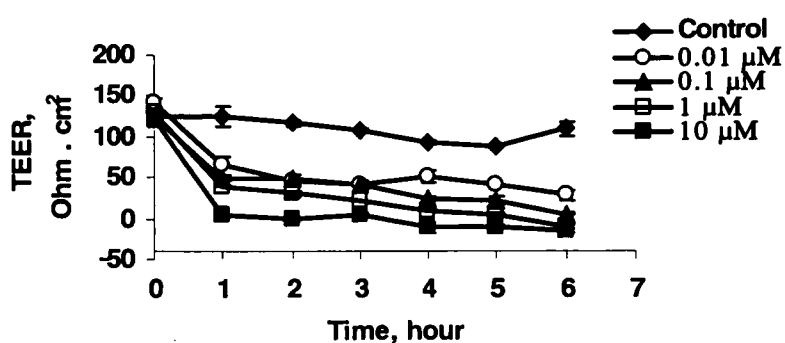


Figure 5.4. Time – dependent transendothelial electrical resistance (TEER) values of PBMECs for α -endosulfan.

Table 5.2. Time – dependent transendothelial electrical resistance (TEER) values of PBMECs for β - endosulfan. Data are measured for quadruplicate samples ($n = 4$) from three independent experiments (Mean $\pm$ SD).

Time (Hour)	Transendothelial electrical resistance (TEER) values (Ohm $\cdot$ cm ²)				
	Control	0.01 μ M	0.1 μ M	1 μ M	10 μ M
0	157 $\pm$ 6	159 $\pm$ 10	174 $\pm$ 18	168 $\pm$ 5	172 $\pm$ 9
1	166 $\pm$ 7	97 $\pm$ 2	89 $\pm$ 25	75 $\pm$ 11	77 $\pm$ 12
2	169 $\pm$ 8	105 $\pm$ 15	99 $\pm$ 15	82 $\pm$ 13	54 $\pm$ 13
3	155 $\pm$ 3	83 $\pm$ 3	85 $\pm$ 5	64 $\pm$ 2	41 $\pm$ 2
4	157 $\pm$ 10	76 $\pm$ 2	65 $\pm$ 3	51 $\pm$ 5	42 $\pm$ 4
5	159 $\pm$ 8	65 $\pm$ 6	60 $\pm$ 1	51 $\pm$ 6	34 $\pm$ 2
6	128 $\pm$ 8	49 $\pm$ 10	55 $\pm$ 1	19 $\pm$ 3	11 $\pm$ 2

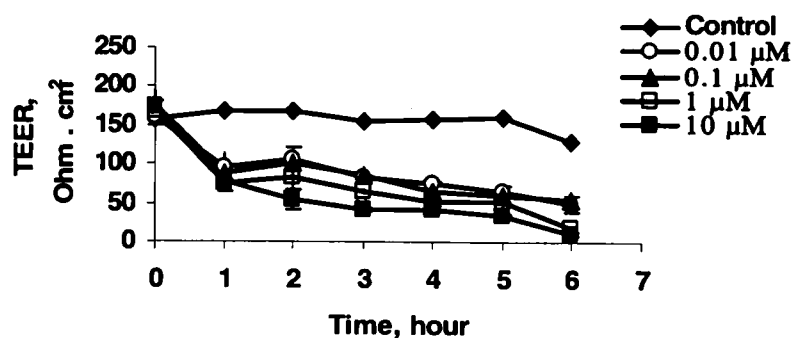


Figure 5.5. Time – dependent transendothelial electrical resistance (TEER) values of PBMECs for β - endosulfan.

Table 5.3. Time – dependent transendothelial electrical resistance (TEER) values of PBMECs for endosulfan sulfate. Data are measured for quadruplicate samples ($n = 4$) from three independent experiments (Mean $\pm$ SD).

Time (Hour)	Transendothelial electrical resistance (TEER) values (Ohm $\cdot$ cm ²)				
	Control	0.01 μ M	0.1 μ M	1 μ M	10 μ M
0	210 $\pm$ 7	210 $\pm$ 1	214 $\pm$ 3	225 $\pm$ 3	233 $\pm$ 1
1	213 $\pm$ 7	175 $\pm$ 6	146 $\pm$ 13	147 $\pm$ 6	120 $\pm$ 3
2	183 $\pm$ 4	144 $\pm$ 2	133 $\pm$ 3	126 $\pm$ 2	100 $\pm$ 3
3	155 $\pm$ 10	135 $\pm$ 3	124 $\pm$ 7	99 $\pm$ 2	64 $\pm$ 2
4	154 $\pm$ 8	111 $\pm$ 1	94 $\pm$ 2	85 $\pm$ 4	47 $\pm$ 2
5	148 $\pm$ 3	108 $\pm$ 6	101 $\pm$ 7	77 $\pm$ 3	39 $\pm$ 3
6	172 $\pm$ 16	102 $\pm$ 3	94 $\pm$ 4	74 $\pm$ 2	39 $\pm$ 2

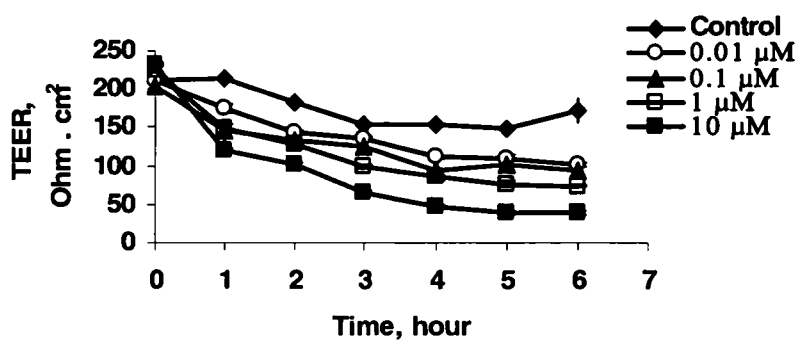


Figure 5.6. Time – dependent transendothelial electrical resistance (TEER) values of PBMECs for endosulfan sulfate.

5.3.2 Cytotoxicity analysis of endosulfan – treated PBMECs

To test the possibility that changes in TEER resulted from the death of cells and the subsequent formation of holes in the monolayer, the cell viability was measured using CCK – 8. The concentrations of 0.001 nM, 0.01 nM, 0.1 nM, 1 nM, 0.01 μ M, 0.1 μ M, 1 μ M, and 10 μ M for α – endosulfan, β – endosulfan and endosulfan sulfate did not decrease cell viability as compared to the control (Table 5.4, Figure 5.7). This indicates that the concentrations of 10 μ M and below did not cause cell death.

Table 5.4. Percentage of viability of PBMECs after α – endosulfan, β – endosulfan and endosulfan sulfate treatments. PBMECs were grown for 96 h were exposed to several concentrations of α – endosulfan, β – endosulfan and endosulfan sulfate individually and cell viability was measured by WST – 8 assay as detailed in Section 5.2.7. Cell viability is expressed as percentage of control. Data are measured for six to eight samples ($n = 6 - 8$) from three independent experiments (Mean $\pm$ SD).

Concentration (μ M)	Cell Viability (% of Control)		
	α - Endosulfan	β - Endosulfan	Endosulfan sulfate
0.000001	93.9 $\pm$ 7.2	97.3 $\pm$ 7.0	104.5 $\pm$ 4.5
0.00001	91.8 $\pm$ 1.2	95.2 $\pm$ 4.0	99.1 $\pm$ 8.1
0.0001	92.4 $\pm$ 3.5	87.7 $\pm$ 4.4	95.6 $\pm$ 4.8
0.001	97.9 $\pm$ 6.3	106.2 $\pm$ 5.0	99.4 $\pm$ 3.5
0.01	96.0 $\pm$ 7.3	98.7 $\pm$ 7.2	102.6 $\pm$ 7.1
0.1	92.3 $\pm$ 3.9	98.7 $\pm$ 2.6	95.9 $\pm$ 4.9
1	89.9 $\pm$ 4.4	97.9 $\pm$ 7.0	96.7 $\pm$ 4.9
10	89.4 $\pm$ 3.1	112.9 $\pm$ 10.9	96.3 $\pm$ 5.2

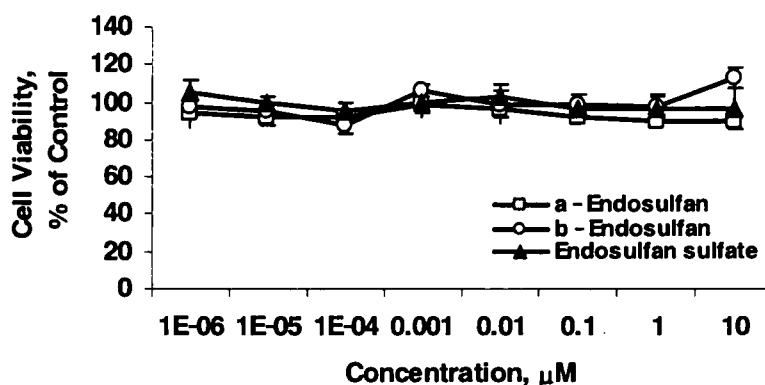
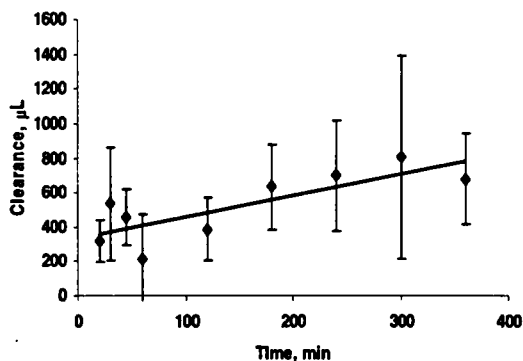


Figure 5.7. Concentration – response curves of cytotoxicity induced by exposure of PBMECs to α - endosulfan, β – endosulfan and endosulfan sulfate.

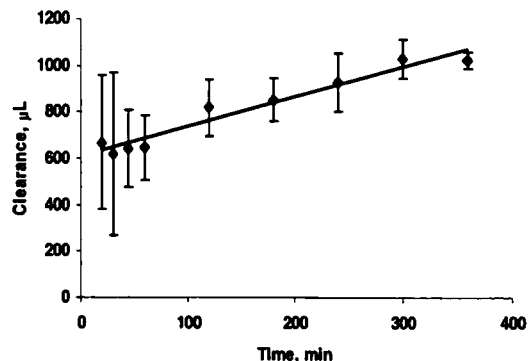
5.3.3 Transendothelial transport (inner – to – outer) study with ^{14}C – Endosulfan

Figure 5.8 shows the plots of clearance per centimeter squared versus time for different concentrations of ^{14}C – Endosulfan. Figure 5.9 and Table 5.5 show the average permeability of the filter grown endothelial cell monolayer to endosulfan (P_e) for different concentrations of ^{14}C – Endosulfan. P_e for 10 μM was high [0.47 ± 0.09 ($\times 10^{-3}$) cm / min] as compared to 1 μM , 0.1 μM and 0.01 μM which remained almost plateau. This indicates that no noticeable divergence of P_e value existed for the concentrations of 1 μM and below.

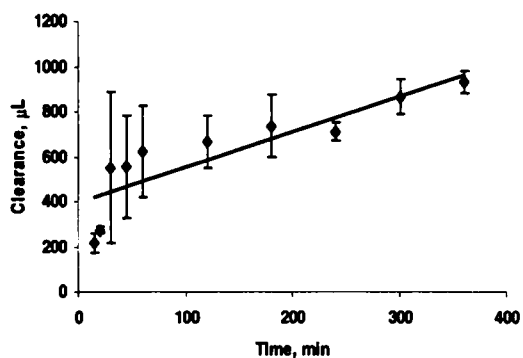
A : 0.01 μ M



B : 0.1 μ M



C : 1 μ M



D : 10 μ M

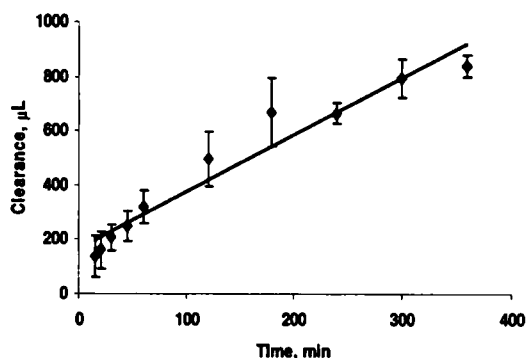


Figure 5.8. Transport profiles of ^{14}C – Endosulfan for (A) 0.01 μM , (B) 0.1 μM , (C) 1 μM and (D) 10 μM from inner - outer compartment. Data are measured for triplicate samples ($n = 3$) from three independent experiments. Each value is the Mean $\pm$ SD of triplicate experiments.

Table 5.5. Effect of inner concentrations of ^{14}C – Endosulfan on the average permeability (Pe) of the filter grown endothelial cell monolayer. Data are measured for triplicate samples ($n = 3$) from three independent experiments. Each value is the Mean $\pm$ SD of triplicate experiments.

^{14}C – Endosulfan (μM)	Average permeability, Pe ($\times 10^{-3}$ cm / min)
0.01	0.31 ± 0.19
0.1	0.29 ± 0.14
1	0.35 ± 0.13
10	0.47 ± 0.06

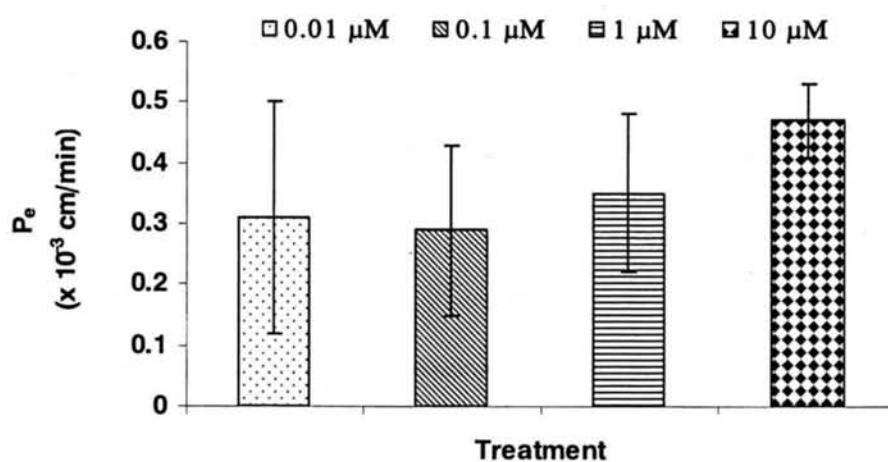


Figure 5.9. Average permeability of the filter grown endothelial cell monolayer to ^{14}C – Endosulfan (Pe) (0.01 μM , 0.1 μM , 1 μM and 10 μM) across PBMEC monolayers from inner – outer compartment.

5.3.4 PBMECs reversible transport (outer – to – inner) study with ^{14}C – Endosulfan

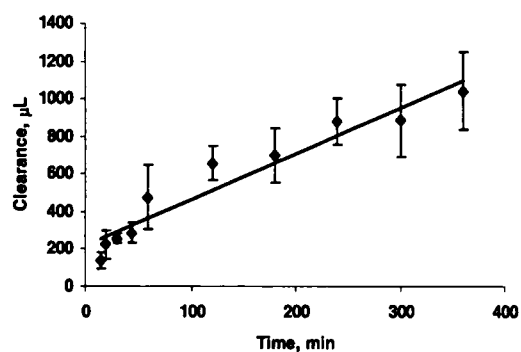
Figure 5.10 shows the plots of clearance per centimeter squared versus time for different concentrations of ^{14}C – Endosulfan. Figure 5.11 and Table 5.6 show the average Pe for different concentrations of ^{14}C – Endosulfan. Pe for 0.01 μM was low [0.50 ± 0.22 ($\times 10^{-3}$) cm / min] as compared to 0.1 μM , 1 μM and 10 μM . The ratio between the outer – to – inner and inner – to – outer compartments for the transport study of ^{14}C – Endosulfan in the concentration range of 0.01 – 10 μM was approximately 1.2 – 2.1.

5.4 Discussion

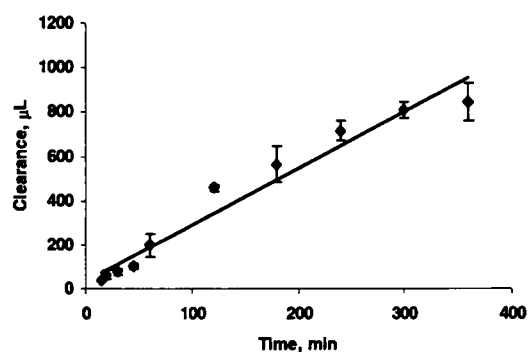
The homeostasis of the microenvironment of central nervous system (CNS), which is essential for normal function, is maintained by blood – brain barrier (BBB). However, the lack of reliable *in vitro* models is one of the major issues in BBB research to examine in an easy and fast manner cellular and molecular mechanisms of BBB function under normal and pathological conditions (Boveri et al., 2005; Cecchelli et al., 1999). Transendothelial electrical resistance (TEER), an important indication of the BBB integrity, is a useful physiological marker and is well – known that the establishment of tight junction correlates with development of TEER (Gonzales – Marisca et al., 1985). In a review by Madara (1998) on the regulation of the movement of solutes across (epithelial) tight junctions for inulin and mannitol, the relationship between electrical resistance and transport of solutes is non – linear. He explained that solute transport is essentially dependent on the sum of transport across all junctional pathways, whereas total electrical resistance is essentially dependent on

areas with the lowest electrical resistance between single cells, even when these low resistance areas are present at a low density.

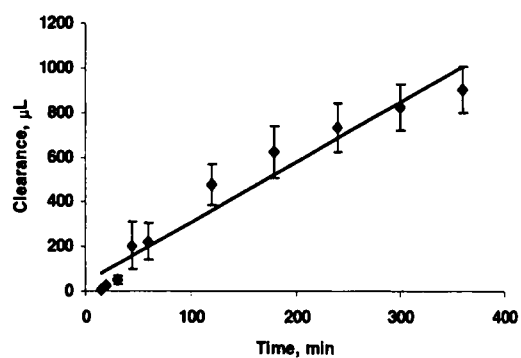
A : 0.01 μ M



B : 0.1 μ M



C : 1 μ M



D : 10 μ M

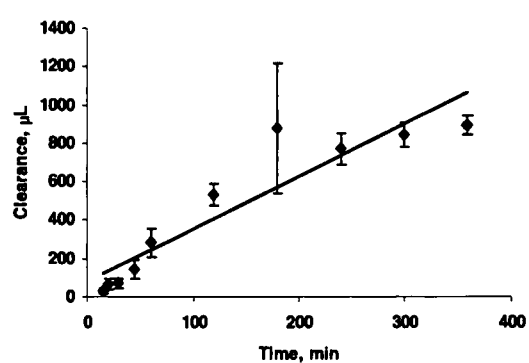


Figure 5.10. Transport profiles of ^{14}C – Endosulfan for (A) 0.01 μM , (B) 0.1 μM , (C) 1 μM and (D) 10 μM from outer - inner compartment. Data are measured for triplicate samples ($n = 3$) from three independent experiments. Each value is the Mean $\pm$ SD of triplicate experiments.

Table 5.6. Effect of outer concentrations of ^{14}C – Endosulfan on the average permeability (Pe) of the filter grown endothelial cell monolayer. Data are measured for triplicate samples ($n = 3$) from three independent experiments. Each value is the Mean $\pm$ SD of triplicate experiments.

^{14}C – Endosulfan (μM)	Average permeability, Pe ($\times 10^{-3}$ cm / min)
0.01	0.50 ± 0.21
0.1	0.60 ± 0.05
1	0.61 ± 0.06
10	0.58 ± 0.09

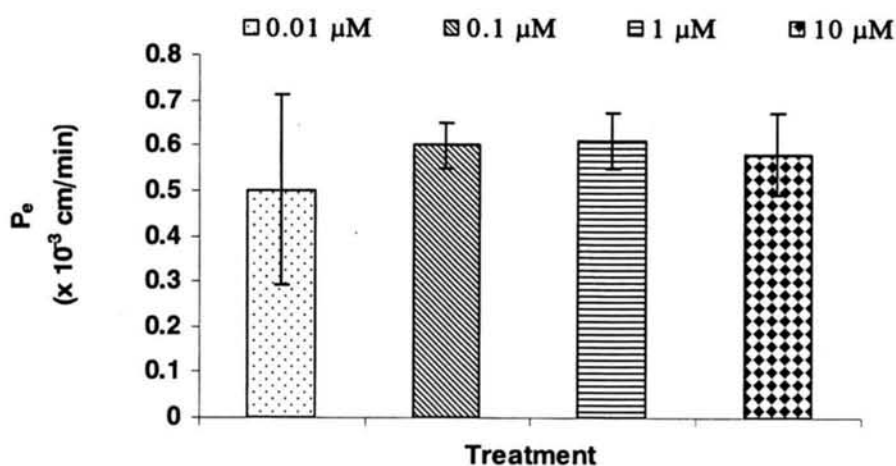


Figure 5.11. Average permeability of the filter grown endothelial cell monolayer to ^{14}C – Endosulfan (Pe) (0.01 μM , 0.1 μM , 1 μM and 10 μM) across PBMEC monolayers from outer - inner compartment.

Recent research has developed methods of cultivating the PBMECs or bovine brain microvascular endothelial cells (BBMECs) with astrocyte co – cultures that results in higher junctions (Boveri et al., 2005; Jeliaskova – Mecheva et al., 2003; Megard et al., 2002). We decided to use the PBMECs without astrocyte co – culture to evaluate the permeability effects of endosulfan. The resistance of $120 \text{ Ohm} \cdot \text{cm}^2$ in our BBB model is adequate to show BBB function as in other model using primary cultures of BBMECs grown as monolayers on polycarbonate filters (Raub et al., 1992).

The present study focuses on how PBMECs respond to the treatment of endosulfan. We studied the effects of α – endosulfan, β – endosulfan and endosulfan sulfate on the TEER across cultured monolayers of PBMECs. It is also the first report demonstrating the effects of α – endosulfan, β – endosulfan and endosulfan sulfate in PBMECs cultures. Concentrations of $10 \mu\text{M}$ and below did not affect cell viability as demonstrated by the WST – 8 assay (Table 5.4). These data suggest that endosulfan is not toxic to the PBMECs at the concentrations evaluated. The BBB permeability, measured as TEER, decreased significantly in a dose – and time – dependent manner when monolayers were treated with α – endosulfan, β – endosulfan and endosulfan sulfate (0.01 , 0.1 , 1 and $10 \mu\text{M}$) as shown in Figures 5.4 – 5.6. TEER reached the bottom level after 6 h of exposure to different concentrations of α – endosulfan, β – endosulfan and endosulfan sulfate. In Figure 5.4, $10 \mu\text{M}$ α – endosulfan exerted the most severe and instantaneous effect, with significant decrease in TEER as compared to other concentration levels and compounds. TEER dropped to $4 \text{ Ohm} \cdot \text{cm}^2$ after 1 h of the initiation of treatment and remained at the bottom level throughout the exposure period. Generally, TEER declined as concentration levels and exposure periods increased (Tables 5.1 – 5.3, Figures 5.4 – 5.6). This phenomenon

demonstrates that the alterations of the endothelial permeability are dependent on the length and severity of endosulfan.

The role of the brain endothelial monolayer is to limit the nonspecific exchanges between blood and brain compartments, behaving as a physical barrier not only to ions, but also to hydrophilic molecules (Boveri et al., 2005; Rubin and Staddon, 1999; Ohtsuki, 2004). When BBB is disrupted (e. g., by disruption of the tight junctions, or by transendothelial channel or pore formation, induced by drugs or diseases), changes in the Pe of the drug are expected to be observed. Hence, large changes in Pe will be measured with a leaky *in vitro* BBB (i. e., when TEER values become low) (Gaillard and de Boer, 2000). We further studied the integrity of the brain endothelium by measuring the transendothelial permeability of the filter grown endothelial cell monolayer to ^{14}C – Endosulfan.

In the present study, we observed that the average Pe for the inner – to – outer transport study after exposure to 0.01, 0.1, and 10 μM ^{14}C – Endosulfan was lower (Table 5.5) than the average Pe for the outer – to – inner transport study (Table 5.6), thus suggesting that the brain – to – blood efflux transport rate is faster than the blood – to – brain influx transport rate. This may be accounted for by active transport by P – glycoprotein (P – gp) in the BBB (Megard et al., 2002; Pardridge, 1995). The low Pe from the inner – to – outer compartment may be attributed to the high lipophilic nature of this compound ($\log P_{\text{octanol} / \text{water}} = 3.55$) [Agency for Toxic Substances and Disease Registry (ATSDR), 2000]. However, the reason for this phenomenon is not clear, thus suggesting that further research is necessary to elucidate this phenomenon.

It was unknown whether endosulfan crosses the cell monolayers via the transcellular route (i. e., diffusing through the cytoplasm of the cells, partitioning into and out of the apical and basolateral cell membranes) or through the paracellular route by means of the aqueous pores between cells. Since endosulfan is lipophilic, we speculated that transcellular route is the most probable transport route as has been reported elsewhere for lipophilic drugs (Megard et al., 2002; Yoo et al., 2003). Further studies are necessary to identify transporters and tight junction proteins at the BBB as well as its mechanism.

5.5 Conclusions

The BBB permeability, measured as TEER, decreased significantly in a dose - and time - dependent manner when treated with α - endosulfan, β - endosulfan and endosulfan sulfate individually. Cytotoxicity test revealed that α - endosulfan, β - endosulfan and endosulfan sulfate did not cause cell death at 0.001 nM, 0.01 nM, 0.1 nM, 1 nM, 0.01 μ M, 0.1 μ M, 1 μ M, and 10 μ M. The average Pe for the inner - to - outer transport study after exposure to 0.01, 0.1, and 10 μ M ^{14}C - Endosulfan was lower than the average Pe for the outer - to - inner transport study. The ratio between the outer - to - inner and the inner - to - outer compartments for the transport study of ^{14}C - Endosulfan in the concentration range of 0.01 - 10 μ M was approximately 1.2 - 2.1.

To our knowledge, this is the first study that reports on the development of an *in vitro* BBB model to study the ability of endosulfan to open tight junctions in PBMECs cultures. It is hoped that the current model may proved useful and as an initial step to developing more complex models to understand the mechanism at the tight junctions

of the BBB. Additional research is necessary to improve the current model until optimal conditions with respect to the medium composition, attachment factors, seeding density and pore sizes of the Millicell filters are archived as well as the possibility of co – culture models. It is hoped that the improved models will further increase our understanding on the pharmacology and toxicology effects of endosulfan as well as its influences on the barrier under *in vitro* conditions.

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CHAPTER 6

A COMPARATIVE STUDY ON THE NEUROTOXIC EFFECTS OF ENDOSULFAN ON GLIAL AND NEURONAL CELL CULTURES FROM RAT AND HUMAN

6.0 Introduction

As has been demonstrated in the *in vitro* experiments in Chapter 5, endosulfan, a chlorinated hydrocarbon insecticide, penetrated through the blood – brain barrier (BBB). Residues of endosulfan have been detected in the brain of rats treated with endosulfan (Ansari et al., 1984; Chan et al., 2005; Dikshith et al., 1984; Gupta, 1978; Nath et al., 1978) and humans (Boereboom et al., 1998; Coutselinis et al., 1978; Eyer et al., 2004). Although toxicity is dependent on the sensitivity and developmental stage of target cells, concentration of test compound, and duration of exposure, it has been proposed that any chemical that passes the BBB is neurotoxic (Jean Harry et al., 1997; Walnum et al., 1990). Hence, endosulfan is classified as a neurotoxic agent based on the above mentioned hypothesis. This also raises more debate since the actual mechanism remained unknown although endosulfan has been extensively studied for the past decades. Currently, the GABA – antagonism mechanism of toxicity is the most widely accepted hypothesis [Agency for Toxic Substances and Disease Registry (ATSDR), 2000].

6.0.1 Principles of the nervous system – Potential sites of neurotoxic attack

The autonomic nervous system coordinates the regulation and integration of body functions. The nervous system exerts its influence by the rapid transmission of electrical impulses over nerve fibers that terminate at effector cells, where specific

effects are caused due to the release of a neuromediator substance (Mycek et al., 2000). The nervous system comprises of two components: the central nervous system (CNS), which is composed of the brain and spinal cord, and the peripheral nervous system (PNS), which comprises the ganglia and the peripheral nerves lying outside of the brain and spinal cord inclusive of the autonomic nervous system. Two general types of cells are predominant in the nervous system: neurons and neuroglial cells (oligodendrocytes, astrocytes, microglia; and in the PNS, the Schwann cells) (Jean Harry et al., 1990; Mycek et al., 2000). The principal neurotoxic action of chlorinated hydrocarbon insecticide is on the axons of nerve cells in the central nervous system (CNS). They interfere with the normal flux of sodium and potassium ions across the axon membrane and neural conduction and do not depress cholinesterase enzymes (Lo et al., 1995).

Neurons are similar to all other cells of the body in general structure, but they have additional anatomical features of axons and dendrites that allow conduction of nerve impulses for communication with other neural cells and between neuronal populations. Neurons are highly specialized cells and are responsible for the reception, integration, transmission, and storage of information. Afferent neuronal pathways carry information into the nervous system for processing; efferent pathways carry commands to the periphery. In addition, there are interneurons that process local information and transfer data within the nervous system. Within the CNS, neurons are segregated into functionally related nuclei that form interconnecting bundles of axonal fibers. Higher organizational levels consisting of several functionally related neurons are frequently called systems, e. g., motor, visual, associative, and neuroendocrine systems. The CNS consists of a number of systems responsible for

the coordination of receiving and processing information from the environment, maintaining the balance of all other organs systems, and responding to changes in the environment.

Glial cells compose a voluminous “support” system that is essential to the proper operation of nervous tissue / the nervous system. Unlike neurons, glial cells have no true synaptic contacts; however, receptors for several neurotransmitters are present and functional on various glial cells. Cell – cell contacts exist between the glial cells and neurons that may regulate both neuronal and glial differentiation and are critical for the glial – guided migration of neurons during development. Glial cells have a dynamic nature and perform a plethora of functions in the nervous system. They provide critical processes necessary to maintain normal functioning of the nervous system (e. g., regulation of local pH and ionic balances, and tropic support for neurite extension in the form of growth factors and cell adhesion factors). They can also be the target for a toxic response.

6.0.2 Mode of action of endosulfan on the CNS

Information indicates that the CNS is the major target of endosulfan – induced in humans and animals following acute exposure by any routes (Aleksandrowicz, 1979; Blanco – Coronado et al., 1992; Boereboom et al., 1998; Boyd and Dobos, 1969; Boyd et al., 1970; Gupta and Chandra, 1975; Shemesh et al., 1988; Terviez et al., 1974). The proposed modes of action of endosulfan include a global action on all presynaptic neurons in the CNS, inhibition of the calmodulin – dependent Ca^{2+} – ATPase activity (Srikanth et al., 1989), alterations in the serotonergic system (Agrawal et al., 1983) and inhibited GABA receptors (Abalis and Eldefrawi, 1986;

Rosa et al., 1996). Brain damage may also be due to the hypoxemia of seizures (Shemesh et al., 1988).

6.1 Objective of the study

Taking into account the present concern regarding the environmental impact of endosulfan on public health, the present study was aimed to study the *in vitro* neurotoxic effects of α - endosulfan, β - endosulfan and endosulfan sulfate by comparing the ability of α - endosulfan, β - endosulfan and endosulfan sulfate to cause cell death in glial and neuronal cell cultures from rat and human. The evaluation was based on the percentage of cell viability determined with WST – 8 assay.

6.2 Materials and methods

6.2.1 Chemicals

All reference analytical standards of α - endosulfan, β - endosulfan and endosulfan sulfate (> 99 % purity), phosphate buffered saline (PBS) powder and dimethyl sulfoxide (DMSO) were supplied by Wako Pure Chemical Industries Ltd. (Osaka, Japan). Trypsin – EDTA, fetal bovine serum (FBS) and Ham's 10 medium were purchased from Gibco BRL (Grand Island, NY, USA). RPMI 1640 medium and Dulbecco's modified Eagle's medium (DMEM) were obtained from Nikken Biomedical Laboratory (Kyoto, Japan). Horse serum was supplied by ICN Biomedicals Inc. (Ohio, USA). Cell Viability Kit (CCK – 8) was obtained from Dojindo Laboratories (Kumamoto, Japan). All chemicals used in the present study were of the highest grade available.

6.2.2 Materials

Falcon BIOCOAT™ Poly D – Lysine tissue culture flasks were obtained from Becton – Dickinson (NJ, USA). Iwaki 96 – well micro plates and tissue culture flasks were purchased from Wakenyaku Co. Ltd. (Kyoto, Japan).

6.2.3 Preparations of chemicals

α – endosulfan, β – endosulfan and endosulfan sulfate were dissolved in DMSO individually to obtain concentrations of 1 nM, 0.01 μ M, 0.1 μ M, 1 μ M, 10 μ M, 100 μ M, 1 mM, 10 mM. These standard solutions will be used for the cytotoxicity studies of rat (PC12, C6) and human (NT2 and CCF – STTG1) cells.

6.2.4 Rationale for dose selection

The concentrations of ^{14}C – Endosulfan in the brain following single administration of 5 mg / kg ^{14}C – Endosulfan to male Sprague rats were 0.11 mg / L (equivalent to 0.27 μ M) after 30 min and 0.27 mg / L (equivalent to 0.66 μ M) after 8 h as detailed and shown in Chapter 3 (Section 3.4.3, Table 3.1). The concentration of ^{14}C – Endosulfan in the brain following three – time repeated administration of 5 mg / kg ^{14}C – Endosulfan to male Sprague rats was in the range of 0.74 μ M to 3.88 μ M as discussed in Chapter 3 (Section 3.4.3, Table 3.2). Since the concentration levels in the brain were low in the experiments in Chapter 3 and in order to maintain maximum survival of the cells, the dose range of 0.001 nM to 10 μ M was chosen for all the experiments in Chapter 6.

6.2.5 Cell cultures

Rat PC12 cell line was purchased from American Type Culture Collection (ATCC) while rat C6, human NT2 and human CCF – STTG1 cell lines were purchased from Dainippon Pharmaceutical Co. Ltd. (Osaka, Japan). PC12 cells were grown to confluence in Falcon BIOCOAT™ Poly D – Lysine tissue culture flasks in RPMI 1640 medium supplemented with 5 % FBS and 10 % horse serum. C6 cells were cultured in Ham's 10 medium containing 2.5 % FBS and 15 % horse serum. NT2 cells were grown in DMEM supplemented with 10 % FBS while CCF – STTG1 cells were cultured in RPMI 1640 medium containing 10 % FBS. All cells were maintained in 95 % air and 5 % CO₂ at 37 °C.

6.2.6 Cytotoxicity studies of PC12, C6, NT2 and CCF – STTG1 cells after treatment with α – endosulfan, β – endosulfan and endosulfan sulfate

Viability of all cells after treatment of α – endosulfan, β – endosulfan and endosulfan sulfate was assessed using a commercially available Cell Viability Kit (CCK – 8) as described previously in Chapter 5, Section 5.2.7. Upon reaching confluence, all cells were briefly trypsinized to remove them from the tissue culture flasks and plated onto the 96 – well microplates in respective mediums at a density of 2×10^5 cells / mL together with α – endosulfan, β – endosulfan and endosulfan sulfate at different concentrations of 0.001 nM, 0.01 nM, 0.1 nM, 1 nM, 0.01 μ M, 0.1 μ M, 1 μ M, 10 μ M respectively and incubated for 96 h. CCK – 8 solution was added to each well of the plate and incubated for another 4 h prior to measurements. The absorbance was measured at wavelength of 450 nm using Bio – Rad Model 550 microplate reader (Bio – Rad Laboratories, Inc., CA, USA). Viability was expressed as a percentage of control (Mean $\pm$ SD) of three independent experiments.

6.2.7 Statistical analysis

The study of each cell line consisted of three independent experiments with six to eight samples ($n = 6 - 8$) on each concentration level. The means and standard errors (Mean $\pm$ SD) of independent tests were calculated on each concentration level. Results were expressed as Mean $\pm$ SD of three independent experiments. Any significant differences between test groups were assessed with one – way ANOVA followed by Tukey's multiple comparison post test at significance level, $P < 0.05$. The median toxic concentration (LC_{50}) values were calculated by linear regression analysis and evaluated by one – way ANOVA followed by Tukey's multiple comparison test at significance level, $P < 0.05$ ([®]SPSS for Windows software package, Release 10.0, SPSS, Inc).

6.3 Results

6.3.1 Cytotoxicity study of rat C6 glial cells

Cell viability for the rat C6 glial cells as compared to control was above 80 % for all concentrations as shown in Table 6.1. Figure 6.1 shows the concentration – response curves of cytotoxicity for α -endosulfan, β – endosulfan and endosulfan sulfate. This indicates that the endosulfan concentrations of 10 μ M and below did not cause cell death. One – way ANOVA showed significant effect of α – endosulfan, β – endosulfan and endosulfan sulfate concentrations [$F = 17.663$, $P < 0.05$ (α – endosulfan); $F = 3.639$, $P < 0.05$ (β – endosulfan); $F = 2.805$, $P < 0.05$ (endosulfan sulfate)].

6.3.2 Cytotoxicity study of rat PC12 neuronal cells

The viability of all cells was tested by WST – 8 assays. The concentrations of 0.001 nM, 0.01 nM, 0.1 nM, 1 nM, 0.01 μ M, 0.1 μ M, 1 μ M, and 10 μ M for α – endosulfan, β – endosulfan and endosulfan sulfate did not decrease cell viability as compared to the control for rat PC12 neuronal cells (Table 6.2). Figure 6.2 shows the concentration – response curves of cytotoxicity for α – endosulfan, β – endosulfan and endosulfan sulfate. Cell viability was above 80 % for all concentrations except that a slightly below 80 % of cell viability was observed for the 10 μ M concentration level with 78.9 % for β – endosulfan and 71.9 % for endosulfan sulfate respectively. One – way ANOVA showed significant effect of β – endosulfan and endosulfan sulfate concentrations [$F = 5.985$, $P < 0.05$ (β – endosulfan); $F = 7.401$, $P < 0.05$ (endosulfan sulfate)].

6.3.3 Cytotoxicity study of human CCF – STTG1 glial cells

Figure 6.3 shows the concentration – response curves of cytotoxicity for α – endosulfan, β – endosulfan and endosulfan sulfate. Cell viability for the human CCF – STTG1 glial cells was above 80 % for all concentrations except that after treatments of 0.1 μ M, 1 μ M, and 10 μ M, cell viability decreased to 72.3 %, 68.2 % and 51.6 % for α – endosulfan, and 75.6 %, 70.4 % and 63.2 % respectively for endosulfan sulfate (Table 6.3). One – way ANOVA showed significant effect of α – endosulfan and endosulfan sulfate concentrations [$F = 5.225$, $P < 0.05$ (α – endosulfan); $F = 7.412$, $P < 0.05$ (endosulfan sulfate)].

6.3.4 Cytotoxicity study of human NT2 neuronal cells

Figure 6.4 shows the concentration – response curves of cytotoxicity for α – endosulfan, β – endosulfan and endosulfan sulfate. Cell viability for the human NT2 neuronal cells as compared to control was above 80 % for all concentrations except that after treatment with 10 μ M, cell viability was approximately 50 % for 52.2 % for α – endosulfan, 49.0 % for β – endosulfan and 46.5 % for endosulfan sulfate respectively (Table 6.4). One – way ANOVA showed significant effect of α – endosulfan, β – endosulfan and endosulfan sulfate concentrations [$F = 6.318$, $P < 0.001$ (α – endosulfan); $F = 5.787$, $P < 0.05$ (β – endosulfan); $F = 5.980$, $P < 0.05$ (endosulfan sulfate)].

Table 6.1. Percentage of viability of C6 cells after exposure to α – endosulfan, β – endosulfan and endosulfan sulfate. Cell viability is expressed as percentage of control. Data are Mean $\pm$ SD from six – eight samples ($n = 6 - 8$) of three independent experiments. The significance compared with the controls is expressed as * $P < 0.05$.

Concentration (μ M)	Cell Viability (% of Control)		
	α – Endosulfan	β – Endosulfan	Endosulfan sulfate
0.000001	88.7 $\pm$ 1.5 *	102.4 $\pm$ 10.6	101.3 $\pm$ 7.7
0.00001	79.2 $\pm$ 5.0 *	92.1 $\pm$ 8.6	87.4 $\pm$ 10.5
0.0001	76.6 $\pm$ 2.0 *	87.1 $\pm$ 9.0	87.7 $\pm$ 9.2
0.001	74.6 $\pm$ 3.5 *	87.2 $\pm$ 7.7	88.5 $\pm$ 7.8
0.01	75.9 $\pm$ 1.9 *	84.7 $\pm$ 5.3	85.3 $\pm$ 7.6
0.1	73.9 $\pm$ 0.3 *	85.3 $\pm$ 2.2	83.0 $\pm$ 6.8
1	73.1 $\pm$ 0.8 *	79.1 $\pm$ 6.1	80.8 $\pm$ 4.7
10	78.5 $\pm$ 0.1 *	84.9 $\pm$ 5.7 *	81.7 $\pm$ 9.7

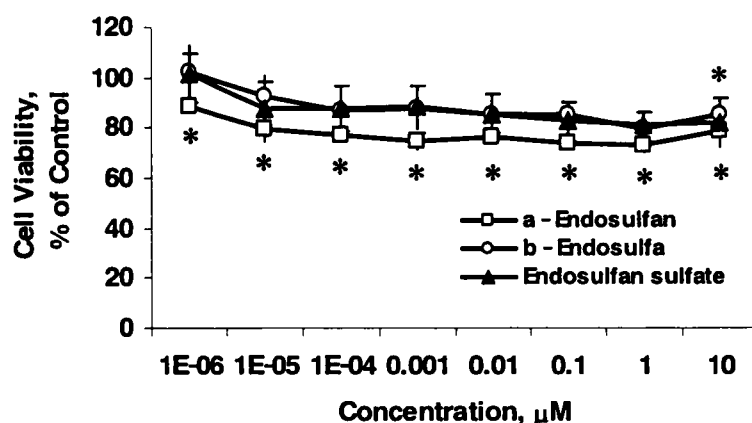


Figure 6.1. Concentration – response curves of cytotoxicity induced by exposure of C6 cells to α - endosulfan, β – endosulfan and endosulfan sulfate.

Table 6.2. Percentage of viability of PC12 cells after exposure to α – endosulfan, β – endosulfan and endosulfan sulfate. Cell viability is expressed as percentage of control. Data are Mean $\pm$ SD from six – eight samples ($n = 6 - 8$) of three independent experiments. The significance compared with the controls is expressed as * $P < 0.05$.

Concentration (μ M)	Cell Viability (% of Control)		
	α - Endosulfan	β - Endosulfan	Endosulfan sulfate
0.000001	100.3 $\pm$ 9.8	105.1 $\pm$ 1.5	126.5 $\pm$ 16.9
0.00001	93.3 $\pm$ 4.4	113.3 $\pm$ 9.6	126.7 $\pm$ 20.0
0.0001	97.9 $\pm$ 8.2	112.2 $\pm$ 5.3	114.4 $\pm$ 7.1
0.001	98.1 $\pm$ 10.3	98.2 $\pm$ 9.1	123.3 $\pm$ 4.8
0.01	93.6 $\pm$ 5.1	99.8 $\pm$ 10.0	107.8 $\pm$ 10.1
0.1	94.8 $\pm$ 5.8	96.8 $\pm$ 4.6	100.1 $\pm$ 4.8
1	94.6 $\pm$ 3.0	91.7 $\pm$ 5.4	91.3 $\pm$ 17.1
10	84.1 $\pm$ 3.6	78.9 $\pm$ 11.7	71.9 $\pm$ 6.1

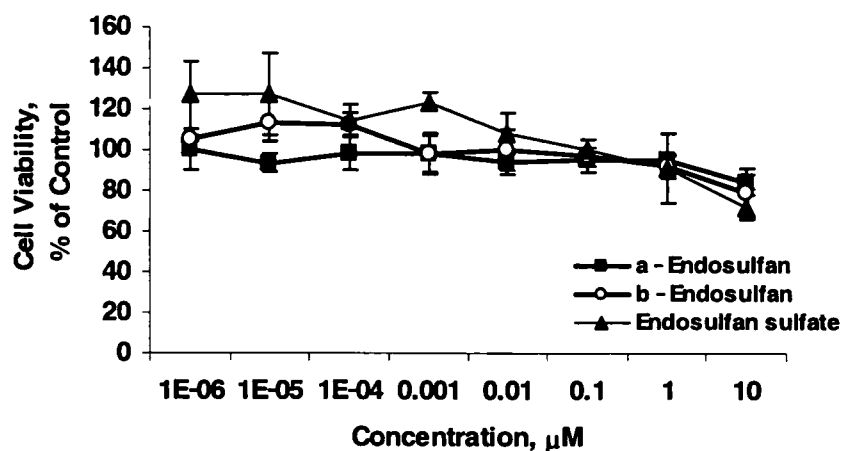


Figure 6.2. Concentration – response curves of cytotoxicity induced by exposure of PC12 cells to α - endosulfan, β - endosulfan and endosulfan sulfate.

Table 6.3. Percentage of viability of CCF – STTG1 cells after exposure to α - endosulfan, β - endosulfan and endosulfan sulfate. Cell viability is expressed as percentage of control. Data are Mean $\pm$ SD from six – eight samples ($n = 6 - 8$) of three independent experiments. The significance compared with the controls is expressed as * $P < 0.05$.

Concentration (μ M)	Cell Viability (% of Control)		
	α - Endosulfan	β - Endosulfan	Endosulfan sulfate
0.000001	86.6 $\pm$ 2.2	94.8 $\pm$ 13.2	108.8 $\pm$ 4.6
0.00001	83.3 $\pm$ 5.7	98.6 $\pm$ 9.8	97.9 $\pm$ 14.0
0.0001	83.9 $\pm$ 11.6	96.2 $\pm$ 8.2	97.1 $\pm$ 7.8
0.001	79.6 $\pm$ 16.0	87.1 $\pm$ 4.2	87.9 $\pm$ 9.6
0.01	82.2 $\pm$ 11.0	88.1 $\pm$ 7.5	81.9 $\pm$ 4.8
0.1	72.3 $\pm$ 14.8	85.2 $\pm$ 8.3	75.6 $\pm$ 7.2
1	68.2 $\pm$ 11.1 *	82.2 $\pm$ 9.2	70.4 $\pm$ 11.9 *
10	51.6 $\pm$ 9.0 *	98.0 $\pm$ 11.5	63.2 $\pm$ 16.2 *

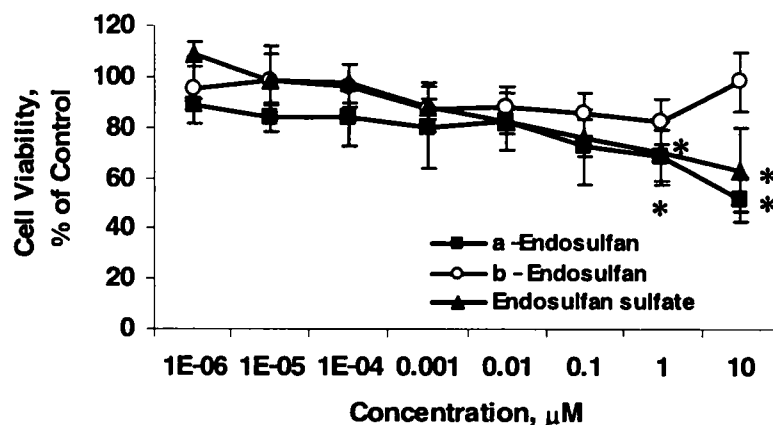


Figure 6.3. Concentration – response curves of cytotoxicity induced by exposure of CCF – STTG1 cells to α - endosulfan, β – endosulfan and endosulfan sulfate.

Table 6.4. Percentage of viability of NT2 cells after exposure to α - endosulfan, β – endosulfan and endosulfan sulfate. Cell viability is expressed as percentage of control. Data are Mean $\pm$ SD from six – eight samples ($n = 6 - 8$) of three independent experiments. The significance compared with the controls is expressed as * $P < 0.05$.

Concentration (μM)	Cell Viability (% of Control)		
	α - Endosulfan	β - Endosulfan	Endosulfan sulfate
0.000001	107.1 $\pm$ 5.5	104.1 $\pm$ 11.0	99.2 $\pm$ 12.6
0.00001	105.2 $\pm$ 12.4	99.6 $\pm$ 17.2	97.3 $\pm$ 9.4
0.0001	100.9 $\pm$ 11.2	101.3 $\pm$ 15.3	100.5 $\pm$ 15.4
0.001	104.2 $\pm$ 17.9	99.1 $\pm$ 15.3	99.4 $\pm$ 17.8
0.01	100.7 $\pm$ 7.6	94.5 $\pm$ 14.3	99.0 $\pm$ 15.6
0.1	99.6 $\pm$ 16.2	89.7 $\pm$ 8.6	97.9 $\pm$ 13.7
1	97.1 $\pm$ 14.1	87.0 $\pm$ 9.2	88.4 $\pm$ 9.5
10	52.2 $\pm$ 8.2 *	49.0 $\pm$ 9.6 *	46.5 $\pm$ 7.0 *

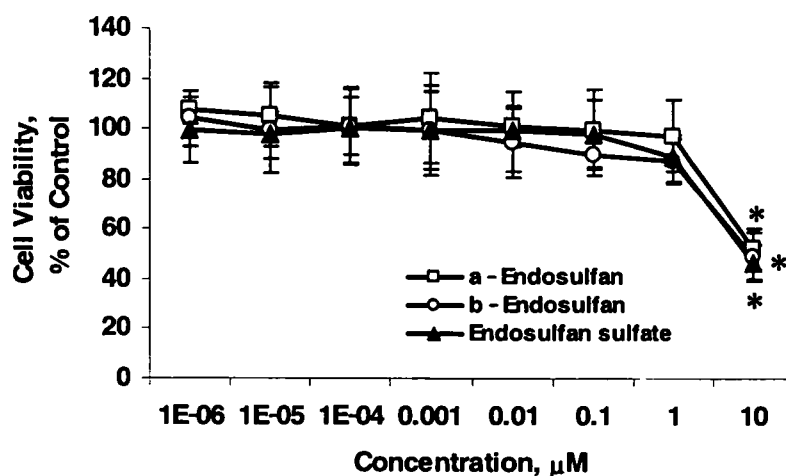


Figure 6.4. Concentration – response curves of cytotoxicity induced by exposure of NT2 cells to α - endosulfan, β - endosulfan and endosulfan sulfate.

6.3.5 Lethal concentration to cause 50 % cell death (LC_{50}) values for glial and neuronal cell cultures from rat and human

Table 6.5 shows the LC_{50} values of 96 h exposure of rat glial and neuronal, human glial and neuronal cell to α - endosulfan, β - endosulfan and endosulfan sulfate. After 96 h exposure to α - endosulfan, the LC_{50} values obtained in human glial (CCF – STTG1) and human neuronal (NT2) cell lines were statistically significant compared with rat neuronal PC12 cell line. α - endosulfan was less cytotoxic in rat neuronal (PC12) cell line than in human CCF – STTG1 and human NT2 cell lines. β - endosulfan showed a higher potency in human NT2 cell line than in other cultures. Endosulfan sulfate was more cytotoxic in human CCF – STTG1 cell line than in human NT2, rat C6 and rat PC12 cell lines.

Table 6.5. LC_{50} for the cytotoxic effects of α - endosulfan, β - endosulfan and endosulfan sulfate in rat glial and neuronal, human glial and neuronal cell lines. Cell cultures were exposed to several concentrations of α - endosulfan, β - endosulfan and endosulfan sulfate individually and cell viability was measured by WST – 8 assay as detailed in Section 6.2.6. LC_{50} values are Mean $\pm$ SD calculated from the individual concentration – response curves from three independent experiments. The significance compared with the controls is expressed as * $P < 0.05$ (Tukey's multiple comparison test).

Cell cultures		LC_{50} (μ M)		
		α - Endosulfan	β - Endosulfan	Endosulfan sulfate
Rat glial	C6	22.26 $\pm$ 4.46	22.27 $\pm$ 6.13	21.63 $\pm$ 4.68
Rat neuronal	PC12	48.03 $\pm$ 23.45	17.76 $\pm$ 5.37	12.97 $\pm$ 2.61 ^d
Human glial	CCF - STTG1	11.21 $\pm$ 4.08 ^a	30.17 $\pm$ 7.54	10.43 $\pm$ 2.57 ^e
Human neuronal	NT2	13.45 $\pm$ 2.40 ^b	11.37 $\pm$ 0.94 ^c	12.64 $\pm$ 1.69 ^f

^a $P < 0.05$ compared to rat neuronal PC12 cell line

^b $P < 0.05$ compared to rat neuronal PC12 cell line

^c $P < 0.05$ compared to human glial CCF – STTG1 cell line

^d $P < 0.05$ compared to rat glial C6 cell line

^e $P < 0.05$ compared to rat glial C6 cell line

^f $P < 0.05$ compared to rat glial C6 cell line

6.4 Discussion

In this study, the effects of α - endosulfan, β - endosulfan and endosulfan sulfate were compared and evaluated in PC12 (neuronal) and C6 (glial) cells from rat and NT2 (neuronal) and CCF – STTG1 (glial) from human with WST – 8 assay. The present study shows that α - endosulfan, β - endosulfan and endosulfan sulfate cause cytotoxicity in neuronal and glial cell cultures from rat and human in a concentration – dependent manner (Figures 6.1 – 6.4). It is also the first report demonstrating the cytotoxic effects of α - endosulfan, β - endosulfan and endosulfan sulfate in neuronal and glial cultures from rat and human. The toxicity of these compounds varied in different cell lines as shown in LC_{50} values (Table 6.5). α - endosulfan effects were highly selective as shown by the wide range of LC_{50} values found in the different cultures, ranging from 11.2 μ M for human CCF – STTG1 glial cells to 48.0 μ M for rat PC12 neuronal cells. In contrast, selective neurotoxicity was not so manifested in glial and neuronal cell cultures after exposure to endosulfan sulfate, as LC_{50} values were in the range of 10.4 – 21.6 μ M.

Human CCF – STTG1 glial cells were the most sensitive cell type to α - endosulfan toxicity compared to human NT2 neuronal cells, rat C6 glial cells and rat PC12 neuronal cells. Human NT2 neuronal cells were the most sensitive cell type to β - endosulfan and endosulfan sulfate toxicities compared to human CCF – STTG1 glial cells, rat C6 glial cells and rat PC12 neuronal cells.

It is known that endosulfan concentration attained in rat brain after a single administration of 5 mg / kg 14 C – Endosulfan was 0.11 mg / L (equivalent to 0.27 μ M) after 30 min and 0.27 mg / L (equivalent to 0.66 μ M) after 8 h as detailed and shown

in Chapter 3 (Section 3.4.3, Table 3.1). The concentration of ^{14}C – Endosulfan in the brain following three – time repeated administration of 5 mg / kg ^{14}C – Endosulfan to male Sprague rats was in the range of 0.74 μM to 3.88 μM (Chapter 3, Section 3.4.3, Table 3.2). Cerebral congestion and edema are often observed at necropsy in animals that die following acute ingestion of endosulfan (ATSDR, 2000; Boyd and Dobos, 1969; Boyd et al., 1970; Terziev et al., 1974). Increased brain weights were observed in rats following consumption of 4.59 mg / kg / day of endosulfan for 13 weeks. However, the significance of the increases in brain weight is unknown, but it could have been related to edema (ATSDR, 2000).

In our *in vitro* study, α – endosulfan and endosulfan sulfate damaged human CCF – STTG1 glial cells and NT2 neuronal cells in a very similar potency (LC_{50} = 11.21 and 13.45 μM respectively for α – endosulfan and 10.43 and 12.64 μM respectively for endosulfan sulfate). Our results also indicate that α – endosulfan and endosulfan sulfate are very toxic to human glial and neuronal cells whereas β – endosulfan is toxic to human neuronal cells, thus suggesting that human cultures may be a useful tool for studying and predicting the neurotoxic effects of these compounds. In general, the use of human cells may lead to an easier extrapolation from *in vitro* models to the clinical situation and facilitate the accuracy of the human risk assessment.

6.5 Conclusions

Several end points have been proposed as simple and rapid methods to assess neurotoxicity of chemicals *in vitro*. Cytotoxicity and viability end points provide information on the intrinsic toxicity of chemicals. They have limited ability, if any, to predict the neural – specific effects. However, these end points must be included to

determine the health status of the cells at the time of process evaluation and possibly to differentiate specific effects from general cytotoxicity (Jean Harry et al., 1998).

In summary, the results demonstrate that α - endosulfan, β - endosulfan and endosulfan sulfate cause cell death in glial and neuronal cell cultures from rat and human brain. α - endosulfan produces a manifest selective neurotoxicity. Human glial cells were the most sensitive cell type to the cytotoxic effects of α - endosulfan followed by human neuronal, rat glial and rat neuronal cells. Human neuronal cells were the most sensitive cell type to the cytotoxic effects of β - endosulfan followed by rat neuronal, rat glial and human glial cells. Human glial cells were the most sensitive cell type to the cytotoxic effects of endosulfan sulfate followed by human neuronal, rat neuronal and rat glial cells. This study also shows that human glial and neuronal cells are more sensitive compared to rat glial and neuronal cells and could be used as a good tool for studying the neurotoxic effects of these chemicals in the CNS, and thus, predicting their impact on human health.

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CHAPTER 7

ASSESSMENT OF THE HEALTH RISKS FOLLOWING EXPOSURE TO ENDOSULFAN

7.0 An overview

Risk assessment is an important tool in deciding on how to allocate resources to controlling risks. In most cases, it is based on hazard data derived from animal experiments and on exposure data from an assessment of the likely or actual exposure of the population of interest. The process of assessing the risk associated with human exposure to environmental chemicals inevitably relies on a number of assumptions, estimates and rationalizations. Some of the greatest challenges result from the necessity to extrapolate outside the range of conditions found in experimental studies. For risk assessments based on animal data, the most obvious extrapolation that has to be performed is from the tested animal species to humans (i. e., the conversion of the animal dose – response relationship to a predicted human dose – human relationship). Furthermore, there is a need to extrapolate beyond the bounds of the animal data, outside of the known dose – response region, to areas of likely human exposure both in terms of level of exposure as well as other factors such as route (e. g., oral versus inhalation) and duration of exposure. An additional difficulty often associated with environmental health assessment is that there may not be adequate toxicity information available on some of the chemicals of concern (Aarons et al., 1999; Clewell, 1995).

A key consideration for such extrapolation is how and at what rate a chemical is absorbed, distributed, metabolized and eliminated (i. e., the pharmaco - or

toxicokinetics of the chemical) in different species. Various approaches have been developed and employed depending upon the situation in question and the amount of data available. The “allometric” relationships may be used which assume that the rates of absorption, distribution,, metabolism and elimination of a substance are a function of body size; as the body size varies between species, adjustments to allow for this can be made.

Assessment of the risk following continuous exposure to very low levels of a chemical in the environment is always problematic and can normally be estimated from laboratory animal data with the application of appropriate safety margins to allow for the uncertainties involved. Frequently, the animal data will have been obtained at considerably higher dose levels with different route of exposure.

PBPK modeling could prove an important tool for improving the accuracy of human health risk assessments for hazardous chemicals in the environment. Proper use of this technique can reduce uncertainties that currently exist in risk assessment procedures by providing more scientifically credible extrapolations across species and routes of exposures, and from high experimental doses to potential environmental exposures. Current applications of PBPK models range from relatively straightforward uses for the extrapolation of chemical kinetics across species, route and duration of exposure to much more demanding chemical risk assessment applications requiring a description of complex pharmacodynamic phenomena. PBPK modeling helps to identify the factors that are most important in determining the health risks associated with exposure to a chemical and provides a means for

estimating the impact of those factors on the average risk to a population and on the specific risk to an individual.

Figure 7.1 shows the elements of risk assessment and risk management [US National Research Council (US NRC), 1983]. Four components of the risk assessment process are identified as hazard identification, dose – response assessment, exposure assessment and risk characterization.

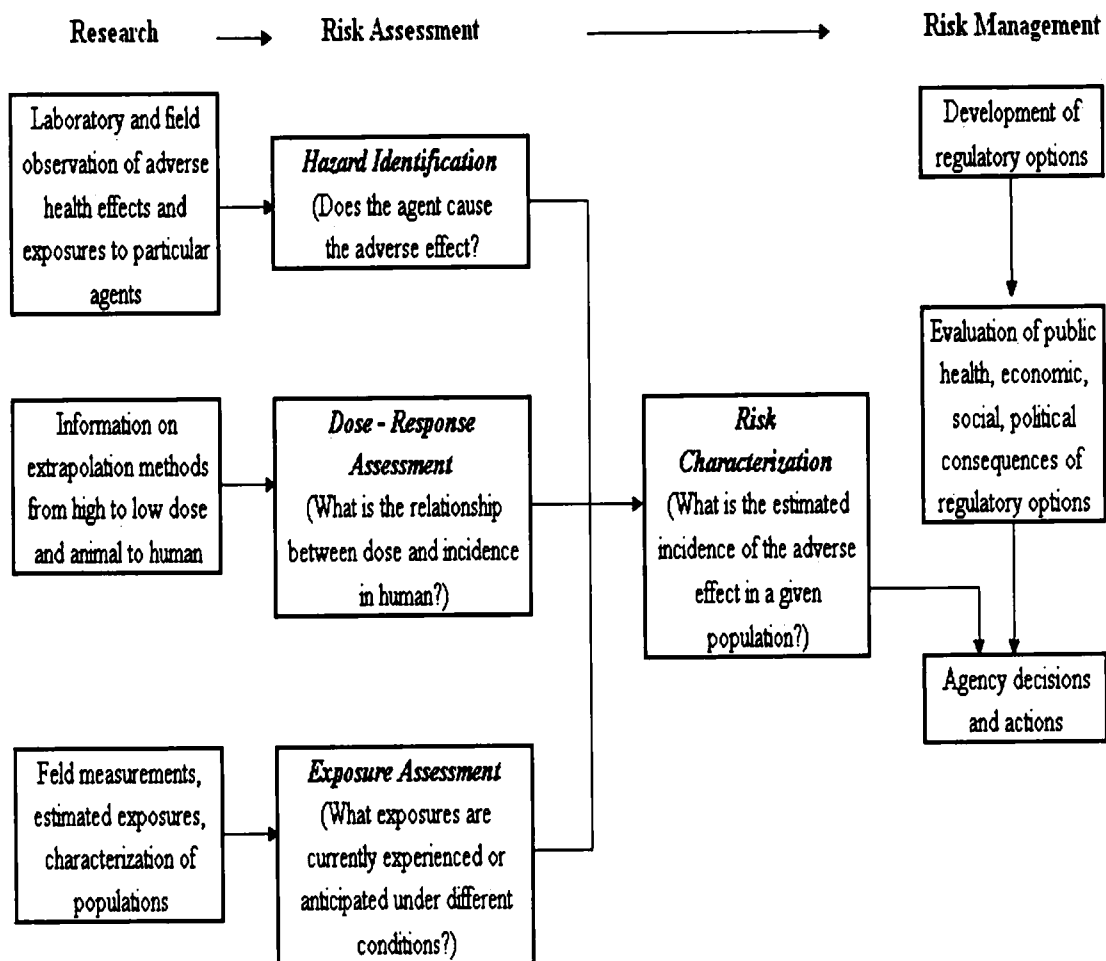


Figure 7.1. Elements of risk assessment and risk management [US National Research Council (US NRC), 1983].

7.1 Neurotoxicity

Involvement of endosulfan, a neurotoxic agent, in the central nervous system (CNS) has been described previously in Chapter 2. The blood – brain barrier (BBB) permeability, measured as transendothelial electrical resistance (TEER), decreased significantly in a dose – and time – dependent manner when treated with α – endosulfan, β – endosulfan and endosulfan sulfate individually (Chapter 5, Section 5.3.1). Cytotoxicity studies (Chapter 6, Sections 6.3.1 – 6.3.4) indicated that α – endosulfan, β – endosulfan and endosulfan sulfate cause cell death in glial and neuronal cell cultures from rat and human brain. No effect was observed when male rats were treated with 12.5 mg / kg bw per day (McGregor, 1998).

7.2 Reproductive toxicity

Adverse reproductive effects of endosulfan include testicular impairment *in vivo* (Sinha et al., 1995; Sinha et al., 1997; Chitra et al., 1999; Dalsenter et al., 1999; Dalsenter et al., 2003; Dikshith et al., 1984; Singh and Pandey, 1989), daily sperm production along with increased sperm abnormalities and altered activities of testicular marker enzymes in both mature and immature rats [Pandey et al., 1990; National Cancer Institute (NCI), 1978] as well as reduction in serum testosterone levels (Choudhary and Joshi, 2003; Wilson and LeBlanc, 1998). In addition, vacuolation of Sertoli cells was observed histologically (Sinha et al., 1999). Testicular cells, especially Sertoli – germ cells coculture have been used by many researchers to investigate the mechanisms by which recognized toxicants exert their effect (Reader and Foster, 1990; Sundaran and Witorsch, 1995). Metabolic cooperation exists between Sertoli cells and germ cells and any disturbance in this interaction may lead to testicular dysfunction (Foster, 1988). Cytotoxic changes

induced by endosulfan in mixed cultures of Sertoli and germ cells were seen after 24 hour and 48 hour of treatment (Sinha et al., 1999). The No – Observed – Adverse – Effect – Level (NOAEL) for reproductive effects was 75 ppm, equal to 6 mg / kg bw per day (McGregor, 1998).

7.3 Objective of the study

The purpose of this chapter is to present a quantitative risk assessment of endosulfan, which utilizes principles of PBPK modeling as well as *in vitro* experiments of cytotoxicity. The neurotoxic, and reproductive risks will be estimated since the brain and testes are among the target organs for toxicity [Agency of Toxic Substances and Disease Registry (ATSDR), 2000].

7.4 Bioassay data and PBPK model simulations

The brain compartment in the PBPK model, which was newly – developed for endosulfan (Chapter 4) will be modeled to estimate the neurotoxic risk while the testes compartment will be modeled to estimate the reproductive risk in humans.

For estimation of the neurotoxic risk, cytotoxic experiments on the glial and neuronal cell cultures from rat and human, which have been described in Chapter 6 will be used. For estimation of the reproductive risk, cytotoxicity experiment describing the cytotoxic effects of endosulfan on rat Sertoli – germ cell coculture (Sinha et al., 1999) will be used.

7.5 Results and discussion

7.5.1 PBPK model simulations

Figure 7.2 illustrates the brain concentration of endosulfan in rats exposed to 12.5 mg / kg / day and testes concentration of endosulfan in rats exposed to 6 mg / kg / day for 5 days respectively, estimated by the rat PBPK model which was developed in Chapter 4. Steady state has been reached in both brain and testes compartments by the end of exposure and post – exposure the concentrations of endosulfan decline with half – lives of 6.5 hour in brain and 6.3 hour in testes. Since steady state has been reached, brain and testes levels would not expected to increase with continued exposure beyond 5 days.

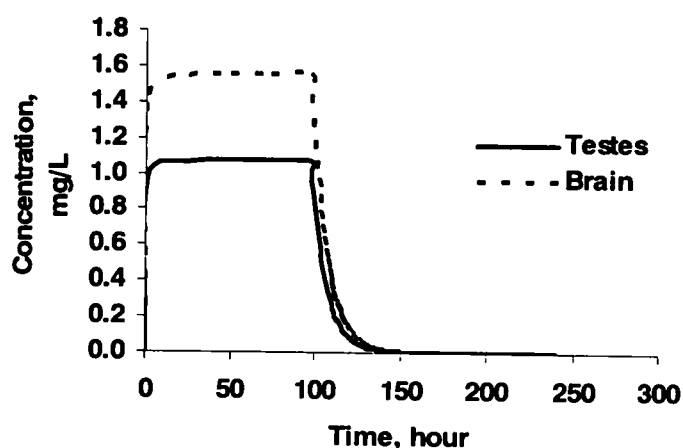


Figure 7.2. Predicted concentrations of endosulfan in rat brain during and post – exposure to 12.5 mg / kg and testes during and post – exposure to 6 mg / kg for 5 days, estimated by the rat PBPK model which was developed in Chapter 4.

7.5.2 Neurotoxic risk

The cytotoxic data on the percentage of viability cells of glial and neuronal cell cultures from rat and human after exposure to endosulfan were similar to those

presented in Chapter 6 (Tables 6.1 – 6.4). The data from Tables 6.1 – 6.4 were calculated by linear regression analysis and replotted in Figures 7.3 – 7.6. Figures 7.3 and 7.4 show the plots of percentage of viability cells (expressed as percentage of control) versus time for glial (C6) and neuronal (PC12) cell cultures from rat after exposure to α – endosulfan, β – endosulfan and endosulfan sulfate. Figures 7.5 and 7.6 show the plots of percentage of viability cells (expressed as percentage of control) versus time for glial (CCF – STTG1) and neuronal (NT2) cell cultures from human after exposure to α – endosulfan, β – endosulfan and endosulfan sulfate.

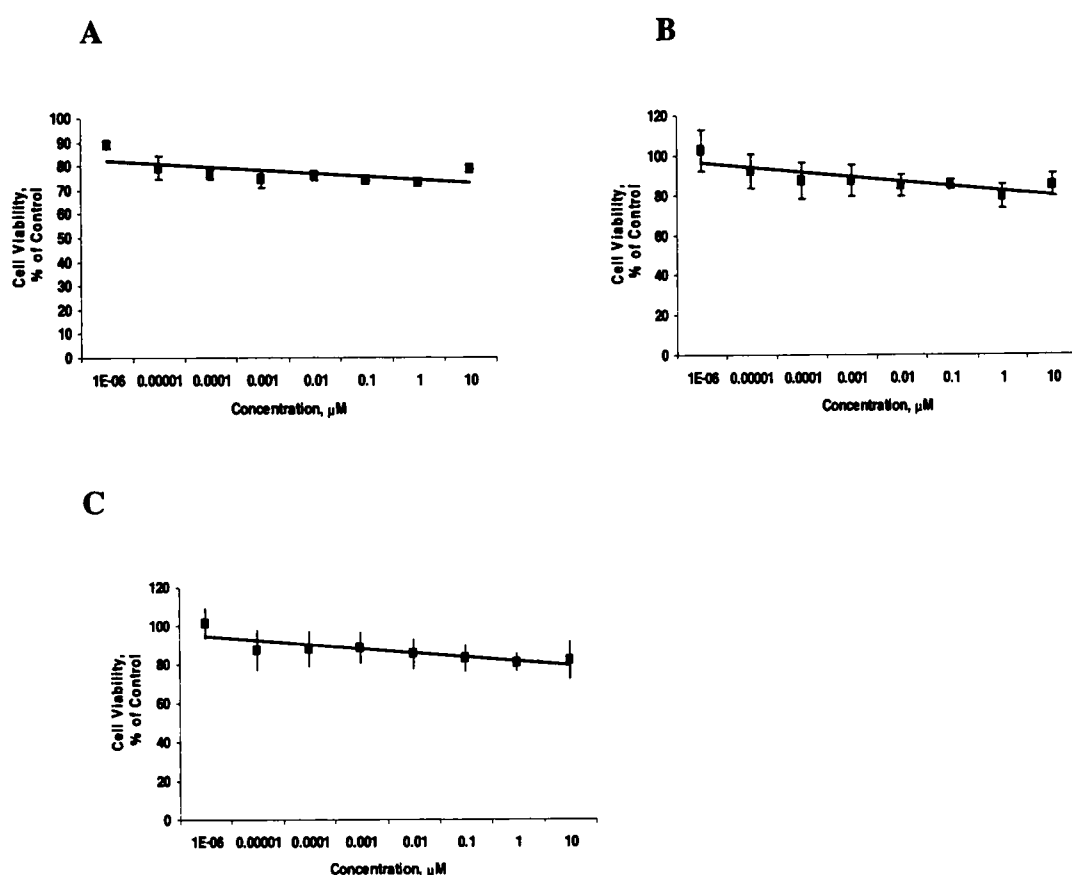


Figure 7.3. Concentration – response curves of cytotoxicity induced by exposure of rat C6 glial cells to (A) α – endosulfan, (B) β – endosulfan and (C) endosulfan sulfate. Data are Mean $\pm$ SD from six – eight samples ($n = 6 - 8$) of three independent experiments.

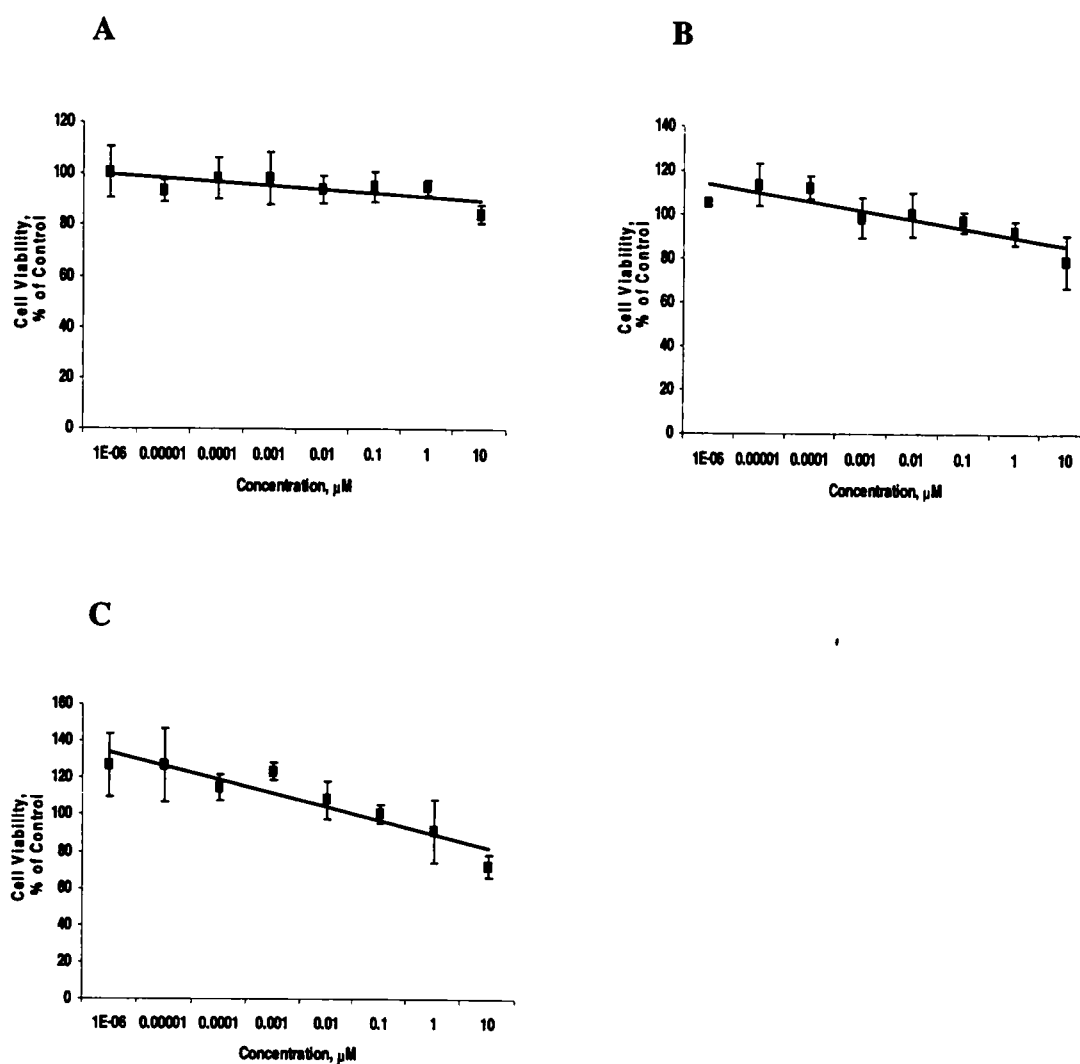


Figure 7.4. Concentration – response curves of cytotoxicity induced by exposure of rat PC12 neuronal cells to (A) α – endosulfan, (B) β – endosulfan and (C) endosulfan sulfate. Data are Mean $\pm$ SD from six – eight samples ($n = 6 - 8$) of three independent experiments.

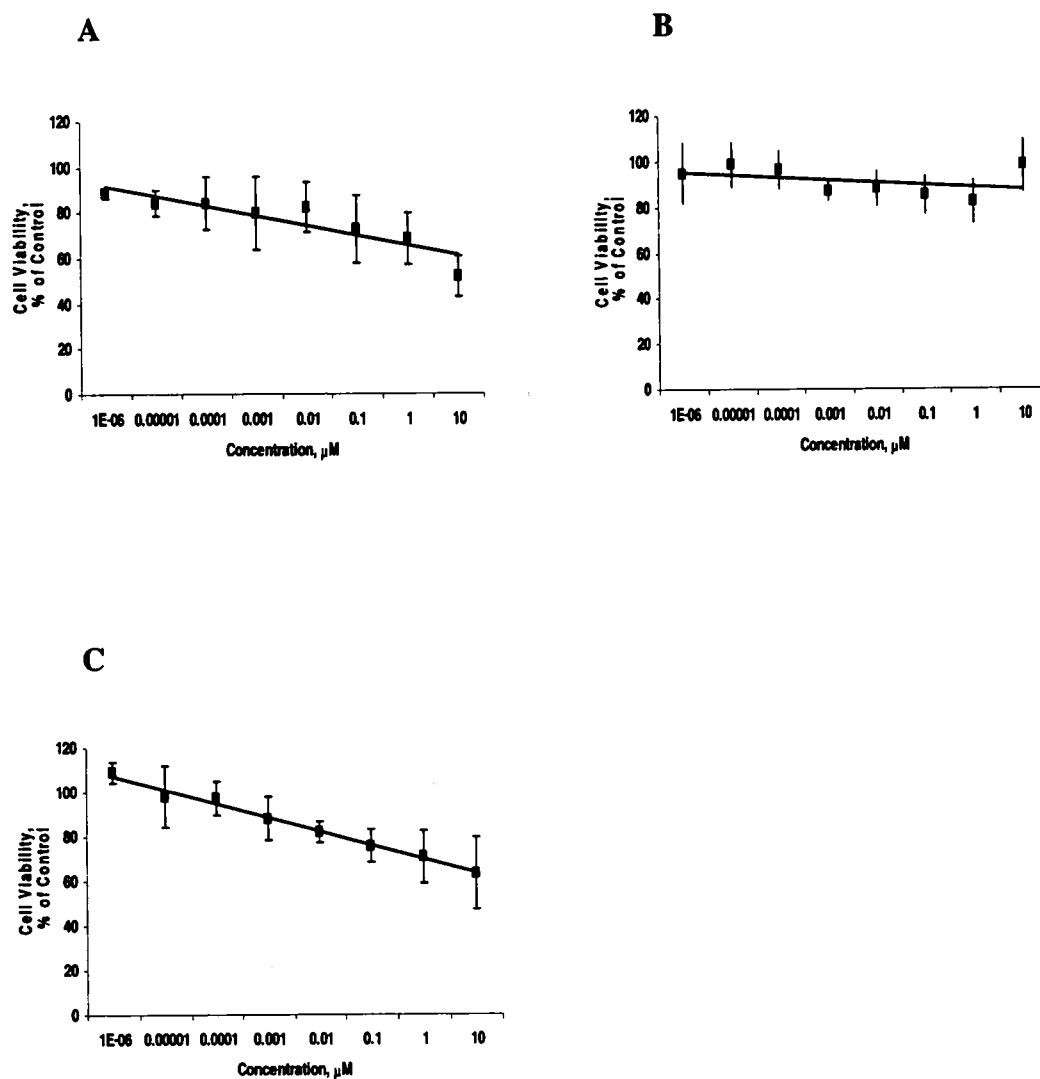


Figure 7.5. Concentration – response curves of cytotoxicity induced by exposure of human CCF – STTG1 glial cells to (A) α – endosulfan, (B) β – endosulfan and (C) endosulfan sulfate. Data are Mean $\pm$ SD from six – eight samples ($n = 6 - 8$) of three independent experiments.

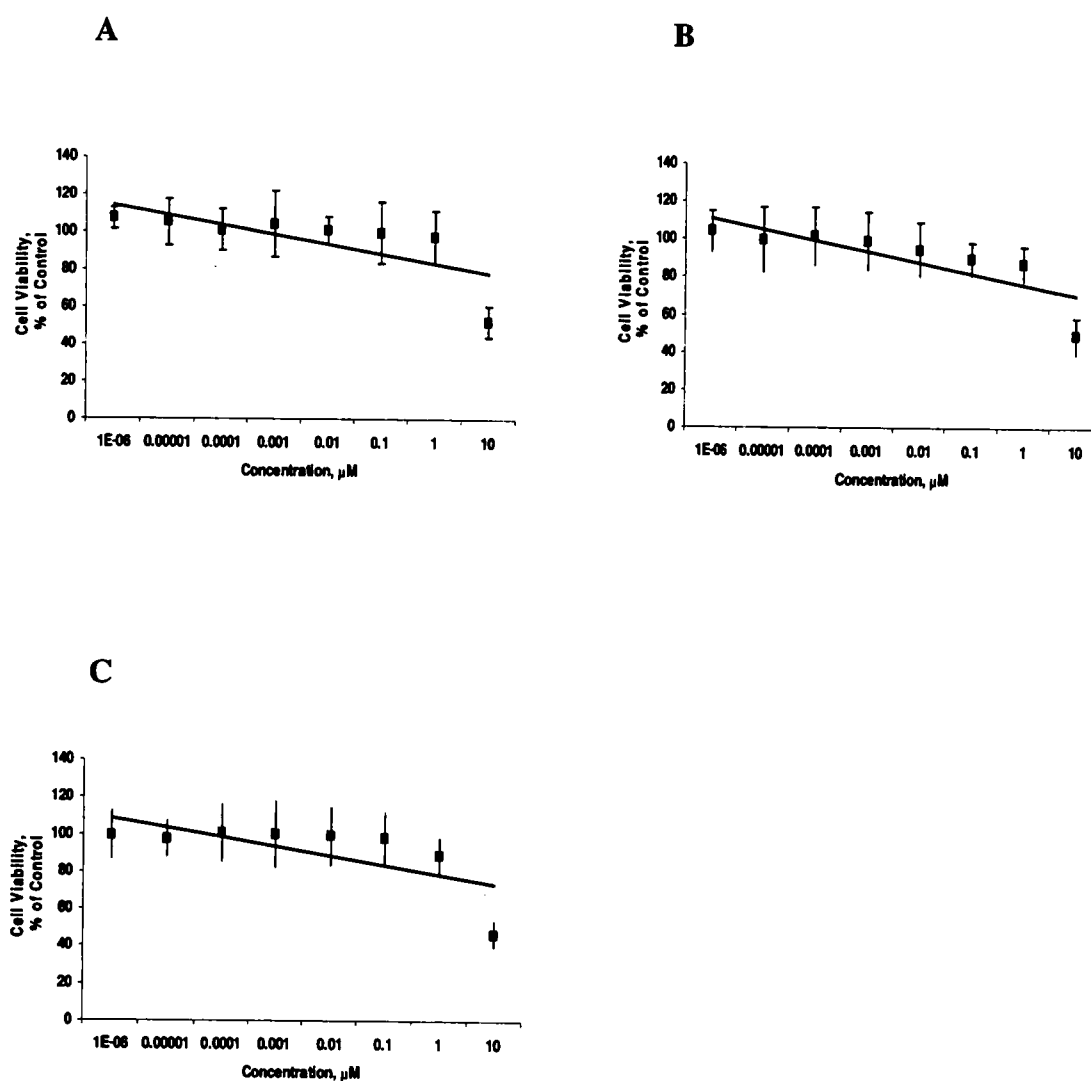


Figure 7.6. Concentration – response curves of cytotoxicity induced by exposure of human NT2 neuronal cells to (A) α – endosulfan, (B) β – endosulfan and (C) endosulfan sulfate. Data are Mean $\pm$ SD from six – eight samples ($n = 6 - 8$) of three independent experiments.

7.5.2.1 PBPK model predictions

In order to assess the likelihood that neurotoxicity would occur in humans following exposure to endosulfan, the model (Chapter 4) was first used to predict the concentration of endosulfan in the brain at the No – Observed – Effect – Level (NOEL) of 12.5 mg / kg /day (equivalent to 30.72 μ M) for neurotoxicity in rats (McGregor, 1998). Exposure at this concentration gave a brain concentration of 1.56 mg / L. The no effect level for neurotoxicity in the rat equates, therefore, to a target organ dose of 1.56 mg / L. To produce the same concentration of endosulfan in the human brain, the model calculates that humans would have to be continuously exposed to 1.27 ppm endosulfan. An application of a safety margin of 100 to account for cross – species (inter – species) and intra – species variability for non – carcinogenic and reproductive effects resulted in an exposure of 12.7 ppb (equivalent to 0.0312 μ M).

Table 7.1 summarizes the estimated percentage of cell viability of glial and neuronal cell cultures from rat and human after exposure to α – endosulfan, β – endosulfan and endosulfan sulfate at a dose level of 12.5 mg / kg (rat) and 1.27 mg / kg (human) respectively. It was observed that high exposure level of 12.5 mg / kg (30.72 μ M) induced glial and neuronal cell death for cell cultures from rat and the estimated percentage of cell viability was below 50 % for all compounds except for α – endosulfan in the rat neuronal PC12 cell cultures. Endosulfan generally induced 50 % of glial and neuronal cell death for cell cultures from rat at LC₅₀ of as low as 12.97 μ M (Chapter 6, Section 6.3.5, Table 6.5). It seems that differences occur in the response between *in vivo* and *in vitro* experiments as 12.5 mg / kg endosulfan was established as a NOEL in rat (McGregor, 1998). However, *in vitro* experiments can

provide a basis for risk assessment since experimental data for humans is extremely difficult to obtain.

Exposure of 0.0312 μM endosulfan to glial and neuronal cell cultures from human did not cause cell death and the estimated percentage of cell viability for all cell lines were above 90 % (Table 7.1).

Table 7.1. Percentage of cell viability estimated from the concentration – response curves of cytotoxicity after exposure of endosulfan to glial and neuronal cell cultures from rat and human.

Dose (Equivalent dose)	Species	Cell type	Cell culture	Compound	% Cell viability (% of control)
12.5 mg / kg ^a (30.72 μM)	Rat	Glial	C6	α - Endosulfan	43.5
				β - Endosulfan	26.9
				Endosulfan sulfate	28.6
		Neuronal	PC12	α - Endosulfan	57.0
				β - Endosulfan	-5.3
				Endosulfan sulfate	-84.91
1.27 mg / kg ^b (0.0312 μM)	Human	Glial	CCF - STTG1	α - Endosulfan	95.9
				β - Endosulfan	96.2
				Endosulfan sulfate	113.4
		Neuronal	NT2	α - Endosulfan	118.9
				β - Endosulfan	116.5
				Endosulfan sulfate	113.5

a : No observed effect level (McGregor, 1998).

b : Dose predicted from the PBPK model simulation in order to obtain the brain concentration similar to those observed in rat.

7.5.3 Reproductive risk

The cytotoxic data on the percentage of viability of detached cells in rat Sertoli – germ cell cocultures after exposure to endosulfan (Technical grade; 95.32 % purity) were retrieved from Sinha et al. (1999). Table 7.2 shows the effect of endosulfan on the viability of detached cells in rat Sertoli – germ cell cocultures after 24 h and 48 h exposure periods.

Table 7.2. Effect of endosulfan on the viability of detached cells in rat Sertoli – germ cell cocultures (Sinha et al., 1999).

Concentration (μM)	% Viability of detached cells	
	Treatment time (h)	
	24	48
2	67.45	60.86
20	50.41	42.31
40	39.86	31.27
80	30.85	26.96

Figure 7.7 shows the plot of percentage of viability of detached cells versus log concentrations of endosulfan after 24 h and 48 h exposure periods.

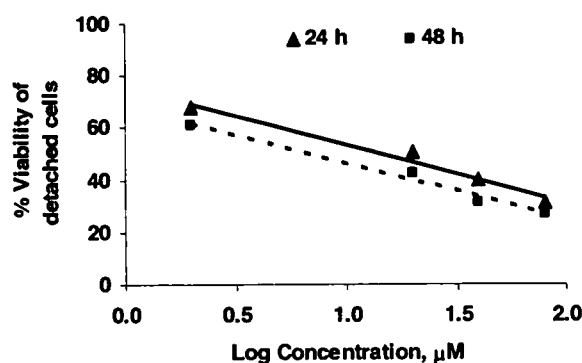


Figure 7.7. Percentage of viability of detached cells versus log concentrations of endosulfan after 24 h and 48 h exposure periods.

7.5.3.1 PBPK model predictions

The PBPK model which was developed in Chapter 4 was first used to predict the concentration of endosulfan in the testes at the No – Observed – Adverse – Effect – Level (NOAEL) of 6 mg / kg /day (equivalent to 14.74 μM) for reproductive toxicity in rats (McGregor, 1998). Exposure at this concentration gave a testes concentration of 1.07 mg / L. The no effect level for reproductive toxicity in the rat equates, therefore, to a target organ dose of 1.07 mg / L. To produce the same concentration of endosulfan in the human testes, the model calculates that humans would have to be continuously exposed to 0.614 ppm endosulfan. An application of a safety margin of 100 to account for cross – species (inter – species) and intra – species variability for non – carcinogenic and reproductive effects resulted in an exposure of 6.14 ppb (equivalent to 1.51 μM).

It was observed that the estimated percentages of viability of detached cells exposure to 1.51 μM (human equivalent dose) endosulfan for 24 h and 48 h were 71.6 % and 64.0 % respectively. Speculation that the shedding of germ cells *in vitro* may be a possible reason for low sperm production observed in *in vivo* studies on mature and immature rats (Sinha et al., 1999). Disturbance on the normal interaction between Sertoli and germ cells may lead to testicular dysfunction.

7.6 Conclusions

At the no effect level of 12.5 mg / kg for neurotoxicity in rat, the brain concentration was calculated by the PBPK model to be 1.56 mg / L. In humans, the same concentration would be achieved following exposure to 12.7 ppb endosulfan.

At the No – Observed – Adverse – Effect – Level (NOAEL) of 6 mg / kg for reproductive toxicity in rat, the testes concentration was calculated by the PBPK model to be 1.07 mg / L. In humans, the same concentration would be achieved following exposure to 6.14 ppb endosulfan.

Based on these results, the estimated exposure level for neurotoxicity in humans was 1.6 – fold higher and exceeded the acceptable daily intake (ADI) of 0.008 mg / kg (equivalent to 8 ppb) (WHO, 1984). This could be attributed to interspecies differences such as receptor binding as this factor was not introduced in the current PBPK model. This parameter is very important in sensitivity and should be introduced to the model in subsequent studies. In contrast, the estimated exposure level for reproductive toxicity in humans was 6.14 ppb, which was below the ADI.

As has been discussed in Chapter 4, Section 4.4.4.1, the pregnant women from Chiba and Kyushu, Japan were estimated to be exposed to endosulfan through dietary intake of 0.76×10^{-5} mg / kg / day (equivalent to 0.0076 ppb) and 9.09×10^{-5} mg / kg / day (0.0909 ppb) respectively whereas school children from Malaysia were estimated to intake endosulfan for 1.06×10^{-5} mg / kg / day (0.0106 ppb). The estimated exposure levels for these groups of people were below the no effect level of 12.7 ppb for neurotoxicity and 6.14 ppb for reproductive toxicity in human, which were estimated in Sections 7.5.2.1 and 7.5.3.1 respectively, thus suggesting that these people were unlikely to develop any serious health problems. However, other possibilities cannot be ruled out.

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CHAPTER 8

8.0 Conclusions

With regards to the objectives of this research as mentioned in Chapter 1 (Section 1.1), the findings are summarized as follow:

Chapter 1

Chapter 1 compares the extent of contamination of endosulfan between the Malaysian and Japanese environment. This chapter also describes the physical and chemical properties of endosulfan followed by the possible routes of exposure, human exposures, effects on the environment and its environmental fate.

Chapter 2

Chapter 2 describes the general toxic effects of endosulfan in mammals (rats) including the neurological, reproductive and endocrine effects.

Chapter 3

The present study in Chapter 3 indicates that ^{14}C – Endosulfan was rapidly excreted in the feces and urine after 96 h following single oral administration of 5 mg / kg ^{14}C – Endosulfan with fecal elimination as the major elimination route in male rats. Elimination was essentially complete within a few days. The cumulative excretions in the urine and feces for four days were 12.4 % and 94.4 % respectively of the total administered dose. The total radioactivity recovered in the excreta for four days was 106.8 %, thus suggesting that elimination was essentially complete within four days.

Following single and three – time repeated oral administration of 5 mg / kg ^{14}C – Endosulfan, it was observed that the radioactivity levels in all tissues decreased after administration was terminated, rats will recover once administration is terminated. The tissue concentrations of residues after 8 h as observed in the present study were generally highest in the liver and kidneys and lower in other tissues such as brain, testes and muscle.

The basic toxicokinetic parameters for ^{14}C – Endosulfan was established following single oral administration of 5 mg ^{14}C – Endosulfan / kg, with k_a of 3.07 h^{-1} and C_{max} of $0.36 \pm 0.08\text{ mg – eq. / L}$ with T_{max} of 2 h. The terminal elimination half life, $T_{1/2}$ of the radioactivity in blood was 193 h.

Chapter 4

Given that no calibration or validation of PBPK model predictions of concentrations of endosulfan in various tissues exist, the first and new PBPK model for endosulfan in male rats has been developed in Chapter 4 and can predict tissue dosimetries following single and multiple oral dosages of endosulfan. Generally, results show reasonable concordance between model predictions and measured values in all target tissues (liver, kidneys, brain, testes and blood) following oral administration. The validity of the model was further verified with experimental data retrieved from the literature. The simulations of those studies from the literature generally agree with experimental data. Sensitivity analyses allow for a quantitative assessment of input parameters on the model simulations of tissue concentrations. The present results indicate that the influence of PBPK model input parameters on total endosulfan tissue dosimetry varies, as expected, across parameter.

The PBPK model for endosulfan in rats was extrapolated to humans, without adjusting the previously established model parameters to test the ability of the model to predict the pharmacokinetic behavior of endosulfan in humans. The ability of the model was tested by predicting the daily intake of endosulfan per individual by comparing the residue levels of endosulfan in selected tissues for both the parent isomers (endosulfan) and its metabolites (endosulfan metabolites). From the PBPK model simulations, the estimated dietary intake for the pregnant women from Chiba and Kyushu, Japan were 0.76×10^{-5} mg / kg / day and 9.09×10^{-5} mg / kg / day respectively, whereas the estimated dietary intake for the Malaysian school children was 1.06×10^{-5} mg / kg / day. Generally, reasonable agreement was observed between model predictions and experimental data, indicating that the model was / could be partially validated since data concerning human exposures are scarce and difficult to obtain experimentally.

Chapter 5

Chapter 5 focuses on the effects of α - endosulfan, β - endosulfan and endosulfan sulfate on the tight junctions of the blood – brain barrier (BBB) by investigating the transendothelial electrical resistance (TEER) and permeability effects across cultured monolayers of porcine brain microvascular endothelial cells (PBMECs). Following exposure to a series of concentrations of endosulfan (0.01 μ M to 10 μ M), TEER declined significantly and reached the bottom level as concentrations and exposure periods increased. Cytotoxicity tests indicated that the concentrations of 10 μ M and below did not cause cell death for all compounds. The integrity of the brain endothelium was further investigated by measuring the transendothelial permeability to 14 C – Endosulfan. It was observed that the transport of endosulfan through the

BBB was reversible, in which endosulfan transported from the blood – brain compartment and from the brain – blood compartment, thus suggesting that residues of endosulfan has little potential to accumulate in the brain, although other possibilities could not be ruled out. The ratio between the outer – to – inner and the inner – to – outer compartments for the transport study of ^{14}C – Endosulfan in the concentration range of 0.01 – 10 μM was approximately 1.2 – 2.1.

Chapter 6

In Chapter 6, a study was carried with *in vitro* assays to assess the neurotoxic effects of α – endosulfan, β – endosulfan and endosulfan sulfate by comparing the ability of the compounds to cause cell death in glial and neuronal cell cultures from rat and human. The results demonstrate that α – endosulfan, β – endosulfan and endosulfan sulfate cause cell death in glial and neuronal cell cultures from rat and human brain in a concentration – dependent manner. α – endosulfan produces a manifest selective neurotoxicity with LC_{50} ranging from 11.2 μM to 48.0 μM . In contrast, selective neurotoxicity was not so manifested in glial and neuronal cell cultures after exposure to endosulfan sulfate, as LC_{50} values were in the range of 10.4 – 21.6 μM .

Human glial CCF – STTG1 cells were the most sensitive cell type to the cytotoxic effects of α – endosulfan followed by human neuronal NT2, rat glial C6 and rat neuronal PC12 cells. Human neuronal cells were the most sensitive cell type to the cytotoxic effects of β – endosulfan followed by rat neuronal, rat glial and human glial cells. Human glial cells were the most sensitive cell type to the cytotoxic effects of endosulfan sulfate followed by human neuronal, rat neuronal and rat glial cells. This study also shows that human glial and neuronal cells are more sensitive compared to

rat glial and neuronal cells and could be used as a good tool for studying the neurotoxic effects of these chemicals in the central nervous system (CNS), and thus, predicting their impact on human health.

Chapter 7

In Chapter 7, a quantitative risk assessment of endosulfan was presented by utilizing the principles of PBPK modeling (as discussed previously in Chapter 4) as well as *in vitro* experiments of cytotoxicity (as discussed previously in Chapter 5). The neurotoxic and reproductive risks were estimated since the brain and testes were among the target organs for toxicity.

At the no effect level of 12.5 mg / kg for neurotoxicity in rat, the brain concentration was calculated by the PBPK model to be 1.56 mg / L. In humans, the same concentration would be achieved following exposure to 12.7 ppb (0.0312 μ M) endosulfan. Exposure of 0.0312 μ M endosulfan to glial and neuronal cell cultures from human did not cause cell death and the estimated percentages of cell viability for all cell lines were above 90 %

At the No – Observed – Adverse – Effect – Level (NOAEL) of 6 mg / kg for reproductive toxicity in rat, the testes concentration was calculated by the PBPK model to be 1.07 mg / L. In humans, the same concentration would be achieved following exposure to 6.14 ppb (1.51 μ M) endosulfan. The estimated percentages of viability of detached cells in the Sertoli – germ cells cocultures exposed to 1.51 μ M endosulfan for 24 h and 48 h were 71.6 % and 64.0 % respectively, indicating

disturbance on the normal interaction between Sertoli and germ cells may lead to testicular dysfunction.

Based on these results, the estimated exposure level for neurotoxicity in humans was 1.6 – fold higher and exceeded the acceptable daily intake (ADI) of 0.008 mg / kg (equivalent to 8 ppb). In contrast, the estimated exposure level for reproductive toxicity in humans was 6.14 ppb, which was below the ADI.

8.1 Recommendations for future study

It has been proposed that humans and wildlife have suffered adverse effects on health as a result of environmental exposure to toxic chemicals. The objectives of this study as mentioned in Chapter 1 (Section 1.2) were achieved. However, some issues and research gaps need to be addressed for future investigation.

A more reliable and sensitive analytical method would need to be incorporated into the future experiments to enhance the separation and identification of the parent compounds and its metabolites present in different matrices. Further research is also necessary to expand the current model for endosulfan to include the two isomers of the parent compounds and its major oxidation metabolites.

The current *in vitro* blood – brain barrier (BBB) model to study the effects of endosulfan on the BBB needs to be improved until optimum conditions are achieved in order to increase our understanding on the pharmacology and toxicology effects of endosulfan as well as its influences on the barrier under *in vitro* conditions.

In order to develop a risk assessment paradigm to assess the health risks induced by endosulfan, the following strategy is proposed:

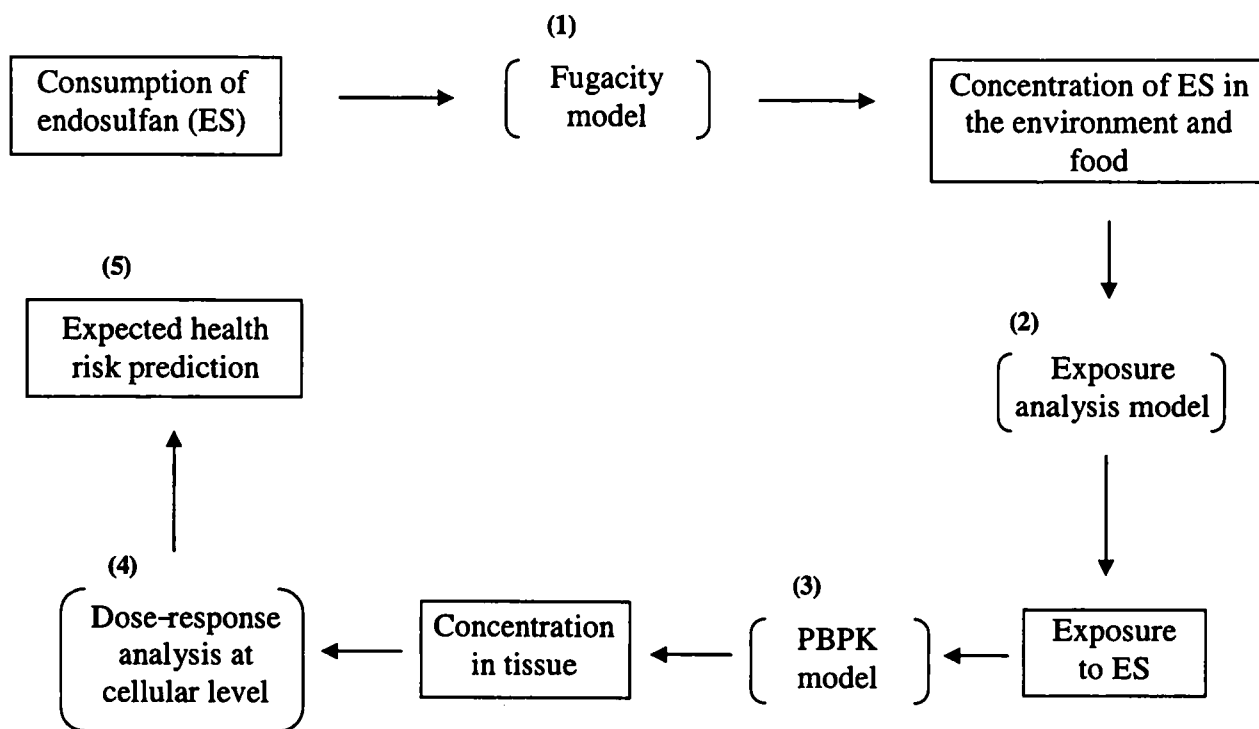


Figure 8.1. Proposed risk assessment paradigm for future study.

- (1) In order to estimate the future trends of exposure to endosulfan, the mathematical models / fugacity models for the evaluation of the dynamic performances of the environmental pollutants need to be developed. Necessary data (i. e. dietary / food intake survey data; consumption of endosulfan data; environmental monitoring data) needs be collected and compiled.

- (2) To develop the exposure analysis models, exposure analysis of people to the environmental pollutants through dietary and respiratory pathways need to be conducted and the data of the concentration of endosulfan in the environment and food intake survey data will be applied. An exposure model is an empirical framework, which allows estimation of exposure parameters from available input data. Chemical release estimates, fate and transport modeling, and exposure potentials based on life habits can be employed to estimate exposure for wildlife and / or humans by way of air, food, water or in total.
- (3) The PBPK model should be revised from time to time to accommodate for any changes until the optimum conditions are achieved. More surveys on the human exposures to endosulfan are necessary in order to validate the model.
- (4) A dose response analysis for endosulfan at the cellular level needs to be established in order to evaluate the magnitude of internal exposure to the target organs. In addition, a reliable biomarker and appropriate endpoints need to be identified before any risk assessment is attempted.
- (5) By integrating and coupling all the necessary information, it is hoped that the proposed strategy could be used to relate / evaluate possible adverse health risk with the environmental pollutants with the hope that effective risk reduction options / measures could be proposed subsequently.