# A STUDY ON THE EFFECT OF PHLORACETOPHENONE ON BILIARY EXCRETION OF METHYLMERCURY IN RATS

**SUPAPORN KONGSAWAT**

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Thesis
entitled

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for the degree of Master of Science (Toxicology)
on
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A STUDY ON THE EFFECT OF PHLORACETOPHENONE ON BILIARY EXCRETION OF METHYLMERCURY IN RATS.

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ABSTRACT

Methylmercury (MeHg) is a worldwide pollutant and environmental toxicant which causes public disasters in many countries. Liver is one of the main organs responsible for the elimination of the ingested MeHg via biliary excretion. In this study, the effect of phloracetophenone or 2,4,6-trihydroxyacetophenone (THA), a choleretic agent, on biliary excretion of MeHg was investigated in adult male Wistar rats. Administration of MeHg (20-80 μmol/kg BW) into the portal vein immediately decreased bile secretion in a dose-related manner. Concurrent with the decreased bile flow rate by the MeHg, activities of plasma alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP) in both plasma and bile were increased whereas hepatic and biliary glutathione (GSH) contents were decreased. The initial excretion rate of mercury (Hg) increased with the administered dose during the first 30 min. After 30 minutes, the biliary Hg excretion rate was steadily maintained and not dependent on the dose, indicating a limitation of the liver to dispose. Administration of THA (300 and 600 μmol/kg BW, intraduodenally) enhanced bile secretion in the MeHg-treated animals but markedly reduced the biliary excretion of the mercury and GSH. However, the liver GSH and Hg content were not significantly altered ($P<0.05$). Furthermore, the activities of plasma ALT, AST, ALP in both bile and plasma, and cytosol glutathione *S*-transferase (GST) in the liver were not altered as compared with those in the MeHg-treated rats. The involvement of THA with cellular GSH content was supported by a reduction of its stimulatory activity after giving the GSH depletors, buthionine sulfoximine (BSO) or acivicin. The biliary secretion of GSH and Hg were further decreased after administration of THA in the GSH-depleted rats. However, THA induced an increase in urine flow rate and enhanced Hg excretion in the urine. It is suggested that THA modulated the hepatic excretion of mercury by diverting the excretion to other routes such as via urinary excretion. An understanding on the excretory mechanism of mercury, and the actual interactions to the transport systems are essential for setting detoxification strategies.

KEY WORDS : METHYLMERCURY / PHLORACETOPHENONE / BILIARY EXCRETION / HEPATOTOXICITY

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การศึกษาผลของสารฟลออะซีโตฟีโนนต่อการขับสารเมทิลเมอร์คิวรี่ออกทางน้ำดีในหนูแรท
(A STUDY ON THE EFFECT OF PHLORACETOPHENONE ON BILIARY EXCRETION OF METHYLMERCURY IN RATS)

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บทคัดย่อ

เมทิลเมอร์คิวรี่เป็นสารประกอบอินทรีย์ของปรอทซึ่งเป็นสารพิษที่พบทั่วไปในสิ่งแวดล้อม ก่อให้เกิดมลภาวะเป็นพิษและความเสียหายในหลายประเทศทั่วโลก ตับเป็นอวัยวะสำคัญที่ทำหน้าที่ขับสารเมทิลเมอร์คิวรี่ที่รับประทานเข้าไปให้ออกจากร่างกายโดยผ่านทางน้ำดี การศึกษาครั้งนี้มีวัตถุประสงค์เพื่อทดสอบฤทธิ์ของสารอะซีโตฟีโนน (2,4,6-ไตรไฮดรอกซีอะซีโตฟีโนน, THA) ซึ่งมีฤทธิ์กระตุ้นการหลั่งน้ำดี ต่อการขับสารเมทิลเมอร์คิวรี่ออกทางน้ำดีในหนูแรท ผลการทดลองพบว่าเมื่อให้สารเมทิลเมอร์คิวรี่ (20-80 μmol/kg BW) เข้าทางเส้นเลือดดำพอทัลที่เข้าสู่ตับ ทำให้การหลั่งน้ำดีลดลงทันทีโดยผลที่เกิดมีความสัมพันธ์กับขนาดของสารเมทิลเมอร์คิวรี่ที่ฉีดให้สัตว์ทดลอง ในขณะที่อัตราการหลั่งของน้ำดีลดลง ระดับของเอนไซม์ alanine aminotransferase (ALT), aspartate aminotransferase (AST) ในพลาสมา และ alkaline phosphatase (ALP) ในพลาสมาและในน้ำดีเพิ่มขึ้น โดยที่ปริมาณของ glutathione (GSH) ในตับและที่หลั่งออกมาในน้ำดีลดลง ได้พบว่าการขับสารปรอทออกทางน้ำดีใน 30 นาทีแรกจะเพิ่มขึ้นเมื่อฉีดสารเมทิลเมอร์คิวรี่เข้าไปเพิ่มขึ้น อย่างไรก็ตามปริมาณการขับสารปรอทในน้ำดีหลังจาก 30 นาทีเป็นต้นไปไม่เปลี่ยนแปลงตามขนาดที่ให้ แสดงให้เห็นว่าตับมีความจำกัดในการขับสารปรอท เมื่อฉีดสาร THA (300 และ 600 μmol/kg BW, เข้าทางลำไส้เล็กส่วนต้น) พบว่าสารดังกล่าวสามารถกระตุ้นการขับน้ำดีในหนูที่ได้รับสารเมทิลเมอร์คิวรี่มาก่อนได้ แต่ปริมาณสารปรอทและ GSH ซึ่งเคยถูกขับออกทางน้ำดีกลับลดลงอย่างมาก อย่างไรก็ตามปริมาณสารปรอทและ GSH ในตับไม่ได้เปลี่ยนแปลงอย่างมีนัยสำคัญทางสถิติ ($p<0.05$) นอกจากนี้ระดับของเอนไซม์ ALT, AST ในพลาสมา, ALP ในพลาสมาและในน้ำดี และเอนไซม์ glutathione *S*-transferase (GST)ในไซโตพลาสซึมของตับไม่เปลี่ยนแปลงเมื่อเปรียบเทียบกับหนูที่ได้รับสารเมทิลเมอร์คิวรี่อย่างเดียว แสดงว่าความเป็นพิษไม่ได้เพิ่มขึ้น เมื่อให้ THA ในสัตว์ทดลองที่มีระดับของ GSH ในตับต่ำ โดยให้ Buthionine sulfoximine, Acivicin นำไปก่อน พบว่าการหลั่งน้ำดีหลังจากถูกกระตุ้นลดต่ำลงมาก ปริมาณของ GSH และสารปรอทในน้ำดีก็ลดลงมากขึ้น แสดงให้เห็นว่าฤทธิ์การกระตุ้นการหลั่งน้ำดีของ THA ส่วนหนึ่งขึ้นอยู่กับระดับของ GSH ในเซลล์ นอกจากนี้ THA ยังสามารถกระตุ้นการขับปัสสาวะพร้อมกับการกระตุ้นการขับสารปรอทออกทางปัสสาวะเพิ่มมากขึ้นอีกด้วย แสดงให้เห็นว่า THA มีผลเปลี่ยนการขับทิ้งสารปรอททางตับโดยหันไปใช้ทางอื่น เช่น การขับออกทางปัสสาวะ ความเข้าใจต่างๆ เกี่ยวกับกลไกการขับสารเมทิลเมอร์คิวรี่โดยตับ และปฏิกิริยากับระบบการขนส่งของสารเมทิลเมอร์คิวรี่มีความสำคัญในการกำหนดวิธีการในการกำจัดสารพิษเพื่อลดอาการพิษที่เกิดขึ้น

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# LIST OF ABBREVIATIONS

| | |
|---|---|
| ALT | alanine aminotransferase |
| ALP | alkaline phosphatase |
| AST | aspartate aminotransferase |
| ATP | adenosine 5′ -triphosphate |
| ANOVA | analysis of variance |
| ATSDA | The Agency for Toxic Substances and Disease Registry |
| BSO | buthionine sulfoximine |
| BSP | sulfobromophthalein |
| BW | body weight |
| $^{o}C$ | degree Celsius |
| $Cl^{-}$ | chloride ion |
| CDNB | 1-chloro-2,4-dinitrobenzene |
| DMSA | dimercaptosuccinic acid |
| DMSO | dimethylsulfoxide |
| DTNB | 5-5′-dithiobis-(2-nitro-5-thiobenzoic acid) |
| DW | distilled water |
| et. al., | and colleagues |
| EtOH | ethanol |
| g | gram |
| GFAAS | graphite furnace atomic absorption spectrophotometer |
| GSH | glutathione |
| GSH-PX | glutathione peroxidase |
| GSH-RX | glutathione reductase |
| γ-GT | γ-glutamyltransferase |
| GST | glutathione *S*-transferase |
| GSSG | glutathione disulfide |

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# LIST OF ABBREVIATIONS (Cont.)

| | |
|---|---|
| h | hour |
| HCl | hydrochloric acid |
| $HCO_3^-$ | hydrogen carbonate ion |
| Hg | mercury |
| $Hg^{++}$ | mercuric ion |
| $Hg_2^{++}$ | mercurous ion |
| IU/ml | international unit per milliliter |
| i.p. | intraperitoneal |
| KCl | potassium chloride |
| kg | kilogram |
| kDa | kilodalton |
| $LD_{50}$ | medial lethal dose, lethal dose for 50% of population |
| M | molar |
| MeHg | methylmercury chloride |
| mg | milligram |
| mg/kg BW | milligram per kilogram body weight |
| mM | millimolar |
| mmol/kg BW | millimole per kilogram body weight |
| mmol/g liver | millimole per gram liver |
| min | minute |
| ml | milliliter |
| Mrp2 | multidrug resistance-associated protein |
| MT | metallothionein |
| MW | molecular weight |
| µl | microliter |
| µl/kg/min | microliter per kilogram per minute |
| µmol | micromoles |
| µmol/g liver | micromole per gram liver |

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# LIST OF ABBREVIATIONS (Cont.)

| | |
|---|---|
| μmol/kg BW | micromoles per kilogram body weight |
| μmol/mg protein/min | micromoles per miligram protein per minute |
| μM | millimoles |
| $Na^+$ | sodium ion |
| NADPH | nicotinamide adenine dinucleotide phosphate, reduced form |
| NaCl | sodium chloride |
| $Na^+K^+$-ATPase | sodium potassium adenosine $5^/$ -triphosphatase |
| $Na_2S_2O_3$ | sodium dithiosulfate |
| nm | nanometer |
| nmol | nanomoles |
| nmol/kg/min | nanomole per kilogram per minute |
| O.D. | optical density |
| Oatp | organic anion transporter |
| % | percent |
| PE | polyethylene |
| pH | log concentration of $H^+$ |
| rpm | round per minute |
| SEM | standard error of mean |
| THA | 2,4,6-trihydroxyacetophenone |
| TSO | trans-stilbene oxide |
| vol | volume |
| WHO | World Health Organization |
| $\overline{X}$ | arithmetic mean |

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# CHAPTER I
# INTRODUCTION

Mercury is a ubiquitous environmental pollutant and is recognized as potentially hazardous material (The Agency for Toxic Substances and Disease Registry, ATSDA, and United State Environmental Protection Agency, USEPA, 1999). Mercury occurs naturally in the environment. There are three general forms of mercury in the environment; elemental, inorganic and organic mercury. However, the organic mercury compound, especially methylmercury (MeHg), is considered to be one of the greatest toxicological interests as its toxicity has caused several widely publicized diasters. (WHO, 1990; Lipfert et al., 1995)

MeHg is an environmental toxicant that bioaccumulates and bioamplifies in animals and human (Bizily et al., 1999). Ninety five percent of MeHg has been reported to be absorbed via human's gastrointestinal tract after oral administration, which is the major route of exposure (Aberg et al., 1969; Miettinen 1973, Dutczak et al., 1991). The lipophilic nature of MeHg facilitates the penetration into cells. MeHg in the bloodstream is distributed to all tissues in which high concentration is observed in brain, kidney, and liver at 2 hours after injection into rats (Gregus et al., 1986). Liver and kidneys are the primary sites for MeHg excretion. However, liver is the major organ for MeHg excretion, which is primarily determined by biliary excretion It accounts for more than 80 % of the total excretion in rodents Norseth and Brendeford, 1971). The predominant form of MeHg in bile was mainly complexed with glutathione (GSH) and cysteine (Refsvik and Norseth, 1975, Urano, 1990). Within the cells, MeHg binds to sulfhydryl groups on GSH and may bind to a variety of enzyme systems including those of microsome and mitochondria, causing cell injury and cell death (Grehardsson and Skerfving, 1996). In 1995, Ballatori and Truong using perfused rat liver reported that the chloride complex of methylmercury caused hepatoxicity by induction of cholestasis and increase in perfusion pressure at a concentration of 0.25 μM. In the hepatocyte, MeHg is transported across the

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canalicular membrane into bile by a process depending on intracellular level of GSH (Refsvik, 1987, Miura et al., 1994). Depletion of hepatic GSH levels with indocyanine green and sulfobromophthalein (BSP), but not BSP-GSH complex, resulted in a dramatic decrease in the excretion GSH and MeHg into bile (Ballatori and Clarkson, 1985). In contrast, the increment of hepatic GSH levels with phenobarbital, spironolactone and pregnenolone-16-carbonitrile lead to an increase in the biliary excretion of both GSH and MeHg (Klaassen, 1975). The excretory MeHg-GSH complex is transported across the canalicular membrane into bile by GSH transporters (Ballatori et al., 1995a). Recent evidence suggested that GSH is also transported via the multidrug resistance-associated protein Mrp2 which is localized in the canalicular plasma membranes (Ballatori et al., 1998). Based on these lines of evidence, GSH and pathways for the excretion of mercury in the liver play important role in the regulation of the biliary excretion of MeHg (Miura, 1994).

Phloracetophenone or 2,4,6–trihydroxyacetophenone (THA) (Figure A), which is the aglycone part of glucoside from *Curcuma comosa* (Roxb), Zingiberaceae family, has recently been demonstrated to enhanced bile secretion in rats (Piyachaturawat et al., 1998, Piyachaturawat et al., 2000). THA decreased the elevated plasma alkaline phosphatase to control level in the ethinylestradiol-induced cholestatic rat and enhanced the hepatic clearance of sulfobromophthalein (Piyachaturawat, 2002a). Therefore, THA may have potential to enhance the excretion of substances those undergone biliary excretion and for attenuation of cholestasis (Piyachaturawat et al., 1998; Piyachaturawat et al., 2002a; Piyachaturawat et al., 2002b).

From these lines of evidence, the biliary excretion is the important pathway for detoxication of MeHg. As MeHg is mainly eliminated via bile, thus THA, which effectively increase bile secretion, may accelerate the biliary excretion of MeHg and subsequently attenuate its intoxication. Therefore, it is of interest to investigate the excretory function of the liver in handling MeHg particularly when it was exposed to the toxic levels of MeHg and to determine the modulating effect of THA on biliary excretion of MeHg

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The objectives of the present study are as follows

1. To investigate the effect of MeHg in inducing hepatotoxicity and on the biliary excretion of mercury in adult male rats

2. To determine the effect of THA on modulating biliary excretion of mercury and its toxicity

3. To investigate the possible mechanism s of action by which THA modulate the biliary excretion of MeHg.

The results of this study may have benefits not only for understanding the hepatic elimination of MeHg but also THA related health effects. The information obtained from dose-response effect of MeHg is essential for further setting of appropriate standard for environmental and occupational exposures.

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# CHAPTER II
# LITERATURE REVIEW

## 1. *Methylmercury*

### 1.1. Physical and chemical properties

Mercury (Hg) compounds are divided into two main chemical classes, inorganic (including elemental Hg) and organic Hg. In its elemental form, Hg at room temperature is a heavy silvery liquid, which can be readily dissolved in biological fluids. Inorganic Hg compounds also include mercuric ($Hg^{++}$) and mercurous ($Hg_2^{++}$) states. Both states can form numerous inorganic and organic chemical compounds. Organic Hg compounds are those in which Hg is covalently bound to at least one carbon atom, such as methylmercury (MeHg) and phenylmercury. The organic Hg compound that is usually considered to be of the greatest toxicological interest is MeHg (Nickle, 1999). Wood and Wang, 1983 reported the formation of MeHg compounds by which the non-enzymic methylation of inorganic Hg ($Hg^{++}$) by methylcobalamine compounds (analogues of vitamin $B_{12}$). This compound is produced as a result of bacterial synthesis in both aerobic and anaerobic conditions (WHO, 1990). MeHg generally has appreciable vapor pressure at room temperature, which is colorless and inflammable. It has boiling point 92 $^{o}$C and easily soluble in ether, alcohol but insoluble in water (Merck index, 1989)

### 1.2. Sources of human and environmental exposure

MeHg is commonly found in the environment. Their presence could be due to natural occurrence or as a result of human activities. To understand the origins of human exposure to MeHg, it is important to examine the environmental movement of the inorganic species. The major natural source of MeHg in environmental is the methylation of inorganic Hg. The sources of inorganic Hg are degassing of the earth's crust, emissions from volcanoes, and evaporation from natural bodies of water. The earth's crust is also an important source of Hg for bodies of natural water. Some of this Hg is undoubtedly of natural origin, but some may have been generated by human

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activities. The important man-made sources are mining activities resulting in losses of Hg through the dumping of mine tailings and direct discharges to the atmosphere. Other important man-made sources are the combustion of fossil fuels, the smelting of metal sulfide ores, the production of cement, and refuse incineration. Moreover, Hg has been used in a various industries. The major uses of Hg are a cathode in the electrolysis of sodium chloride solution to produce caustic soda and chlorine gas, the electrical industry (lamps, arc rectifiers, and Hg battery cells), in control instruments in the home and industry (switches, thermostats, barometers). It is also widely used for tooth amalgam fillings and other laboratory and medical instruments so the possibility of occupational exposures remains. Although WHO has earned against the use of alkylmercury compounds in seed dressing (WHO, 1974). Hg compounds continue to be used in anti-fouling, mildewproofing paints as well as to control fungal infections of seeds, bulb plants, and vegetation. Interestingly, the releases of Hg into confined areas can be the source of high toxicity risk even though these releases may be small relative to global emissions. This point is relevant to the contamination of Minamata Bay and the Agano River in Niigata, Japan, as well as to inadvertent poisoning via contaminated bread in Iraq (WHO, 1990).

In Thailand, although there is no obvious health problem, but the mercury pollution is becoming a severe problem in the Gulf of Thailand. The data form International Cooperative Study of the Gulf of Thailand indicated that there are some significant environmental consequences associated with petroleum production. Not only their concerns about petroleium itself which is released into the Gulf, but in recent years there has been a great concern about industrial by-products, inflowing water form entering rivers, and agricultural waste. All of which are quite harmful to the marine life and human health effects. As recently reported by Environmental Pressure Group Wild Aid that shark fins in Thailand contain dangerously high levels of Hg.

The change in speciation of Hg from inorganic to methylated forms is the first step in the aquatic bioaccumulation process. Once MeHg is released, it enters the food chain by rapid diffusion and tight binding to proteins. As a result of food-chain biomagnification, high levels are found in the tissues of such predatory species as

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freshwater trout, pike, walleye, ocean tuna, swordfish, and shark. Fish from nonpolluted water areas may contain 0.01 to 0.05 mg/kg BW (Schutz et al., 1994). However, in polluted areas, Hg concentrations may exceed 1 mg/kg (Al-Majed and Preston, 2000; Junghans, 1983; Micttinen, 1973; WHO, 1990). For the general population, exposure to organic Hg from ingestion of food is the most important route of environmental exposure.

### 1.3. Toxicokinetics

#### 1.3.1. Absorption

Absorption of Hg in humans and animals varies, depending on the Hg form and exposure route. Inhalation of some organic Hg compounds could produce intoxication in humans (Friberg et al., 1986), but quantitative data were not available. In small rodents, MeHg is rapidly and completely absorbed from the lung into the systemic circulation (Ostlund, 1969: Fang, 1980). Dermal absorption of MeHg is limited ranging from 2.25 – 5.9 % in guinea pigs (Friberg et al., 1986). However, MeHg is almost completely absorbed in the gastrointestinal tract because of its lipophilicity, which facilitates the penetration into the cell. Aberg et al (1969) and Miettinen (1973) reported that 95 % of MeHg was absorbed via human's gastrointestinal tract after oral administration. After ingestion of MeHg in water or incorporated into food about 97 % to 100 % of MeHg was absorbed from the gastrointestinal tract (Clarkson, 1971; Nielsen and Andersen, 1991; Nielsen, 1992). Nevertheless, no differences between young and adult rats were found for the absorption of organic Hg (Walsh, 1982).

#### 1.3.2. Distribution and metabolic transformation

After exposure to MeHg majority, the dose eventually distributed into the systemic circulation. In blood, the major part is bound to the cellular components of blood (mainly hemoglobin in the erythrocyte), or it can bind onto some ligands in the plasma, especially those containing free sulfhydryl groups. The ratio between the MeHg levels in erythrocytes and plasma is usually about 20:1 (Zalups and Lash, 1996). However, the species differences exist. Once MeHg gains access to the circulatory system, the three primary tissues or organs system that determine the status of MeHg are the blood, liver, and kidneys. The high concentration was observed in

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brain, kidney, and liver 2 hours after injection into rats (Gregus Z et al., 1986). Within these tissues, MeHg may bind to a variety of enzyme systems including those of microsomes and mitochondia, producing cell injury and cell death (Ashour et al., 1993; Zalups and Lash, 1996). It also has a particular affinity for nonspecific ligand containing sulfhydryl groups, in particular glutathione (GSH), which is one of the predominant intracellular nonprotein thiols. In each of these compartments, tissue GSH status appears to be an important determinant in the biological disposition of MeHg (Alexander et al., 1982; Zalups et al., 1997). Rabenstein (1989) notes that the bonding between GSH and methylmercury chloride in a 1:1 ratio. These bonds appear to be quite stable *in vitro* since negligible dissociation has been detected throughout a pH range of 0-14.

**1.3.2.1. Molecular mechanism of MeHg transport in the liver**

The mechanism of hepatic uptake of MeHg is depending on the two points. First, the great affinity between MeHg and sulfhydryl groups suggests that virtually all of the MeHg that is delivered to the liver through the portal circulation will be in the form of complex with proteins such as albumin or with low molecular weight thiols such as cysteine or GSH. Secondary, even though MeHg is lipid soluble, but a simple diffusion plays only a minimal role in the transport of MeHg across biological membranes (Ballatori, 1991). Thus, it appears that specific transport carriers of Hg-containing complexes are present on the sinusoidal plasma membranes that mediate uptake of Hg into the hepatocytes. Inside the hepatocytes, MeHg has numerous potential ligands with which to bind, including metallothionein (MT), other sulfhydryl-containing proteins, and low molecular weight thiols such as cysteine and GSH. Binding to MT plays only a minor role in the intracellular sequestration of MeHg, because the binding affinity of MeHg for MT is much lower than that of inorganic Hg for MT. Because of the predominance of GSH in the cell and the existence of specific plasma membrane transport systems, GSH appears to play a central role in the hepatic disposition of both organic and inorganic Hg. In the hepatocytes, intracellular GSH is present at a concentration of approximately 10 mM, while cysteine is generally found at a concentration less than 0.2 mM (Zalups and

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Lash, 1996). It is probable, therefore, that MeHg is presented to the active site of transport proteins on the canalicular membrane, with at least one GSH molecule bound to each mercuric ion because GSH plays a major role in the biliary secretion of MeHg (Ballatori and Clarkson, 1983, 1984). The MeHg-GSH complex may be transported into the bile across the canalicular membrane by specific transport mechanisms (Dutczak et al., 1992; Ballatori et al., 1995b). Recent study in isolated canalicular plasma membrane vesicles demonstrate that canalicular MeHg-GSH transport is mediated by high- and low-affinity electrogenic carriers that also transport GSH (Ballatori et al., 1995b). However, recent study by Rebbeor et al, (2000; 2002) demonstrated that low affinity ATP-dependent transport of GSH are multidrug resistance-associated proteins 2 (Mrp2), the liver canalicular organic anion transporter. Mrp2 mediates effluxes of GSH, GSSG, and GSH *S*-conjugates from the hepatocyte into bile. The canalicular or biliary epithelial membrane has both γ-glutamyltransferase (γ-GT) and a dipeptidase for degrading GSH to cysteinylglycine *S*-conjugates and cysteine *S*-conjugates, respectively (Ballatori et al., 1986, 1988). These complexes may be recycled back to the liver by a biliary-hepatic recycling mechanism (Dutczak and Ballatori, 1992) or they may be delivered to the intestine for eventual excretion or reabsorption and return to the liver (enterohepatic circulation) and other organ. The complexes of MeHg and the degradative products of GSH catabolism can be reabsorbed by a poorly defined biliary-hepatic recycling mechanism. Exposure of the kidneys to these MeHg complexes could also occur by reabsorption into hepatocytes and efflux across the sinusoidal membrane into plasma (hepatic-renal circulation). Recent studies indicated that GSH derivatives and GSH *S*-conjugates efflux from hepatocytes by function of Oatp1 and Oatp2, the hepatic basolateral organic solute transporter (Li et al., 1998; Li et al., 2000).

**1.3.2.2. Molecular mechanism of MeHg transport in the kidney**

Mechanisms involved in the renal tubular uptake and transport of MeHg are not well understood. MeHg-GSH complexes may move more readily across the basolateral membrane due to the lipophilic nature and can be transported across the basolateral membrane that is dependening on the activity of the

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sodium pump. Organic forms of Hg also accumulate in the kidneys of mammals to a significant degree (Norseth and Clarkson, 1970; Zalups et al., 1992), but to a lesser extent than inorganic or elemental forms of Hg. Regarding the intrarenal distribution of organic Hg, it accumulates primarily in the renal cortex and outer stripe of the outer medulla along the proximal tubule (Zalups and Barfusss, 1990). Tanaka-Kagawa et al. (1993) provided *in vivo* evidence in mice suggesting that some MeHg present in the cytoplasm of renal epithelial cells along the proximal tubule may enter into the lumen by a secretory pathway as a MeHg-GSH complex. It is currently unknown whether there is any significant uptake and/or transport of inorganic or organic forms of Hg along other segments of the nephron or collecting duct. Complexes of Hg bound to cysteine or GSH are filtered freely at the glomerulus and are handled and reabsorbed avidly along the proximal tubule (Silbernagl, 1992). One potential mechanisms involves the reabsorption of GSH and MeHg after the catalytic cleavage of the γ-glutamylcysteine bound by γ-glutamyltransferase, which releases glutamate from GSH for reabsorption by the sodium-dependent amino acid transport system (Zalups and Lash, 1996). Further processing may be required by the dipeptidase on the luminal membrane that is responsible for hydrolysis of the cysteinylglycine bond (Silbernagl, 1992), which allows both cysteine and glycine to be reabsorbed by the sodium-dependent amino acid transport system. There is experimental evidence indicating that γ-glutamyltransferase plays an important role in the proximal tubular reabsorption of MeHg. This evidence comes from studies showing that inhibition of γ-glutamyltransferase with acivicin prior to exposure to MeHg (Naganuma et al., 1988, Tanaka-Kagawa et al. 1993) caused a decrease in the renal burden of Hg and an increase the urinary excretion of Hg. Additional evidence supporting this hypothesis is from a previous study in which it was shown that the urinary excretion of MeHg and GSH increased in rats (Berndt et al., 1985) and mice (Tanaka et al., 1990) when they were pretreated with acivicin. These data again indicate that the brush border enzyme γ-glutamyltransferase is probably involved in the renal uptake and accumulation of MeHg. Zalups and Lash (1997) also showed recently that the renal accumulation of Hg in rat was decreased when intracellular GSH in the liver and renal was depleted with buthionine sulfoximine (inhibiting the synthesis of GSH), diethyl

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maleate (forming a complex with GSH) or in the bile duct ligated-rats prior to treatment with inorganic Hg. These data seem to indicate the presence of a hepato-renal shunt that delivers Hg to the kidneys as a MeHg-GSH conjugate or complex that is first formed in the liver.

**1.3.3. Elimination**

MeHg is cleared from the body via two main routes: urinary and fecal excretion. However, fecal excretion is the major route of MeHg elimination, which is determined primarily by biliary secretion. The fecal pathway accounts for about 90% of the total elimination of Hg in man and other mammals after exposure to MeHg. The biological half-life for MeHg is about 120 days (WHO, 1990). Recent studies in rats with congenital defect in biliary GSH excretion demonstrate that biliary excretion of MeHg was markedly impaired and the biological half-life is increased 2.5 times (Ballatori et al., 1995a).

**1.4. Toxicity**

The toxicity of MeHg has caused widespread public concern as a result of several widely publicized diasters. In the 1953S, an epidemic of MeHg poisoning developed in Minamata, Japan, affecting over 2500 individuals. About 10 years after Minamata poisoning a similar incident occurred in Niigata. The main source of MeHg in both cases was through consumption of contaminated shellfish that has accumulated MeHg dumped into water as an industrial effluent. It also occurred in Iraq, Pakistan, and Guatemala (WHO, 1990). The disaster in Iraq occurred as a result of the ingestion of seed that had been treated with MeHg fungicide. MeHg causes adverse central nervous system effects in adults as well as children, although children clearly appear to be more vulnerable to such effects. Acute high level exposure during development results in a range of neurotoxic effects that include cerebral palsy, mental deficiency, motor retardation, and sensory deficits such as blindness and deafness (Wu et al., 1985; Rice et al., 1990). However, in human the clinical symptoms of neurotoxic effect of MeHg take time. It became evident only after a relatively long latent period of 1 – 2 months (Ballotori et al., 1998). The organs and cells of MeHg accumulation are liver, mucous membranes of the intestinal tract, epithelium of the skin, spleen, interstitial cells of the testicles, and some parts of the

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brain. In the liver, the highest concentrations of MeHg are found in periportal regions of the liver lobule (Berlin and Ullberg, 1963). Acute MeHg overload due to accidental or suicidal ingestion causes abdominal pain, diarrhea, and necrosis of the intestinal mucosa that may lead to circulatory collapse and death. However, if patients survive the gastrointestinal damage, renal failure due to necrosis of the proximal tubular epithelium occurs, leading to anuria and uremia (Zalups and Lash, 1996). In the hepatocytes, MeHg exerts its toxicity by binding to sulfhydryl groups on proteins and inactivates key enzymes in glycolysis and oxidative phosphorylation, leading to inhibition of respiration, depolarization of mitochondria, and depletion of ATP. The degree of Hg-dependent hepatotoxicity depends on the chemical form of the heavy metal. MeHg is far more toxic to rat hepatocytes than mercuric chloride, causing both pathological and biochemical changes in the organ (Stacey and Klaassen, 1981; Ashour et al., 1993). In study by Ashour et al (1993), a different concentration of MeHg toxicity was evaluated by aspartate and alanine transaminase leakage. MeHg cause an increased in serum alanine transaminase activity at concentrations that did not increase aspartate transanimase since the aspartate transaminase is localized mainly to mitochondria (Clampitt, 1987). However, Ashour et al (1993) reported that the liver showed a significant increase of alanine transaminase (ALT) and aspartate transaminase (AST) in dose-and time-dependent manner. Therefore, the authors concluded that the major site of MeHg toxicity was cytosolic held inside the cell. The liver contains the highest concentration of GSH (about 10 mM) than any major organs. GSH acts as a reducing and conjugating agent to detoxify many potential toxicants. In the hepatocytes, GSH forms a complex with MeHg that is rapidly excreted into the bile resulting in the reduction of the hepatic content of MeHg. Therefore, GSH protects hepatocytes from overload with MeHg. Approximately 90% of cellular GSH is found in the cytosol with the remainder sequested in mitochondria. The prominent feature of MeHg intoxication in mice is a marked reduction of GSH in both brain and liver. Especially in the liver, hepatocytes can maintain viability in the face of cytosolic GSH depletion but even small alterations of mitochondrial GSH promote irreversible cell injury (Meredith and Reed, 1982). Mitochondria appear to be a preferential target for MeHg and MeHg may cause rapid mitochondria GSH

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depletion leading to mitochondrial dysfunction and ultimately cell death (Nieminen et al., 1990). Ashour et al. (1993) reported that MeHg induced lipid peroxidation which led to a depletion of GSH in liver cell in culture cells as well as in experimental animals. In addition, glutathione peroxidases (GSH-PX) and glutathione reductase (GSH-RX) were significantly decreased. In 1995c, Ballatori and Truong studied in isolated rat hepatocytes and reported that the chloride complex of MeHg caused hepatoxicity by induction of cholestasis and increase in perfusion pressure at a concentration of 0.25 μM. However, the cholestasis effect of MeHg has not been elucidated *in vivo*.

## *2. Phloracetophenone (2,4,6-trihydroxyacetophenone)*

Phloracetophenone or 2,4,6–trihydroxyacetophenone (THA) (Figure A) is the aglycone part of glucoside from *Curcuma comosa.*(Roxb)*.,* Zingiberaceae family. It is commonly known as Wan Chak Mod Look that has been widely used in folk medicine in the north-eastern and eastern region of Thailand. The biological activities of the crude extracts of *C. comosa* have been extensively studied in our laboratory. However, the choleretic activity was most effectively induced by butanol extract and ethyl acetate extract (Piyachaturawa et al., 1996; Suksamrarn et al., 1997). Choleretic activity of THA and its analogs has recently been studied in rats by Piyachaturawat et al (1998, 2000). The fourteen acetophenone analogs of the compound varying in substituents on the benzene nucleus and in the structure of side chain of acetophenone have been screened for their choleretic activity. The results demonstrated the differences in bile flow, bile acid output, and biliary cholesterol caused by these individual analogs. The variability in the choleretic efficacy of these different compounds is related to their physicochemical properties. Among hydroxylated acetophenone analogs, 2,4,6–trihydroxyacetophenone was the most effective compound in stimulating bile secretion with a high bile flow rate and a high bile salts output and led to lower plasma cholesterol and phospholipid levels. Furthermore, this bile had a low lithogenic index. Therefore, THA enhanced bile secretion by the mechanism, which resulted from increasing in both bile acid-dependent and bile acid-independent flow. Base on this available information, THA may have potential for

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development as a therapeutic agent for dissolving gallstone, reducing plasma cholesterol, and treatment of cholestasis or enhance excretion of substances that undergo biliary secretion (Piyachaturawat et al., 1998; Piyachaturawat et al., 2002a). In a recent study, the possible therapeutic effect of the choleretic THA was evaluated in both the hypercholesterolemic hamsters (Piyachaturawat et al., 2002b) and ethinylestradiol-induced cholestasis rats (Piyachaturawat et al., 2002a). These results suggested that THA exert its cholesterol lowering effect by inducing hepatic cholesterol 7α- hydroxylase activity which increases hepatic conversion of cholesterol to bile acid for disposal via biliary secretion. In addition, THA increased the hepatic clearance of sulfobromophthalein and reversed elevated plasma alkaline phosphatase to a control level. Concurrently, the acute and subacute toxicity of THA was investigated in mice, rats, and hamsters (Piyachaturawat et al., 2002a). $LD_{50}$ value was 489 mg/kg BW after a single oral administration to adult male rats. This result suggested that THA at a biologically active choleretic dose (100 mg/kg BW) had low toxicity.

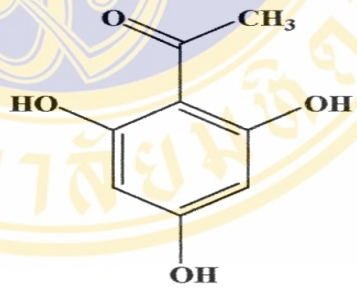


**Figure A**. Structure of phloracetophenone or 2,4,6–trihydroxyacetophenone (THA)

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# CHAPTER III
# MATERIALS AND METHODS

## A. Animals

Adult male Wistar rats weighting between 250 – 300 g were supplied by the National Laboratory Animal Center, Salaya, Mahidol University, Nakornprathom, Thailand. They were housed in a controlled room temperature approximately 25 ± 2 $^{o}$C and a relative humidity of approximately 50 ± 10 % in stainless steel hanging cages. A light-dark cycle of 12-h was maintained. They were fed with regular rat chow (Pokphand Animal Feed Co., Ltd.; Bangkok, Thailand) and tap water *ad libitum*. The studies were conducted in accordance with the International Guiding Principles for Biomedical Research involving Animals (CIOMS/WHO).

## B. Chemicals

Methylmercuric chloride (MeHg) was purchased from Wako Chemical Co (Tokyo, Japan). Phloracetophenone (2,4,6-hydroxyacetophenone, THA) was purchased from Fluka Chemie AG (Buchs, Switzerland) and its purity is over 99 %. Glutathione (GSH), GSH reductase, nicotinamine adenine dinucleotide phosphate (NADPH), buthionine sulfoximine (BSO), acivicin, 1-chloro-2,4-dinitrobenzene (CDNB) were purchased from Sigma Chemical Co (St. Louis, MO, USA). Enzymatic reagent kits for determinations of plasma alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALT) and bile ALP were purchased from Bio-Medical Laboratory (BM-Lab, Bangkok, Thailand). All other reagents were of analytical grade.

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## C. Experimental Procedure

### a) General animal preparations for bile collection.

After an overnight fast, animals were anesthetized with sodium pentobarbital (50 mg/kg, i.p.). A tracheostomy was performed and a midline abdominal incision was made to expose the liver. The common bile duct, left femoral vein and left femoral artery were cannulated with polyethylene (PE)-10 tubing for collection of bile secretion and PE-50 tubing for infusion of normal saline (0.9 % w/v aqueous sodium chloride) and for blood sample collection, respectively (Figure B). In the case of urine collection, both ureters were cannulated for the collection. Body temperature of animal was maintained at 37 °C with an overhead-heating lamp. After 30 minutes of stabilization, bile samples were collected over a 30-min period for 2 hours in chilled tared Eppendorf tubes containing 2 – 4 vol of 0.25 M perchloric acid in order to precipitate proteins and to prevent autoxidation of GSH to GSSG (Akerboom and Sies, 1981). Two hours bile and urine samples were kept for further analyses of GSH, alkaline phosphatase activity, and total mercury contents respectively. Concurrent with bile sample collection, the blood sample was also collected every hour for determinations of AST, ALT, and ALP activities. At the end of the experiment, liver was removed and kept at –80 °C for analysis of GSH and mercury content.

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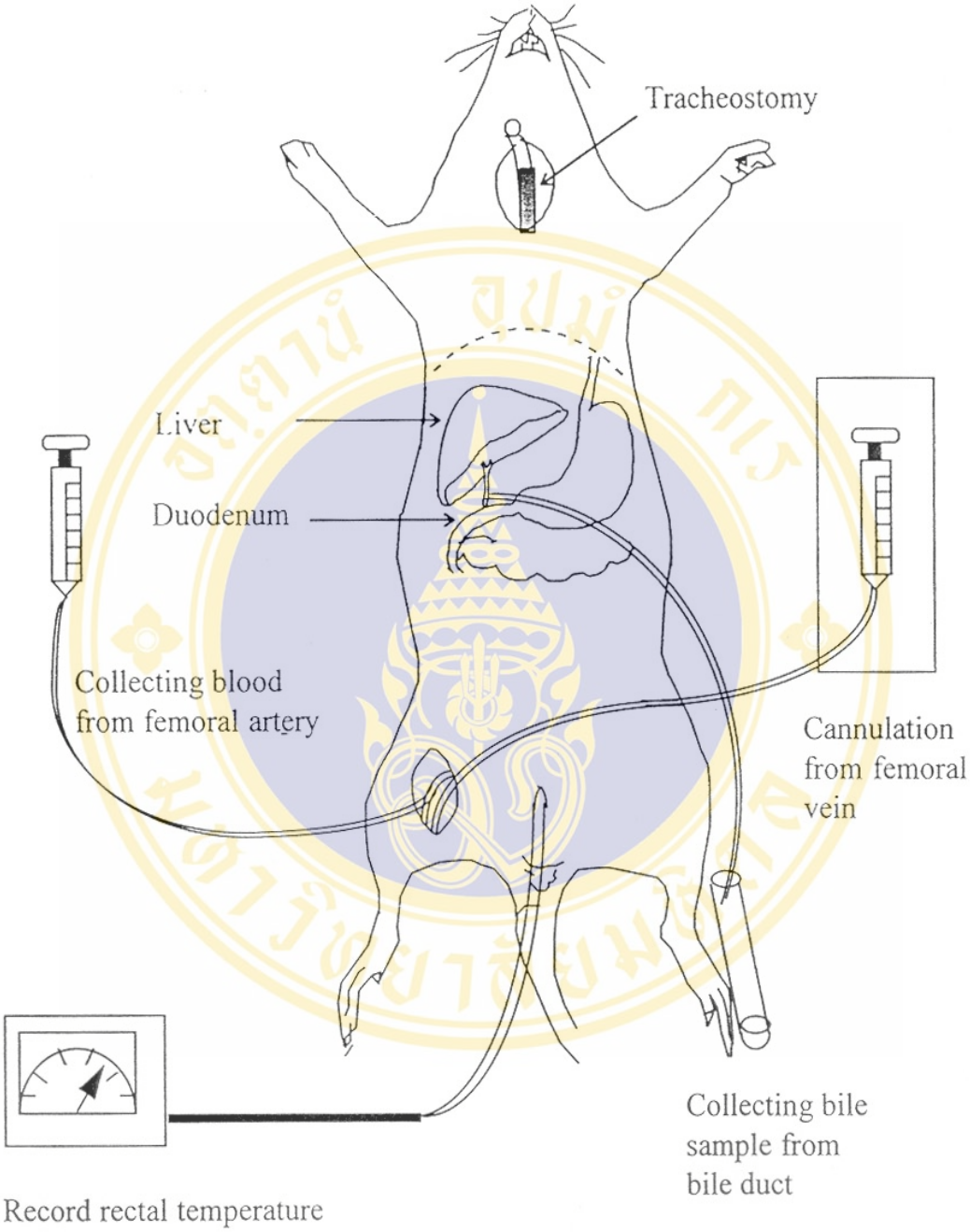


**Figure B.** Experimental scheme showing general animal operation and cannulation.

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**b) Preparation of subcellular fractions**

At the end of experiment, following blood sample collection, rats liver was immediately removed, trimmed of fat, blotted, weighed and immediately immersed in ice-cold 0.9 % NaCl. Liver was perfused with ice-cold 0.9 % NaCl to remove hemoglobin. Liver homogenate was prepared in 4 volume of 1.15 % KCl in 0.1 M phosphate buffer, pH 7.4 using a Teflon-glass potter-Elvehjem. The homogenate was centrifuged at 1,000 x g for 10 min to remove unbroken cells and cell debris. The supernatant fraction was then centrifuged at 10,000 x g at 4 $^{o}$C for 20 min to remove mitochondria and lysosomes. The supernatant fraction was further centrifuged at 100,000x g for 60 minutes by using ultracentrifugation (Beckman, USA.). The supernatant or cytosolic fraction and the pellet or microsomal fraction was obtained. All procedures were conducted at 4 $^{o}$C, and the samples were stored at –80 $^{o}$C until use.

**c) Mercury extraction from liver and bile**

A 5-g portion of finely chopped liver sample was suspended in 5 ml deionized distilled water and was homogenized in a Teflon-glass potter-Elvehjem. Mercury in 0.5 ml of the homogenate as well as 0.5 ml of bile was extracted in a 10-ml glass vial where 5-ml of 1 % NaCl (w/v) in 1 N hydrochloric acid and 1 ml of benzene were added. The vial was capped, shaken in a horizontal shaker for 30 min, and then centrifuged for 5 min at 6000x g. Thereafter, the benzene layer was transferred into a 10-ml vial. The extraction was repeated three times with additional 1-ml aliquots of benzene. The mixture was then shaken for 5 min and recentrifuged as previously described. The final organic layers were combined and stored in a glass vial for determination of total mercury content. One milliliter of 0.01 M $Na_2S_2O_3$ solution was added to the benzene layers for back extraction of mercury to aqueous solution and vortexed for 30 second. Then, the benzene layer was discarded and the aqueous layer stored at –20 °C for determination of mercury by using graphite furnace atomic absorption spectrophotometry (GFAAS).

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**d) Biochemical analyses**

**1) Determination of enzyme alanine aminotransaminase (ALT)**

Alanine aminotransaminase activity was determined as previously described by Reitman and Frankel method (1957) (Appendix A) using commercial diagnostic kits (Bio-Medical Laboratory). The α-ketoglutaric acid and dl-alanine were served as substrates. The transaminase catalyzed reaction leading to the formation of glutamic acid and pyruvic acid. Pyruvic acid reacted with 2,4-dinitrophenylhydrazine, giving the hydrozone. The yellow-brown color of hydrazone was measured colorimetrically by spectrophotometer at 550 nm.

**2) Determination of enzyme aspatate aminotransaminase (AST)**

Aspatate aminotransaminase was determined as previously described for ALT except that the substrate was aspartic acid rather than alanine and the product of reaction was oxaloacetic acid. Oxaloacetic acid was then reacted with 2,4-dinitrophenylhydrazine, producing yellow-brown hydrazone, which was measured colorimetrically by spectrophotometer (Appendix B).

**3) Determination of enzyme alkaline phosphatase (ALP)**

Plasma and biliary ALP was determined according to the method described by Authur in 1996 (Appendix C). Alkaline phosphatase hydrolysed phenolphthalein monophosphate in alkaline solution and produced a phenolphthalein that has a pink color and could be measured at 550 nm.

**4) Determination of protein**

Protein concentration of subcellular preparations was determined using the modified Lowry method (1951) (Appendix D). The protein reacted with copper in alkali and then adding Folin reagent to the copper-treated protein, the original yellow color of the phosphomolybdate will be disappeared. The dissociation of the phosphate from molybdate resulted in blue color product, and the absorbance of the final product was measured at 770 nm.

**5) Determination of bile and urine flow rate**

Bile and urine flow rates were estimated using the gravimetric method, assuming a bile density as well as urine density, which are 1.0 g/ml. The pre-weighed eppendorf was used for the collection. Then this eppendrof filled with

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bile or urine will be weighed again. The difference of the weights between blank eppendrof and the eppendrof-contained bile or urine is assumed to be the weight of bile or urine collected in the indicated periods.

**6) Determination of glutathione *S*-transferase**

The glutathione *S*-transferase was determined according to the method described by Habig et al., (1980) (Appendix E). In this method, 1,2-Dichloro-4-nitrobenzene (DCNB) was used as a substrate. When it was conjugated with glutathione (GSH) which was catalyzed by glutathione-S-transferase in a thermostatted compartment at 25 $^{o}$C resulted in the thioether formation. It was a result from nucleophilic attack at carbon in which the glutathione thiolate anion participates as a nucleophile and was relied on a direct change in the absorbance of the substrate. A complete assay mixture without enzyme was served as the control. The change in absorbance at OD 345 was observed for the enzyme concentration.

**7) Determination of liver and biliary glutathione concentration**

Liver glutathione content was determined by using an enzymatic method described by Theodurus (1981) (Appendix F). Glutathione is conveniently assayed by an enzymatic recycling procedure in which it was sequentially oxidized by 5,5′-dithiobis-(2-nitro-5-thiobenzoic acid) (DTNB) and then was reduced by NADPH in the presence of glutathione reductase. The rate of 2-nitro-5-thiobenzoic acid formations was measured at 412 nm and the glutathione present was evaluated by comparison of those results with a standard curve.

**8) Determination of liver and biliary mercury concentration**

Liver and bile concentration of Hg was determined by using graphite-furnace atomic absorption spectrophotometry (Appendix G). A mercury hollow-cathode lamp operated at 4 mA (spectral band-pass = 0.2 nm) with a deuterium background corrector and at a resonance wavelength of 253.7 nm was used. The optimized experimental furnace conditions were as follows: drying, 100 $^{o}$C for 20 S, ramp time 5 S; ashing, 200 $^{o}$C for 10 S, ramp time 5 S; atomization, 1500 $^{o}$C for 5 S, ramp time 1 S; cleaning, 2000 $^{o}$C for 2 S, ramp time 1 S.

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## D. Statistical Analysis

All results were expressed as means and the standard error of means ($\overline{X}$+SEM). The statistical analysis was conducted by using the statistical software package, SPSS for window version 9.0. The statistical difference among different groups were analyzed by using one way analysis of variance (one-way ANOVA) and the differences in pairs of means were performed by Scheffe's test. Statistical significance of the differences was accepted at the p-values of less than 0.05 or exceeding the 95 % critical value ($p<0.05$).

## E. Experimental Protocol

**Experiment 1 Determination of the dose-response relationship of methyl mercury chloride (MeHg) on acute toxicity.**

This experiment was designed to investigate the acute toxicity of MeHg on the liver. Adult male wistar rats were divided into 5 groups:

Group 1) Vehicle control (0.02 $NaHCO_3$)

Group 2) MeHg 20 μmol/kg BW

Group 3) MeHg 40 μmol/kg BW

Group 4) MeHg 60 μmol/kg BW

Group 5) MeHg 80 μmol/kg BW

Animals were prepared for bile samples collections. After 30 minutes of the control period, MeHg at various doses, ranging from 20 – 80 μmol/kg BW dissolved in 0.02 M $NaHCO_3$, were injected into portal vein at approximately 1 inch away from liver hilum. Bile samples were collected every 30-min period for 2-hours in ice chilled tared Eppendorf tubes containing phosphoric acid to prevent autoxidation of GSH to GSSG (Parvathi et al., 1994). Control rats were given a similar volume of vehicle. Concurrently with bile sample collection, the blood samples (0.5 ml) were also collected very 1-hour period before and after administration of MeHg. At the end of experiment the animals were sacrificed and blood samples were drawn from abdominal aorta for the determination of AST, ALT, and ALP. Liver was immediately removed for determination of GSH and total

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mercury content. Appropriate dose of methyl mercury chloride in inducing hepatotoxicity from the experiment was chosen for the subsequence experiment (Figure C).

**Experimental protocol for experiment 1**

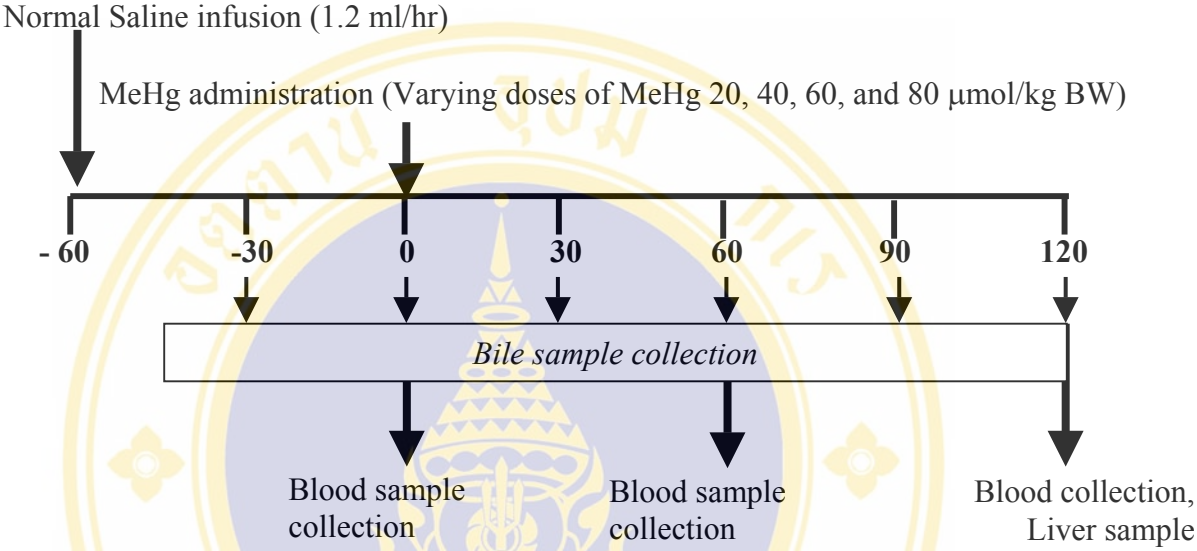


**Figure C.** Schematic diagram showing sample collection and time point for administration of the compound.

**Experiment 2 Effect of THA on biliary excretion of MeHg.**

The purpose of this experiment is to determine the effect of THA, a choleretic agent, in modulating biliary excretion of MeHg. The animals were divided into 5 groups of 8 animals, as follows:

Group (1) Vehicle control for THA treatment with DMSO: EtOH: distilled water, 25:15:60

Group (2) Low dose of THA 300 μmol/kg BW.

Group (3) High dose of THA 600 μmol/kg BW.

Group (4) Vehicle control for THA and vehicle for MeHg

Group (5) MeHg 40 μmol/kg BW + THA 300 μmol/kg BW.

Group (6) MeHg 40 μmol/kg BW + THA 600 μmol/kg BW.

The animals were prepared for bile and urine samples collection for evaluation the capacity of biliary and urinary excretion of MeHg by THA as in the

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Experiment 1. In groups 2 and 3, the animals were given THA both at low and high doses after the third period of collection or at the time of 90 min. In groups 4 and 5, one hour after administration of MeHg (40 μmol/kg BW) via portal vein, THA was intraduodenally injected at a dose of 300 or 600 μmol/kg BW. Bile and urine samples were collected for another two periods for determination of GSH, mercury content, ALP activity and urinary mercury. Blood samples were collected every hour for determination of ALT, AST, and ALP. Control animals were given the similar volume of THA and MeHg vehicles. At the end of experiment, the animal was sacrificed and blood sample was collected from abdominal aorta. Liver was removed for determination of GSH, mercury content, and glutathione-S-transferase (Figure D).

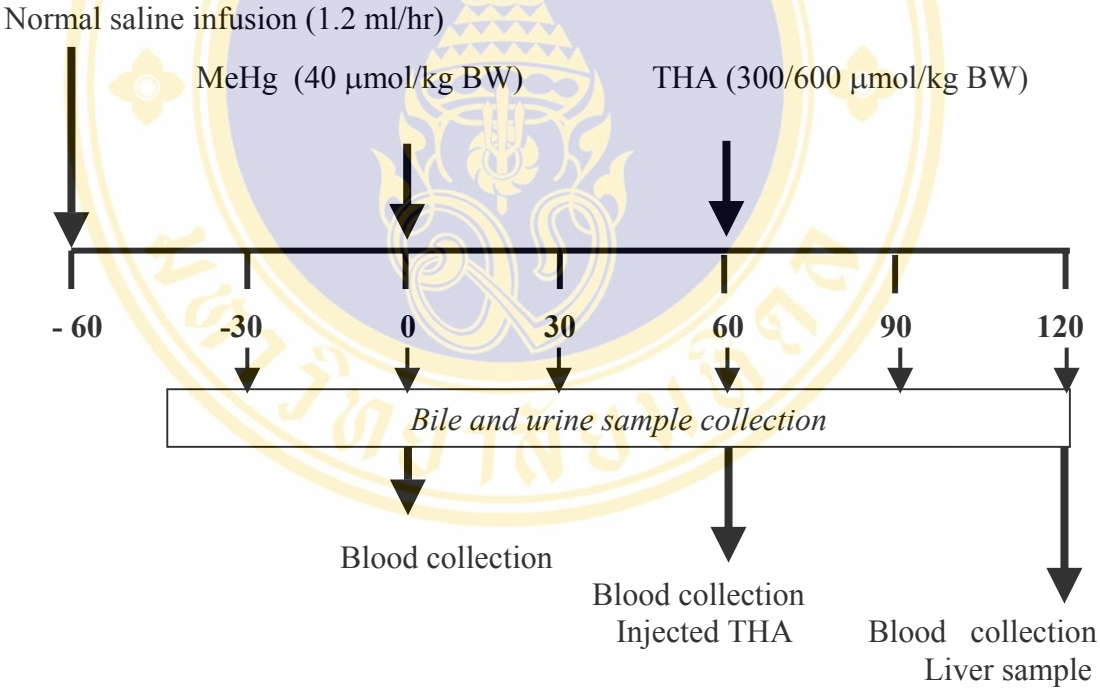


**Figure D.** Schematic diagram showing sample collection and time point for administration of compounds.

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**Experiment 3 Effect of THA administration in GSH-depleted rats**

This experiment was conducted to investigate the possible mechanism(s) by which THA mediated the action. The effect of GSH depletors, buthionine sulfoximine (BSO) and acivicin on the excretion of MeHg after administration of THA was evaluated. The animals were randomly divided into 3 groups of 8 animals per group.

Group (1) Vehicle treated control group (Saline)

Group (2) Pretreatment with acivicin

Group (3) Pretreatment with BSO

The first one was pretreated with normal saline and served as a control. The second group was pretreated with acivicin (to inhibit the γ-glutamyltransferase enzyme), and the third group was treated with BSO which inhibit the γ-glutamylcystein synthetase to deplete GSH in liver and kidney. The protocol used for injection acivicin was done according to that described previously by Zalups and Barfusss, in 1999. The animals received a 10 mg/kg, ip of acivicin dissolve in 2.0 ml/kg normal saline (0.9 % w/v aqueous sodium chloride). Ninety minutes later, the animals received a second 10 mg/kg dose of acivicin that was administered into the left femoral vein (while they were anesthetized). Rats pretreated with BSO were given a 2.0 mmol/kg dose iv in 4.0 ml/kg normal saline. After pretreatment with GSH depletor, MeHg was injected at a dose of 40 μmol/kg BW via portal vein bile samples were collected every 30-min interval for 1-hour. Subsequently, THA was intraduodenally injected at a dose of 300 or 600 μmol/kg BW. At the end of experiment, the animal was sacrificed. Liver was removed for determination of GSH, mercury content, and glutathione-S-transferase activity.

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# CHAPTER IV
# RESULTS

## Experiment I Effect of MeHg on Hepatotoxicity

### 1.1. Dose-response of MeHg in inducing hepatotoxicity.

Dose-response of MeHg in inducing hepatotoxicity was investigated in adult male rats. After a bolus injection of MeHg into the portal vein, bile flow rate (BFR) was abruptly decreased in a dose-related manner (Table 1, Figure 1a). BFR decreased from approximately 63.02 $\pm$ 1.82 – 65.32 $\pm$ 1.15 µl/kg/min in control to 51.87 $\pm$ 1.11 (81.57 % of control), 48.15 $\pm$ 0.57 (75.73 % of control), 42.11 $\pm$ 1.16 (66.23 % of control), and 37.94 $\pm$ 1.71 µl/kg/min (59.67 % of control) within 30 min after administration of MeHg at a dose of 20, 40, 60, and 80 µmol/kg BW, respectively. Concurrent with the decrease in BFR, the biliary secretion of GSH was also significantly decreased ($p<0.05$). As shown in Table 1 and Figure 2b, the biliary secretion of GSH in control animals was steadily maintained at approximately 105.56 $\pm$ 3.05 nmol/kg/min. After administration of MeHg, it immediately reduced to 66.69 $\pm$ 0.63, 58.63 $\pm$ 1.98, 55.33 $\pm$ 2.29, and 50.92 $\pm$ 3.81 nmol/kg/min in rats receiving MeHg 20, 40, 60, and 80 µmol/kg BW, respectively. There was no statistical difference of the secreted GSH among these four different doses of MeHg. The excretion of mercury into bile also showed a dose-related dependent increase with the dose of MeHg administered (Table 3, Figures 2a, b). However, when they were expressed as the percent of the administered dose, the excretion of mercury was found to be decreased with the increasing dose. Two hours after administration of MeHg at a dose of 20, 40, 60, and 80 µmol/kg BW, the excreted amount were 12.6 $\pm$ 0.07 %, 7.95 $\pm$ 0.09 %, 5.58 $\pm$ 0.09 % and 4.77 $\pm$ 0.10 % of the administered doses, respectively. However, the excretory rate of biliary Hg was not altered with the administered doses (Table 3).

Activities of plasma AST, ALT, and ALP, which are indices of liver membrane damage, were markedly increased after receiving MeHg (Table 4).

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The elevation of these enzyme activities showed both a dose- and time-related effects. The plasma activity of AST was increased from 68.42 ± 3.08 IU/ml in the vehicle control to 924.42 ± 93.93, 1679.72 ± 90.80, 1865.04 ± 156.03 and 3215.34 ± 213.88 IU/ml at 60 min after MeHg injection at a dose of 20, 40, 60, and 80 μmol/kg BW, respectively. At 120 min after receiving MeHg, the plasma activity of AST was further increased to 1677.63 ± 176.29, 2348.03 ± 283.84, 2991.00 ± 135.03, and 3510.33 ± 509.30 IU/ml, respectively. In these MeHg-induced hepatotoxicity and cholestasis, the activity of plasma ALT showed a similar increase from 16.38 ± 2.09 IU/ml in the vehicle control to 546.75 ± 107.18, 918.29 ± 126.20, 1182.78 ± 128.10, and 1549.54 ± 199.77 IU/ml at 60-min after MeHg injection at a dose of 20, 40, 60, and 80 μmol/kg BW, respectively. At 120 min after receiving MeHg, the activity of plasma ALT was further increased to 1482.51 ± 143.19, 1787.85 ± 150.17, 2017.29 ± 213.41, and 2296.41 ± 240.81 IU/ml. Likewise at 60 min, plasma ALP activity increased from 43.88 ± 2.39 IU/ml in the vehicle control to 49.74 ± 1.45, 53.18 ± 2.40, 60.64 ± 1.27, and 68.89 ± 1.47 IU/ml, respectively. At 120 min, plasma ALP was further increased to 55.83 ± 1.60, 60.21 ± 1.78, 64.26 ± 2.50, and 75.39 ± 1.84 IU/ml, respectively. The effect of MeHg on biliary levels of ALP was also determined as shown in Table 4. In control rats receiving vehicle, the basal enzyme activity was approximately 5.65 ± 0.89 – 5.91 ± 0.66 IU/ml. After a bolus injection of MeHg at the doses of 20, 40, 60, and 80 μmol/kg BW, they were slightly increased to 7.66 ± 0.45, 8.80 ± 0.32, 9.08 ± 0.17, and 12.82 ± 0.26 IU/ml at 60 min, respectively. At 120 min after MeHg injection, the biliary ALP was further increased to 7.13 ± 0.92, 10.57 ± 0.84, 14.83 ± 0.90, and 15.29 ± 0.76 IU/ml, respectively. All changes were significantly different from their controls ($p<0.05$) and showed dose-related effects.

Table 5 and Figure 3 show the concentrations of hepatic GSH at two hour after MeHg injection. They were significantly decreased from 6.31 ± 0.46 μmol/g liver to 5.08 ± 0.54 (80.51 % of control), 4.50 ± 0.36 (71.32 % of control), 3.75 ± 0.28 (59.43 % of control) and 2.10 ± 0.27 μmol/g liver (32.28 % of control), in MeHg-treated rats at a dose of 20, 40, 60, and 80 μmol/kg BW, respectively ($p<0.05$). The concentrations of mercury found in liver were 9.21 ± 0.97 μmol/g liver (25 % of

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administered dose), 16.02 ± 1.39 (32.1 % of administered dose), 23.81 ± 1.48 (36.13 % of administered
dose) and 32.77 ± 1.59 μmol/g liver (38.25 % of administered dose) after administration of MeHg at dose of 20, 40, 60, and 80 μmol/kg BW, respectively (Table 5, Figure 4). It is evident that, both a decrease in concentration of hepatic GSH and an increase in mercury are dose-related manner.

From this part of the study, it indicated that administrations of MeHg at a dose of 20-80 μmol/kg BW into portal vein are acutely toxic to the liver. It immediately decreased BFR, biliary secretion of GSH, liver GSH content but induced an increase in liver mercury accumulation and liver cell membrane damages. All of them showed dose-related effects to MeHg. A submaximal dose of MeHg at 40 μ mol/kg BW was chosen for subsequent studies for evaluating any modulation by the choleretic THA.

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**Table 1.** Effect of methylmercury chloride (MeHg) on bile flow rate (µl/kg/min).

| Treatment | No. of animal | Bile flow rate (µl/kg/min) | | | | |
|---|---|---|---|---|---|---|
| | | 0 | 30 | 60 | 90 | 120 (min) |
| Control | 8 | 64.25 ± 2.77 | 63.89 ± 2.72 | 62.82 ± 2.78 | 62.88 ± 2.71 | 62.39 ± 2.33 |
| MeHg 20 µmol/kg BW | 8 | 65.15 ± 0.84 | 51.87 ± 1.11* | 52.04 ± 1.25* | 55.12 ± 1.82* | 57.92 ± 1.61* |
| MeHg 40 µmol/kg BW | 8 | 65.32 ± 1.15 | 48.15 ± 0.57* | 50.53 ± 0.84* | 51.21 ± 1.29* | 50.88 ± 1.40* |
| MeHg 60 µmol/kg BW | 8 | 65.17 ± 1.07 | 42.11 ± 1.16* | 45.25 ± 2.13* | 45.44 ± 2.75* | 45.42 ± 2.89* |
| MeHg 80 µmol/kg BW | 8 | 63.02 ± 1.82 | 37.94 ± 1.71* | 38.48 ± 1.92* | 42.00 ± 1.28* | 43.23 ± 3.17* |

MeHg was injected into the hepatic portal vein. The biliary excretion of MeHg was determined at 30-min period. Each value is mean ± SEM.

* $p < 0.05$ significant difference when compared with the vehicle control at the corresponding time.

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**Table 2.** Effect of methylmercury chloride (MeHg) on biliary secretion of glutathione (GSH) (nmol/kg/min).

| Treatment | No. of animal | GSH (nmol/kg/min) | | | | |
|---|---|---|---|---|---|---|
| | | 0 | 30 | 60 | 90 | 120 (min) |
| Control | 8 | 105.56 ± 3.05 | 104.99 ± 4.33 | 105.68 ± 4.11 | 105.89 ± 3.07 | 107.31 ± 2.97 |
| MeHg 20 µmol/kg BW | 8 | 101.73 ± 2.87 | 66.69 ± 0.63* | 64.71 ± 2.52* | 63.68 ± 2.25* | 65.44 ± 5.67* |
| MeHg 40 µmol/kg BW | 8 | 106.88 ± 4.61 | 58.63 ± 1.98* | 58.84 ± 1.72* | 59.82 ± 1.74* | 57.34 ± 2.29* |
| MeHg 60 µmol/kg BW | 8 | 101.81 ± 2.28 | 55.33 ± 2.29* | 56.99 ± 2.14* | 45.44 ± 2.12* | 52.65 ± 0.85* |
| MeHg 80 µmol/kg BW | 8 | 110.81 ± 5.87 | 50.95 ± 3.81* | 43.69 ± 2.31* | 48.47 ± 3.04* | 52.49 ± 1.95* |

MeHg was injected into the hepatic portal vein. The biliary excretion of MeHg was determined at 30-min period.
Each value is mean ± SEM.

* $p < 0.05$ significant difference when compared with the vehicle control at the corresponding time.



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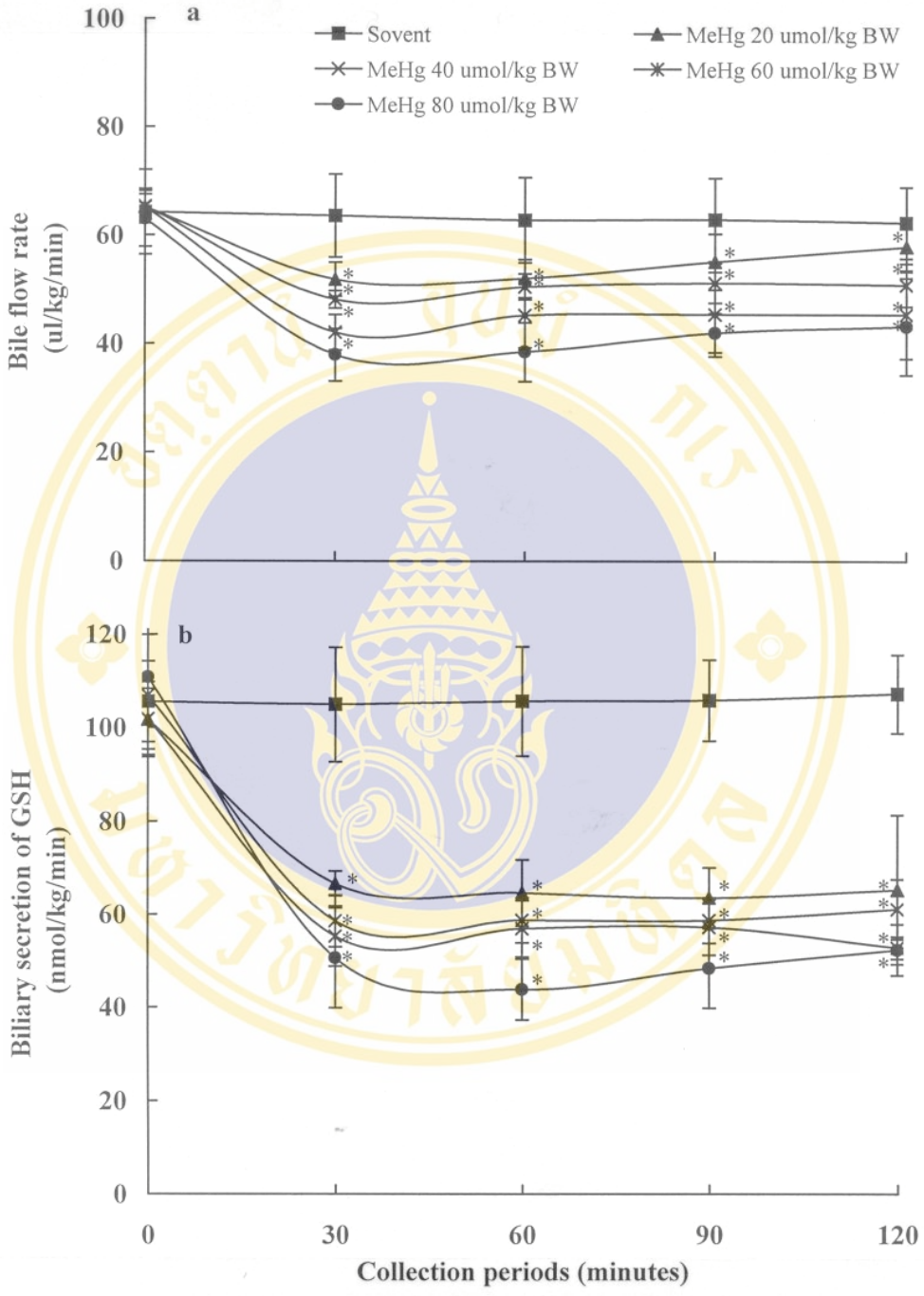


**Figure 1.** Bile flow rate (a) and biliary secretion of glutathione (GSH) (b) after a bolus injection of methylmercury chloride (MeHg) into the hepatic portal vein at a dose of 20-80 μmol/kg BW. Bile sample was collected in 30-min period for 120 min.
Each point represents mean ± SEM of 8 rats/group.
* $p < 0.05$ significant difference when compared with the vehicle control at the corresponding time.

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**Table 3.** Effect of methylmercury chloride (MeHg) on cumulative excretion of mercury in bile.

| Time (min) | MeHg (μmol/kg BW) | | | |
|---|---|---|---|---|
| | 20 | 40 | 60 | 80 |
| **At 30-min after MeHg administration** | | | | |
| Cumulative excretion (μmol/kg BW) | 0.19 ± 0.01 | 0.27 ± 0.01* | 0.31 ± 0.01* | 0.38 ± 0.01* |
| Accumulation (% dose) | 1.00 ± 0.05 | 0.62 ± 0.03* | 0.50 ± 0.02* | 0.50 ± 0.01* |
| Excretion rate (μmol/kg BW/30 min) | 0.19 ± 0.01 | 0.27 ± 0.01 | 0.31 ± 0.02 | 0.38 ± 0.04 |
| **At 60-min after MeHg administration** | | | | |
| Cumulative excretion (μmol/kg BW) | 0.36 ± 0.02 | 0.45 ± 0.01* | 0.50 ± 0.01* | 0.59 ± 0.77* |
| Accumulation (% dose) | 1.74 ± 0.10 | 1.12 ± 0.03* | 0.83 ± 0.02* | 0.75 ± 0.09* |
| Excretion rate (μmol/kg BW/30 min) | 0.18 ± 0.01 | 0.19 ± 0.02 | 0.20 ± 0.02 | 0.21 ± 0.02 |
| **At 90-min after MeHg administration** | | | | |
| Cumulative excretion (μmol/kg BW) | 0.49 ± 0.02 | 0.61 ± 0.02* | 0.66 ± 0.01* | 0.77 ± 0.02* |
| Accumulation (% dose) | 2.49 ± 0.10 | 1.50 ± 0.05* | 1.08 ± 0.02* | 0.93 ± 0.03* |
| Excretion rate (μmol/kg BW/30 min) | 0.13 ± 0.01 | 0.16 ± 0.02 | 0.16 ± 0.02 | 0.18 ± 0.01 |
| **At 120-min after MeHg administration** | | | | |
| Cumulative excretion (μmol/kg BW) | 0.63 ± 0.03 | 0.80 ± 0.02* | 0.84 ± 0.02* | 0.95 ± 0.02* |
| Accumulation (% dose) | 3.42 ± 0.15 | 1.99 ± 0.05* | 1.41 ± 0.03* | 1.18 ± 0.03* |
| Excretion rate (μmol/kg BW/30 min) | 0.14 ± 0.01 | 0.19 ± 0.01 | 0.18 ± 0.02 | 0.19 ± 0.01 |

MeHg was injected into hepatic portal vein. The biliary excretion of MeHg was collected at 30-min period.
Each value is mean ± SEM.

* $p < 0.05$ significant difference when compared with the MeHg-treated rats at dose of 20 μmol/kg BW at the corresponding time.



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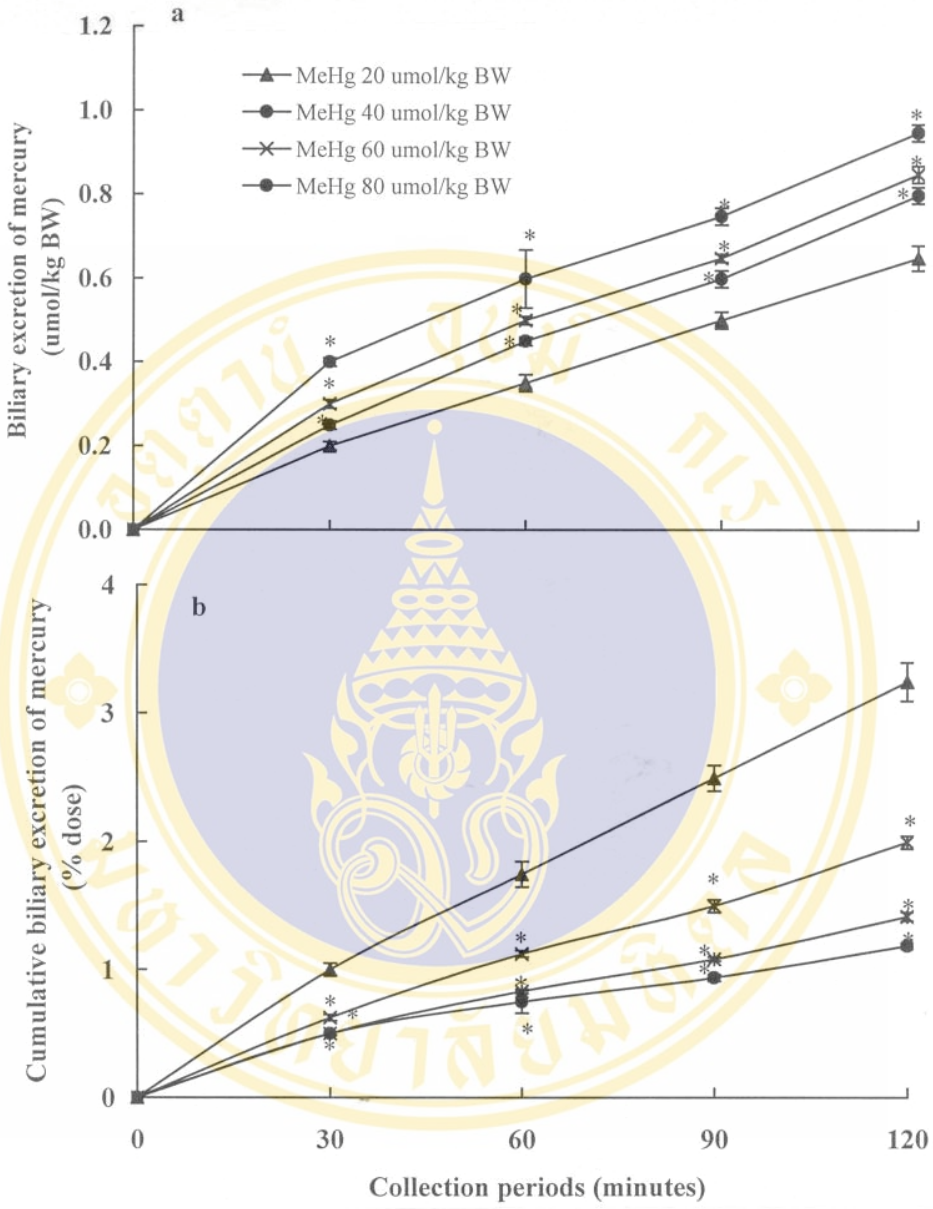


**Figure 2.** Biliary excretion of mercury after a bolus injection of methylmercury chloride (MeHg) into the hepatic portal vein at a dose of 20-80 μmol/kg BW. Bile sample was collected in 30-min period for 120 min.
Each point represents mean ± SEM of 8 rats/group.
* $p < 0.05$ significant difference when compared with that treated with MeHg 20 μmol/kg BW at the corresponding time.

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**Table 4.** Effect of methylmercury chloride (MeHg) administration on plasma levels of AST, ALT, ALP and biliary ALP (IU/ml). The control animals received their respective vehicle. Plasma and bile samples were determined at 30-min periods for 120 min.

| Treatment | | MeHg (μmol/kg BW) | | | | |
|---|---|---|---|---|---|---|
| | | 0 | 20 | 40 | 60 | 80 |
| **Before MeHg administration** | | | | | | |
| Plasma | AST | 65.45 ± 5.51 | 69.22 ± 2.06 | 70.61 ± 7.36 | 67.84 ± 4.31 | 68.99 ± 4.74 |
| | ALT | 16.54 ± 3.29 | 17.39 ± 3.13 | 17.60 ± 2.31 | 18.44 ± 2.09 | 19.54 ± 2.55 |
| | ALP | 43.49 ± 4.07 | 43.38 ± 1.80 | 42.72 ± 1.42 | 43.49 ± 1.64 | 44.74 ± 1.45 |
| Bile | ALP | 5.65 ± 0.89 | 6.07 ± 1.00 | 6.65 ± 0.85 | 6.09 ± 1.49 | 6.08 ± 0.66 |
| **60-min after MeHg administration** | | | | | | |
| Plasma | AST | 68.42 ± 3.08 | 924.42 ± 93.93* | 1679.72 ± 90.80* | 1865.04 ± 156.03* | 3215.34 ± 213.88* |
| | ALT | 16.38 ± 2.09 | 546.75 ± 107.18* | 918.29 ± 126.20* | 1182.78 ± 128.10* | 1549.54 ± 199.77* |
| | ALP | 43.88 ± 2.39 | 49.79 ± 1.45* | 53.18 ± 2.40* | 60.64 ± 1.27* | 68.89 ± 1.47* |
| Bile | ALP | 5.91 ± 0.66 | 7.66 ± 0.45 | 8.8 ± 0.32* | 9.08 ± 0.17* | 12.82 ± 0.26* |
| **120-min after MeHg administration** | | | | | | |
| Plasma | AST | 68.74 ± 3.35 | 1677.6 ± 176.29* | 2348.03 ± 283.84* | 2991.00 ± 135.03* | 3510.33 ± 509.30* |
| | ALT | 15.76 ± 2.51 | 1482.5 ± 143.19* | 1787.85 ± 150.17* | 2017.29 ± 213.41* | 2296.41 ± 240.81* |
| | ALP | 44.69 ± 3.08 | 55.83 ± 1.60* | 60.21 ± 1.78* | 64.26 ± 2.50* | 75.39 ± 1.84* |
| Bile | ALP | 6.45 ± 0.74 | 7.13 ± 0.92 | 10.57 ± 0.84* | 14.83 ± 0.90* | 15.29 ± 0.76* |

Each value is mean ± SEM of 8 rats/group.

* $p < 0.05$ significant difference when compared with vehicle control.



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**Table 5.** Effect of methylmercury chloride administration on hepatic glutathione and mercury contrations in MeHg-treated rats.

| Treatment | No. of animal | Glutathione (μmol/g liver) | Mercury (μmol/g liver) |
|---|---|---|---|
| Control | 8 | 6.31 ± 0.46 | 0.00 |
| MeHg 20 μmol/kg BW | 8 | 5.08 ± 0.54 | 9.22 ± 1.00 |
| MeHg 40 μmol/kg BW | 8 | 4.50 ± 0.36* | 16.00 ± 1.40 |
| MeHg 60 μmol/kg BW | 8 | 3.75 ± 0.28* | 23.83 ± 1.50 |
| MeHg 80 μmol/kg BW | 8 | 2.10 ± 0.27* | 32.75 ± 1.60 |

MeHg was injected into hepatic portal vein. The liver was collected at at 120-min after the injection.
Each value is mean ± SEM.

* $p < 0.05$ significant difference when compared with the vehicle control.

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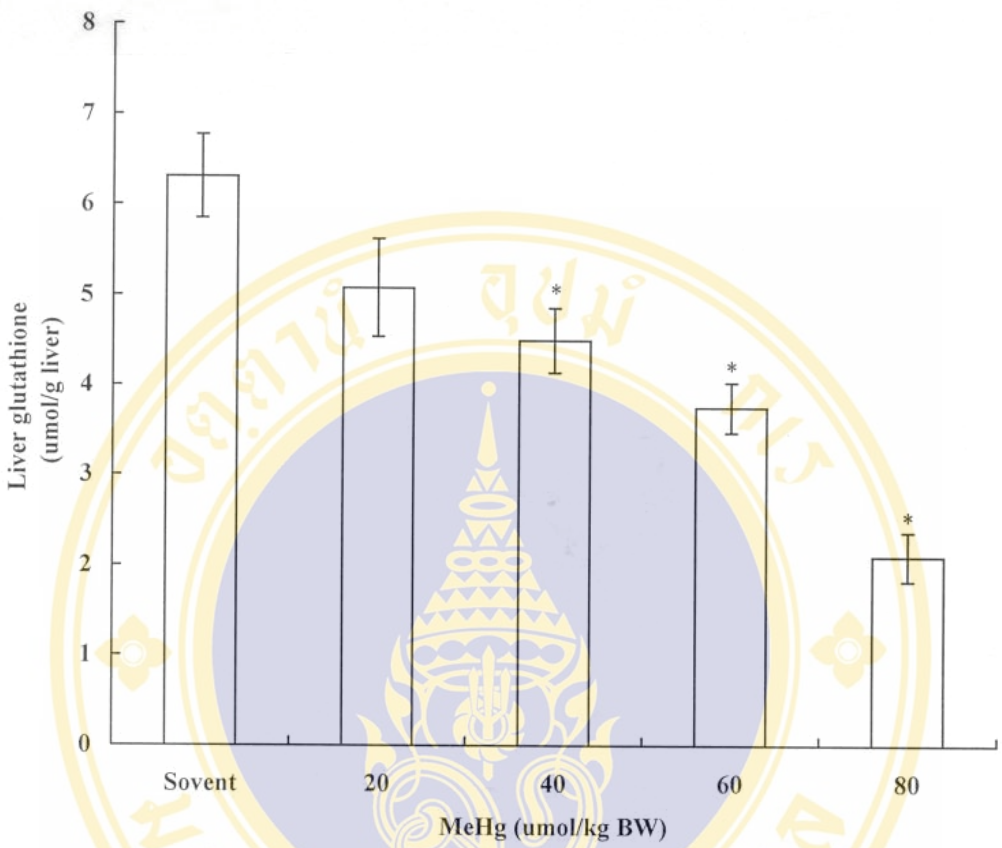


**Figure 3.** Effect of methylmercury chloride (MeHg) on liver glutathione concentration. MeHg was injected into the hepatic portal vein at a dose of 20-80 μmol/kg BW. Liver sample was collected at 120 min after MeHg injection.
Each bar represents mean ± SEM of 8 rats/group.
* $p < 0.05$ significant difference when compared with vehicle control.

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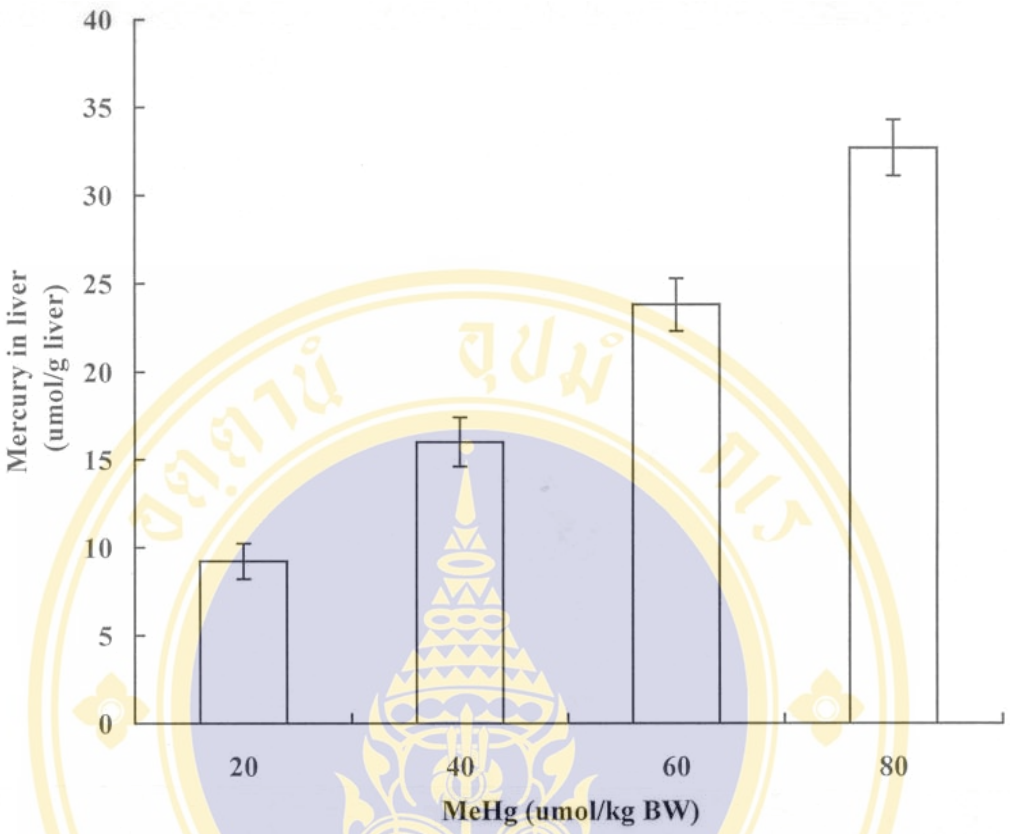


**Figure 4.** Liver mercury concentration after injection of methylmercury chloride (MeHg) into hepatic portal vein at a dose of 20-80 μmol/kg BW. Liver sample was collected at 120 min after MeHg injection.
Each bar represents mean ± SEM of 8 rats/group.

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## Experiment II Effect of THA on the MeHg-induced Hepatotoxicity.

From the experiment 1, a submaximal dose of MeHg at 40 μmol/kg BW was chosen for inducing hepatotoxicity in the subsequent studies by which BFR was maintained at approximately 75 % of control and the hepatic GSH content was significantly decreased. In this study, a single dose of MeHg (40 μmol/kg) was injected into the portal vein for 60 min prior to THA administration. Modulation of hepatotoxicity by THA was followed for another 60 min or 120 min after MeHg administration or 150 min after starting the experiment (Figure D). The animals were sacrificed at 60 min after THA administration for determination of the hepatic mercury and GSH contents.

The stimulating effects of THA on bile flow rate in the MeHg -treated animals (40 μmol/kg) are shown in Table 6 and Figure 5. Both doses of THA (300 and 600 μmol/kg) were able to increase bile secretion in the MeHg-treated animals in which BFR were significantly increased from approximately 46.59 $\pm$ 0.40 μl/kg/min after MeHg injection to 103.68 $\pm$ 0.82 μl/kg/min (222.54 % of control) and from 51.00 $\pm$ 1.50 μl/kg/min to 131.93 $\pm$ 3.83 μl/kg/min (258.69 % of control), respectively ($p<0.05$). In the absence of MeHg, THA at a dose of 300 and 600 μmol/kg BW increased BFR from approximately 61.05 $\pm$ 0.53 μl/kg/min to 168.83 $\pm$ 6.18 μ l/kg/min (276.54 % of control) and from 60.51 $\pm$ 1.13 μl/kg/min to 184.83 $\pm$ 5.28 μ l/kg/min (300.48 % of control), respectively at 30min after administration ($p<0.05$) (Table 6, Figure 5a).

As the MeHg-GSH complex is one of the major forms of hepatic excretion of MeHg, the liver GSH content or GSH status may be an important factor for determination of the MeHg excretion (Ballatori and clarkson, 1982). In the present study, GSH concentration was determined in both bile and liver, as shown in Tables 7 and 9. In control animals, the biliary secretion of GSH was steadily maintained at approximately 108.85 $\pm$ 3.61 nmol/kg/min to 111.95 $\pm$ 1.46 nmol/kg/min during the course of experiment. An administration of THA, which markedly increased bile secretion, significantly decreased biliary secretion of endogenous GSH in a dose-related manner. It decreased from 108.02 $\pm$ 3.07 nmol/kg/min in control period to 88.38 $\pm$ 1.28 nmol/kg/min at peak BFR of THA administration (600 μmol/kg BW) and from 113.98

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± 7.75 nmol/kg/min to 61.16 ± 0.90 nmol/kg/min at peak of THA at 300 μmol/kg BW ($p<0.05$). The biliary secretion of GSH, which had been suppressed in the MeHg-treated rats, was further decreased by THA administration (Table 7, Figure 5b). It was significantly decreased from 54.73 ± 1.44 nmol/kg/min in the MeHg-treated control to 30.36 ± 1.28 nmol/kg/min and from 59.85 ± 0.84 nmol/kg/min to 16.56 ± 0.78 nmol/kg/min after THA administration at a dose of 300 and 600 μmol/kg BW, respectively ($p<0.05$). It is evident that the high dose of THA (600 μmol/kg BW) had more inhibition on the biliary secretion of GSH into bile as compared with that of low dose (300 μmol/kg BW).

Surprisingly, in spite of having capability to increase BFR in the MeHg-treated rats, THA adversely affected the excretion of mercury in bile. Concurrent with the decreased biliary secretion of GSH, the biliary excretion of mercury was significantly decreased by THA administration ($p<0.05$). It was decreased from 0.80 ± 0.02 nmol/kg BW in the MeHg-treated control to 0.60 ± 0.01 nmol/kg BW and 0.55 ± 0.02 nmol/kg BW at 60-min after administration of THA at a dose of 300 and 600 μ mol/kg BW, respectively (Table 8, Figures 6a-6b). It is estimated that rats injected with high and low dose of THA excreted mercury as much as 1.37 ± 0.05 % and 1.50 ± 0.03 % of the administered dose, respectively within the 120 min as compared with 1.99 ± 0.13 % of the administered dose in the absence of THA (Figure 6b).

The concentration of liver GSH in the MeHg-treated animal (40 μmol/kg BW) also significantly decreased as compared with that in control (Table 9 and Figure 7). Although the secretion of biliary GSH was markedly decreased, the concentration of GSH in the liver was not significantly altered by THA ($p<0.05$). Meanwhile, the hepatic mercury concentration was increased (Table 9, Figure 8). It was increased from approximately 16.02 ± 1.39 μmol/g liver in control to 18.76 ± 1.26 and 20.11 ± 0.95 μmol/g liver at 60 min after administration of THA at a dose of 300 and 600 μ mol/kg BW, respectively.

MeHg significantly suppressed glutathione S-transferase (GST) activity in rat liver homogenate (Table 9, Figure 9). MeHg (40 μmol/kg BW) decreased GST activity to 1.56 ± 0.11 μmol/mg protein/min. In contrast, THA administration increased GST activity in a dose-related manner. It increased from 2.19 ± 0.16 μ mole/mg protein/min

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in control to 3.11 ± 0.14 and 4.49 ± 0.18 μmole/mg protein/min at 60 min after THA administration at a dose of 300 and 600 μmol/kg BW, respectively. However, the administration of THA to MeHg-treated rats did not significantly alter GST activities, which had been suppressed in the MeHg-treated rats (40 μmol/kg BW). It remained at the level comparable with that in the MeHg-treated rats.

Hepatotoxicity was also evaluated after administration of THA to MeHg-treated rats. Administration of THA alone had no significant effect on the plasma enzyme level of hepatic enzymes. In contrast to the increase in accumulation of mercury in the liver, plasma levels of AST, ALT, ALP and biliary ALP which had been elevated by MeHg 40 μmol/kg BW, were not significantly increased by THA administrations (Table 10). These results implicated that the toxicity of MeHg was not significantly augmented by THA administration.

From the result of this section, although BFR in the MeHg-treated animals was increased by THA, the biliary excretion of Hg was inhibited. Concurrently, the biliary GSH secretion was also reduced. By contrast, the hepatic GSH concentration was not affected. THA, which increased GST activity, could not increase the GST activity in the MeHg-treated animals. Moreover, the administration of THA did not alter the level of plasma enzyme AST, ALT, ALP, and the level of ALP in bile. It is suggested that THA might modulate the MeHg excretion by shifting from biliary route to the other route of excretion. The inhibition of THA might be due to its competitive in the formation of excretory GSH complex as well as at the canalicular excretion of the complex by sharing the same.

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**Table 6.** Effect of 2,4,6 - trihydroxyacetophenone (THA) treatment on bile flow rate (µl/kg/min) in the MeHg-treated rats.

| Treatment | Bile flow rate (µl/kg/min) | | | | |
|---|---|---|---|---|---|
| | 0 | 30 | 60 | 90 | 120 (min) |
| Control | 61.79 ± 0.85 | 61.37 ± 0.46 | 60.77 ± 0.55 | 60.09 ± 0.85 | 61.65 ± 0.45 |
| THA 300 µmol/kg BW | 62.02 ± 0.92 | 61.29 ± 0.92 | 61.05 ± 0.53 | 168.83 ± 6.18* | 91.48 ± 11.83* |
| THA 600 µmol/kg BW | 60.32 ± 1.03 | 60.64 ± 1.54 | 60.51 ± 1.13 | 184.83 ± 5.28* | 88.13 ± 2.61* |
| MeHg 40 µmol/kg BW | 65.32 ± 1.15 | 48.15 ± 0.57* | 50.53 ± 0.84* | 51.21 ± 1.29* | 50.88 ± 1.4* |
| MeHg 40 µmol/kg BW + THA 300 µmol/kg BW | 59.35 ± 0.59 | 45.35 ± 0.41* | 46.59 ± 0.4* | 103.68 ± 0.82*+ | 78.54 ± 1.2*+ |
| MeHg 40 µmol/kg BW + THA 600 µmol/kg BW | 61.08 ± 0.06 | 48.79 ± 0.79* | 51.00 ± 1.50* | 131.93 ± 3.83*+ | 83.52 ± 4.15*+ |

Rats received MeHg (40 µmol/kg BW) for 30-min prior to an intraduodenal administration of THA at a dose of 300 or 600 µmol/kg BW. Bile sample was collected in 30-min periods for 120 min.

Each value is mean ± SEM of 8 rats/group.

* $p < 0.05$ significant difference when compared with vehicle control at the corresponding time.

+ $p < 0.05$ significant difference when compared with the MeHg-treated control at the corresponding time.

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**Table 7.** Effect of 2,4,6 - trihydroxyacetophenone (THA) treatment on biliary secretion of glutathione (nmol/kg/min) in the MeHg-treated rats.

| Treatment | Biliary secretion of glutathione (nmol/kg/min) | | | | |
|---|---|---|---|---|---|
| | 0 | 30 | 60 | 90 | 120 (min) |
| Control | 108.85 ± 3.61 | 109.24 ± 2.81 | 110.38 ± 3.25 | 108.02 ± 2.43 | 106.85 ± 2.08 |
| THA 300 µmol/kg BW | 105.17 ± 2.14 | 107.86 ± 2.15 | 108.02 ± 3.07 | 88.38 ± 1.28* | 92.46 ± 1.67* |
| THA 600 µmol/kg BW | 111.95 ± 1.46 | 107.65 ± 2.73 | 113.98 ± 7.75 | 61.16 ± 0.9* | 72.22 ± 2.81* |
| MeHg 40 µmol/kg BW | 106.88 ± 4.61 | 58.63 ± 1.98* | 58.84 ± 1.72* | 59.82 ± 1.74* | 57.34 ± 2.29* |
| MeHg 40 µmol/kg BW + THA 300 µmol/kg BW | 103.79 ± 2.03 | 51.27 ± 2.1* | 54.73 ± 1.44* | 30.36 ± 1.28*+ | 33.63 ± 1.84*+ |
| MeHg 40 µmol/kg BW + THA 600 µmol/kg BW | 103.40 ± 1.78 | 59.36 ± 1.10* | 59.85 ± 0.84* | 16.56 ± 0.78*+ | 39.16 ± 1.95*+ |

Rats received MeHg (40 µmol/kg BW) for 30-min before an intraduodenal administration of THA at a dose 300 or 600 µmol/kg BW. Bile sample was determined in 30-min periods for 120 min.
Each value is mean ± SEM of 8 rats/group.

* $p < 0.05$ significant difference when compared with the vehicle control at the corresponding time.

+ $p < 0.05$ significant difference when compared with the MeHg-treated control at the corresponding time.



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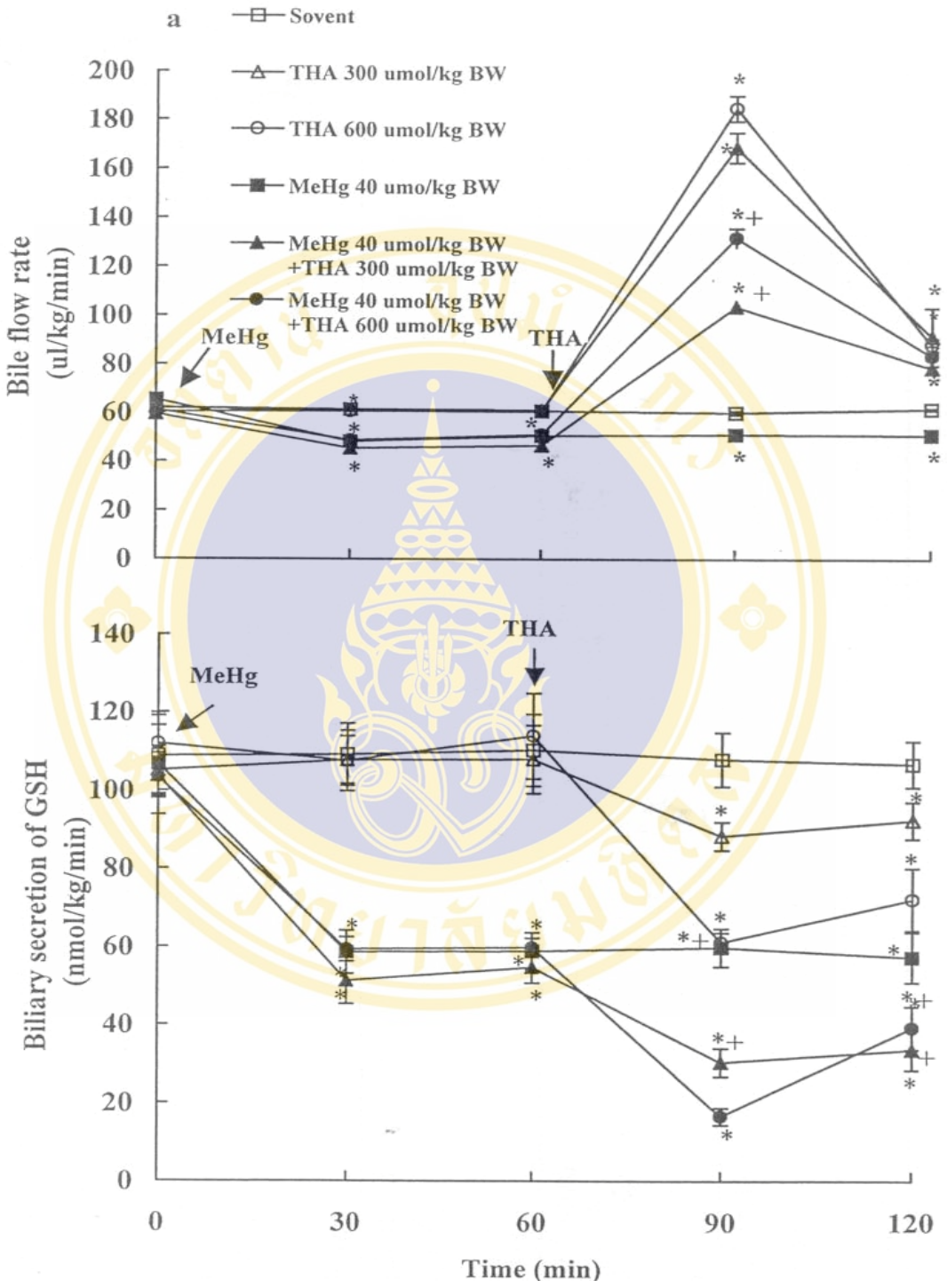


**Figure 5.** Bile flow rate (a) and biliary secretion of glutathione (GSH) (b) in rats treated with MeHg (40 μmol/kg BW) before intraduodenal administration of THA (300 or 600 μmol/kg BW). Bile sample was collected in 30-min period for 120 min. Each point represents mean ± SEM of 8 rats/group.
* $p<0.05$ significant difference when compared with vehicle control.
+ $p<0.05$ significant difference when compared with the MeHg-treated control at the corresponding time.

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**Table 8.** Effect of 2,4,6 - trihydroxyacetophenone (THA) treatment on biliary mercury concentration in MeHg-treated rats.

| Time (min) | MeHg (μmol/kg BW) + THA (μmol/kg BW) | | |
|---|---|---|---|
| | 40 | 40+300 | 40+600 |
| **At 30-min after MeHg administration** | | | |
| Cumulative excretion (μmol/kg BW) | 0.25 ± 0.01 | 0.25 ± 0.06 | 0.25 ± 0.08 |
| Accumulation (% dose) | 0.62 ± 0.05 | 0.62 ± 0.05 | 0.62 ± 0.05 |
| Excretion rate (μmol/kg BW/30 min) | 0.27 ± 0.01 | 0.23 ± 0.01 | 0.23 ± 0.01 |
| **At 60-min after MeHg administration** | | | |
| Cumulative excretion (μmol/kg BW) | 0.45 ± 0.05 | 0.40 ± 0.05 | 0.40 ± 0.09 |
| Accumulation (% dose) | 1.12 ± 0.08 | 1.00 ± 0.08 | 1.00 ± 0.05 |
| Excretion rate (μmol/kg BW/30 min) | 0.19 ± 0.02 | 0.19 ± 0.01 | 0.18 ± 0.01 |
| **At 90-min after MeHg administration** | | **30 min after giving THA** | |
| Cumulative excretion (μmol/kg BW) | 0.60 ± 0.06 | 0.55 ± 0.04 | 0.50 ± 0.05 |
| Accumulation (% dose) | 1.50 ± 0.08 | 1.37 ± 0.10 | 1.25 ± 0.08 |
| Excretion rate (μmol/kg BW/30 min) | 0.16 ± 0.02 | 0.11 ± 0.01 | 0.10 ± 0.005 |
| **At 120-min after MeHg administration** | | **60 min after giving THA** | |
| Cumulative excretion (μmol/kg BW) | 0.80 ± 0.02 | 0.60 ± 0.01* | 0.55 ± 0.02* |
| Accumulation (% dose) | 1.99 ± 0.13 | 1.50 ± 0.03* | 1.37 ± 0.05* |
| Excretion rate (μmol/kg BW/30 min) | 0.19 ± 0.01 | 0.08 ± 0.005 | 0.07 ± 0.005 |

Rats received MeHg (40 μmol/kg BW) for 30-min before intraduodenal administration of THA at a dose of 300 or 600 μmol/kg BW. Bile sample was collected in 30-min periods for 120 min.

Each value is mean ± SEM of 8 rats/group.

* $p < 0.05$ significant difference when compared with MeHg-treated control at the corresponding time.



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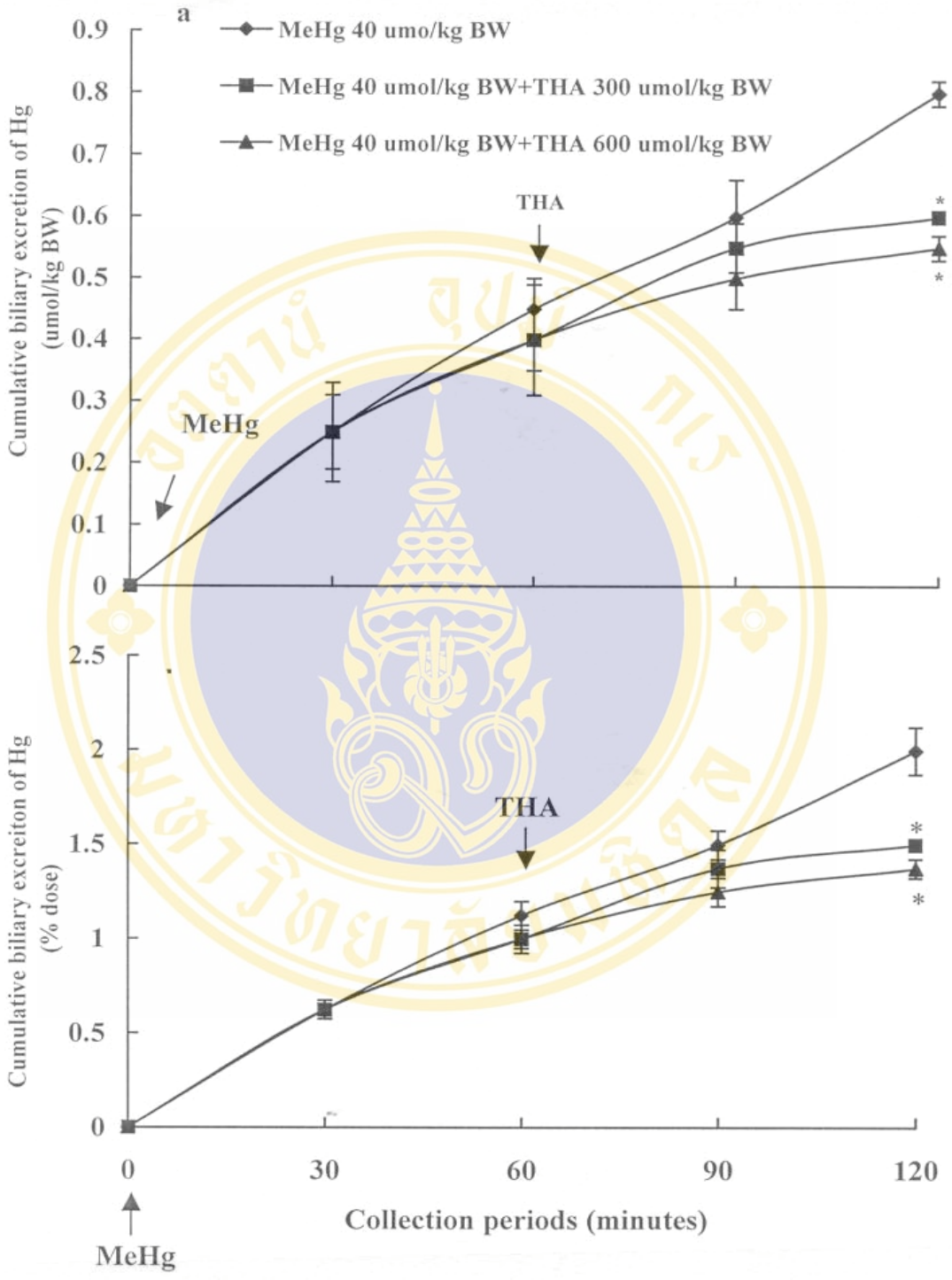


**Figure 6.** The cumulative biliary excretion of mercury in rats treated with MeHg (40 μmol/kg BW) before intraduodenal administration of THA (300 or 600 μ mol/kg BW). Bile sample was collected in 30-min period for 120 min. Each point represents mean ± SEM of 8 rats/group.
* $p<0.05$ significant difference when compared with the MeHg-treated rat at the corresponding time.

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**Table 9.** Effect of 2,4,6 - trihydroxyacetophenone (THA) treatment on liver glutathione and mercury concentrations and glutathion *S* -transferase activity (GST) in the MeHg-treated rats.

| Treatment | Glutathione (µmol/g liver) | Mercury (µmol/g liver) | GST (µmole/mg protein/min) |
|---|---|---|---|
| Control | 5.88 ± 0.22 | 0.00 | 2.71 ± 0.18 |
| THA 300 µmol/kg BW | 5.90 ± 0.16 | 0.00 | 3.11 ± 1.14 * |
| THA 600 µmol/kg BW | 6.10 ± 0.24 | 0.00 | 4.49 ± 0.18 * |
| MeHg 40 µmol/kg BW | 4.50 ± 0.36* | 16.02 ± 1.39 | 1.56 ± 0.11 * |
| MeHg 40 µmol/kg BW + THA 300 µmol/kg BW | 4.78 ± 0.43* | 20.11 ± 0.95 | 1.79 ± 0.13*+ |
| MeHg 40 µmol/kg BW + THA 600 µmol/kg BW | 5.17 ± 0.41* | 18.76 ± 1.26 | 1.86 ± 0.14*+ |

Rats received MeHg (40 µmol/kg BW) for 30-min before intraduodenal administration of THA at a dose of 300 or 600 µmol/kg BW.
Bile sample was collected in 30-min periods for 120 min.
Each value is mean ± SEM of 8 rats/group.
* $p < 0.05$ significant difference when compared with vehicle control.
+ $p < 0.05$ significant different when compared with corresponding THA control.



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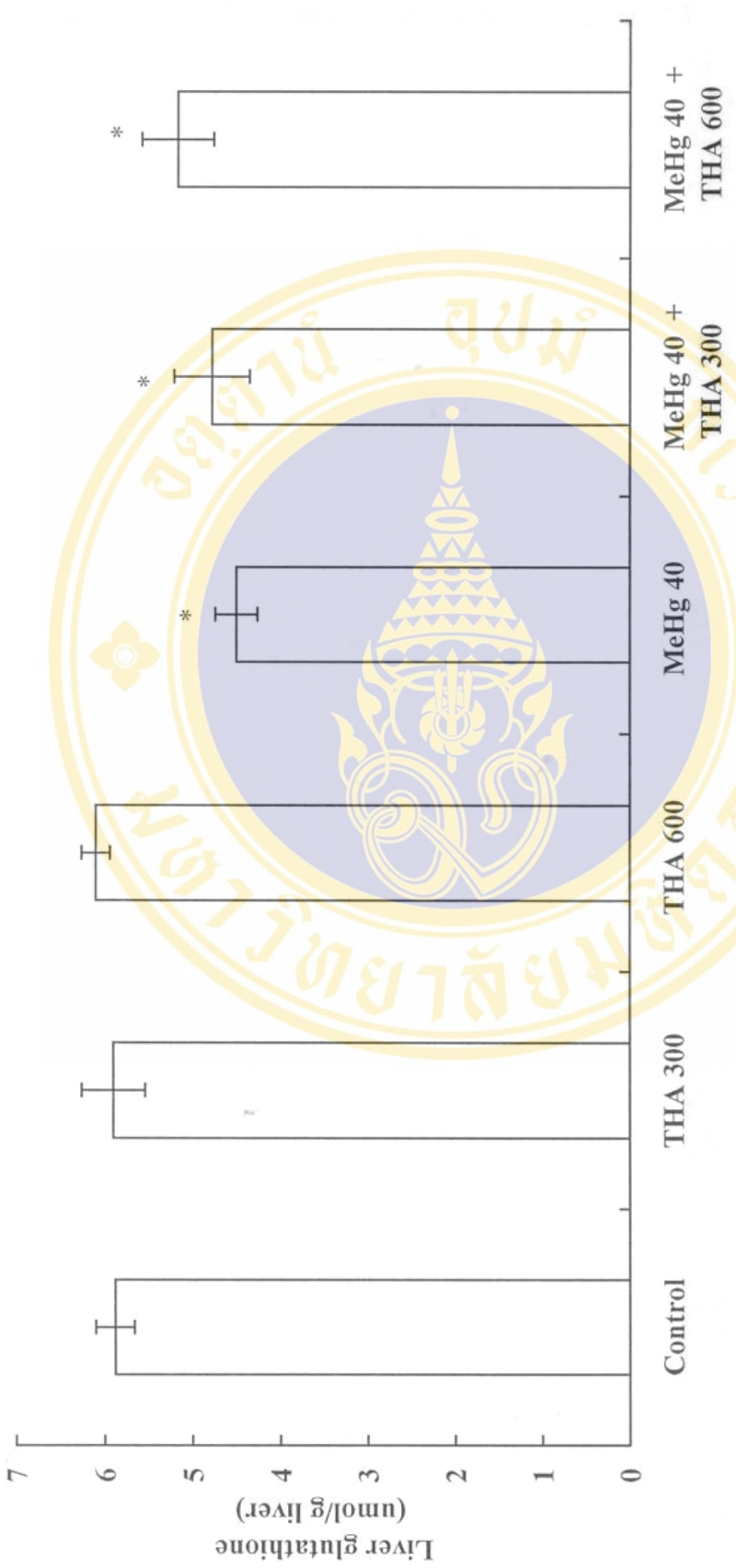


**Figure 7.** Effect of THA (300 or 600 µmol/kg BW) administration on liver glutathione concentration in rats treated with MeHg (40 µ mol/kg BW) at 30 min before intraduodenal administration of THA. The control animals received their corresponding vehicle. Liver sample was collected at the end of experiment, at 120 min after MeHg injection.
Each bar represents mean ± SEM of 8 rats/group.
* $p<0.05$ significant difference when compared with the control.

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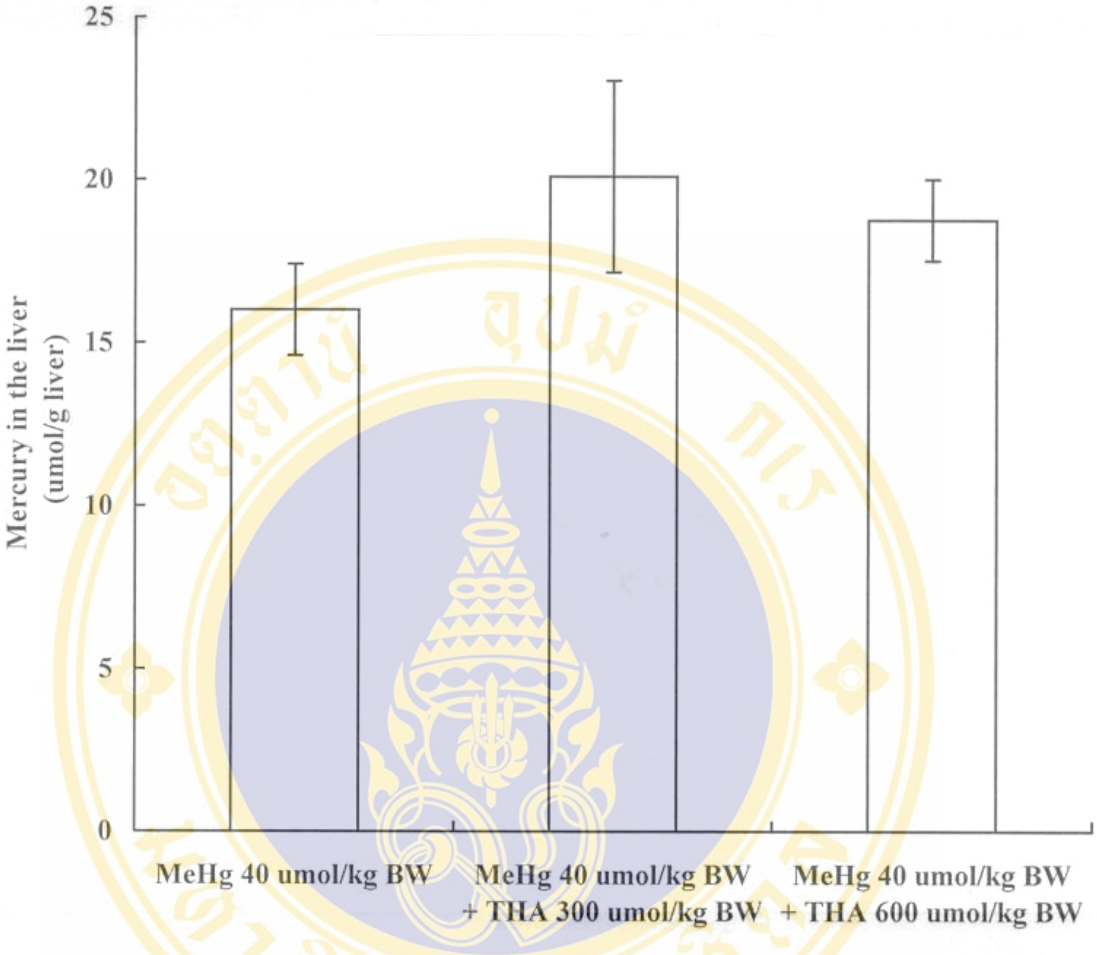


**Figure 8.** Effect of THA (300 or 600 μmol/kg BW) administration on hepatic mercury concentration in rats treated with MeHg (40 μmol/kg BW) at 30 min before intraduodenal administration of THA. The control animals received their corresponding vehicle. Liver sample was collected at the end of experiment, at 120 min after MeHg injection.
Each bar represents mean ± SEM of 8 rats/group.
No significant difference was observed among various group of animal.

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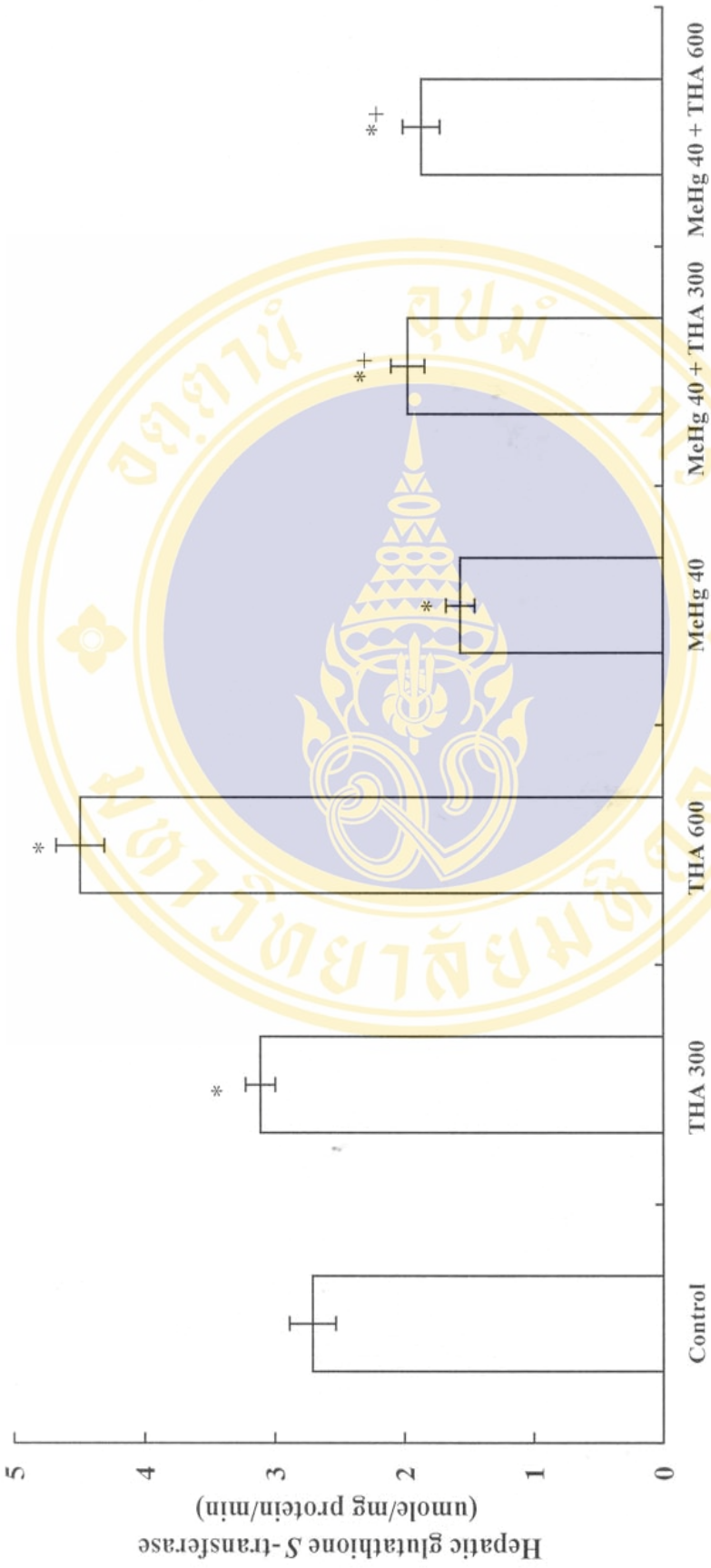


**Figure 9.** Effect of THA (300 or 600 μmol/kg BW) administration on hepatic glutathione S-transferase (GST) activity in MeHg-induced hepatotoxicity in adult male rats. GST activity towards CDNB was measured in fresh liver homogenates. Each bar represents mean ± SEM of 8 rats/group.
* $p<0.05$ significant difference when compared with the control group;
+ $p<0.05$ significant difference when compared with the corresponding THA-control.

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**Table 10.** Effect of THA (300 or 600 µmol/kg BW) administration on plasma levels of AST, ALT, ALP and biliary ALP (IU/ml) in the MeHg treated animal at 30 min before intraduodenally administration of THA. The control animals receiving their corresponding vehicle.

| Parameters | | MMC (µmol/kg BW) | | MMC 40 µmol/kg BW + THA (µmol/kg BW) | |
|---|---|---|---|---|---|
| | | 0 | 40 | 300 | 600 |
| **Before MeHg treatment** | | | | **Before MeHg treatment** | |
| Plasma | AST | 65.45 ± 5.51 | 70.61 ± 7.36 | 65.51 ± 3.93 | 62.34 ± 2.99 |
| | ALT | 16.54 ± 3.29 | 17.60 ± 2.31 | 15.59 ± 2.64 | 17.51 ± 1.58 |
| | ALP | 43.49 ± 4.07 | 42.72 ± 1.42 | 44.81 ± 2.53 | 43.68 ± 1.54 |
| Bile | ALP | 5.65 ± 0.89 | 6.65 ± 0.85 | 6.13 ± 0.31 | 6.08 ± 0.14 |
| **60-min after MeHg treatment** | | | | **60-min after MeHg without THA** | |
| Plasma | AST | 68.42 ± 3.08 | 1679.72 ± 90.80 * | 1332.79 ± 194.76 * | 1562.73 ± 177.13 * |
| | ALT | 16.38 ± 2.09 | 918.29 ± 126.20 * | 1061.96 ± 182.60 * | 1075.91 ± 167.75 * |
| | ALP | 43.88 ± 2.39 | 53.18 ± 2.40 * | 50.42 ± 2.31 * | 49.82 ± 1.53 * |
| Bile | ALP | 5.10 ± 0.66 | 8.8 ± 0.32 * | 7.49 ± 0.55 * | 8.32 ± 0.45 * |
| **120-min after MeHg treatment** | | | | **120-min after MeHg and 60-min after THA** | |
| Plasma | AST | 68.74 ± 3.35 | 2348.03 ± 283.84 * | 2466 ± 173.05 * | 2106.05 ± 170.83 * |
| | ALT | 15.76 ± 2.51 | 1787.85 ± 150.17 * | 1581.88 ± 185.60 * | 1645.05 ± 470.91 * |
| | ALP | 44.69 ± 3.08 | 60.21 ± 1.78 * | 58.68 ± 0.58 * | 59.06 ± 1.53 * |
| Bile | ALP | 6.45 ± 0.74 | 10.57 ± 0.84 * | 8.55 ± 0.52* | 7.95 ± 0.03* |

Each value is mean ± SEM of 8 rats/group.

* $p < 0.05$ significant difference when compared with vehicle control.



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## Experiment III Effect of THA Administration in GSH-Depleted Rats.

Hepatic GSH level has been implicated to be an important factor for determination of biliary excretion of mercury. In the present study, the effects of systemic alteration of GSH by BSO or acivicin administration on mercury excretion were evaluated in the presence of THA. After giving GSH depletors, BSO and acivicin, the basal bile secretion was significantly reduced from 62.25 ± 2.77 µl/kg/min in control to 47.34 ± 1.53 and to 44.79 ± 1.83 µl/kg/min, respectively, as shown in Table 11 and Fig. 10a ($p<0.05$). Meanwhile the biliary secretion of GSH was also markedly decreased (Table 12, Figure 10b). It was maintained at the levels about 23.69 ± 2.06 – 25.17 ± 1.59 µl/kg/min and 22.04 ± 2.59-28.15 ± 0.73 µl/kg/min in BSO and acivicin-treated rats, respectively as compared with 100.58 ± 4.32-112.53 ± 3.26 µl/kg/min in control animals.

The effects of THA in modulating bile flow and biliary secretion of GSH were investigated in the BSO as well as acivicin pretreatment in the MeHg-treated rats. In control rats, THA at a dose of 300 and 600 µmol/kg effectively increased BFR. They could also induce acute choleresis in the MeHg-treated animals even in a lesser extent as compared with those in the non-MeHg-treated rats (Table 11, Figure 11). Concurrent with the increased BFR, the biliary secretion of free GSH concentration was markedly decreased. The biliary GSH secretion was decreased from 108.02 ± 3.07 nmol/kg/min in control rats to 88.38 ± 1.28 nmol/kg/min and from 113.98 ± 7.75 nmol/kg/min to 61.16 ± 2.9 nmol/kg/min by THA at a dose of 300 and 600 µmol/kg, respectively (Table 12, Figure 11b). Similar to that in the untreated-MeHg control rats, the administration of THA to the MeHg-treated rats further reduced the biliary secretion of GSH to 33.46 ± 1.42 nmol/kg/min and 22.32 ± 2.56 nmol/kg/min, respectively (Table 12, Figure 11b).

In the BSO-pretreated rats, BFR was markedly decreased. As shown in Table 11, it was decreased from 62.25 ± 2.77 µl/kg/min in the untreated control to 47.34 ± 1.53 µl/kg/min in the BSO-pretreated rats and was maintained at this level throughout the experimental period. THA administration at a dose of 300 and 600 µmol/kg BW to the BSO-pretreated rats were able to increase BFR from 50.63 ± 1.07 to 99.45 ± 3.23 µl/kg/min and from 51.52 ± 3.72 to 119.84 ± 10.87 µl/kg/min,

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respectively (Table 11,Figure 12a). Furthermore, THA also increased BFR in the MeHg-treated rats, which received BSO. BFR increased from 47.69 ± 1.07 to 75.41 ± 3.23 μl/kg/min and from 44.56 ± 2.45 to 90.15 ± 2.79 μl/kg/min at 30-min after THA administration at the dose of 300 and 600 μmol/kg BW, respectively (Table 11, Figure 12a). Concurrently, the biliary GSH secretion, which was suppressed in the BSO-pretreated rats, was further decreased by THA at a dose of 300, 600 μmol/kg BW (Table 12, Figure 12b). It significantly decreased from 27.78 ± 3.52 to 19.20 ± 2.18 nmol/kg/min and from 21.55 ± 1.47 to 16.21 ± 1.85 nmol/kg/min, respectively ($P < 0.05$). Administration of THA to the MeHg-treated rats further decreased total biliary GSH secretion (Table 12, Figure 12b).

Likewise, in the acivicin-pretreated rats, the suppressed BFR was increased by THA from 46.98 ± 0.96 μl/kg/min in control to 98.59 ± 2.07 μl/kg/min and from 48.73 ± 3.25 to 109.65 ± 3.06 μl/kg/min after THA administration at a dose of 300 and 600 μmol/kg BW, respectively (Table 11, Figure 13a). In the MeHg-treated rats, after pretreated with acivicin, THA could also increase BFR (Table 11, Figure 13a). In accompany with the increased in BFR by THA in the acivicin-pretreated rats, the biliary secretion of GSH was decreased. The GSH secretion was also further decreased in the MeHg-treated rats. It decreased from 18.47 ± 1.68 nmol/kg/min in control to 10.28 ± 1.57 nmol/kg/min and from 15.79 ± 0.98 to 9.87 ± 0.70 nmol/kg/min, after THA administration at a dose of 300 and 600 μmol/kg BW, respectively (Table 12, Figure 13b). Concurrent with the decreased biliary secretion of total GSH, the mercury excretion in bile was decreased in the BSO and the acivicin-pretreated rats after THA administration in the MeHg-treated rats (Table 13, Figure 14). In the BSO-pretreated rats, the total biliary excretion of mercury 120-min period was decreased from 0.6±0.30 μmol/kg BW in the untreated control rats to 0.33 ± 0.04 μmol/kg BW and from 0.55 ± 0.02 μmol/kg BW to 0.25 ± 0.01 μmol/kg BW after THA administration at a dose of 300 and 600 μmol/kg BW, respectively. In the acivicin-pretreated rats, the amount of mercury excretion for 120-min period was decreased to 0.38 ± 0.02 μmol/kg BW and 0.30 ± 0.03 μmol/kg BW after THA administration at a dose of 300 and 600 μmol/kg BW, respectively.

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In the BSO- and the acivicin-pretreated rats, the hepatic GSH concentrations were significantly lower than those in the untreated-control rats ($p<0.05$) (Table 14, Figure 15). It was decreased from approximately 6.10 $\pm$ 0.21 μmol/g liver in the saline-treated control rats to 4.35 $\pm$ 0.36 and 4.18 $\pm$ 0.34 μmol/g liver in the BSO and acivicin-pretreated rats, respectively. However, there were no significant differences in the hepatic GSH content in between the BSO-pretreated and the acivicin-pretreated rats. THA administration did not significantly alter the hepatic GSH concentrations in all GSH-depleted rats. Meanwhile, it increased the liver mercury content in the MeHg-treated rats about two folds (Table14, Figure 16). In the BSO-pretreated animals, the liver mercury content was increased from 24.56 $\pm$ 3.38 μmol/g liver in the saline-treated control rats to 36.46 $\pm$ 2.25 μmol/g liver and from 20.32 $\pm$ 3.49 μ mol/g liver to 32.62 $\pm$ 3.21 μmol/g liver after THA administration at a dose of 300 and 600 μmol/kg BW, respectively. In the acivicin-pretreated animals, the mercury content in the liver was similarly increased to 38.94 $\pm$ 2.10 and 38.99 $\pm$ 4.66 μmol/g liver after the THA administration at a dose of 300 and 600 μmol/kg BW, respectively.

The effects of THA and MeHg on the cytosolic GST activity of the liver were also investigated in rats-pretreated with BSO and acivicin, as shown in Table 14, and Figure 17. The GST activity was not significantly altered in the BSO-pretreated rats. However, it was significantly increased by THA at a dose of 600 μmol/kg BW ($p <0.05$). It increased from approximately 2.90 $\pm$ 0.12 μmole/mg protein/min in the saline-pretreated control rats to 4.60 $\pm$ 0.20 μmole/mg protein/min. However, this dose of THA did not increase GST in the BSO-pretreated rats. Treatment with acivicin gave a similar result. No significant difference of the GST activity was observed in between the BSO-pretreated and acivicin-pretreated rats.

Based on these results, the hepatic GSH status appeared to be an important factor for determination of biliary excretion of mercury. Both BSO and acivicin caused rapid and significant decreases in hepatic and biliary GSH contents. However, administration of THA was able to induce choleresis in these GSH-depleted animals, which indicated that the stimulating bile secretion by THA was partly independent to GSH. On the other hand, it was partly dependent on GSH, which is a bile

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acid-independent bile flow. This probably relate to the decrease in the amount of GSH secretion and mercury excretion into bile by THA. At the same time, the liver mercury content was increased after administration of THA.

**Effect of THA on urine flow rate and urinary mercury content**

In addition to increase BFR, THA administration also significantly increased the urine flow rate in a dose-related manner ($p<0.05$) (Table 15, Figure 18a,b). THA had a rapid onset of actions. The urine flow rate reached peak within 30 min after THA administration. It was increased from 30.66 $\pm$ 6.66 – 33.93 $\pm$ 5.61 μl/kg/min in the vehicle control to 61.04 $\pm$ 6.94 μl/kg/min and 80.23 $\pm$ 1.13 μl/kg/min after THA administration at a dose of 300 and 600 μl/kg/min, respectively. After the peak at 30 min the urine flow rate gradually declined. Moreover, THA also significant increased the urine flow rate in the MeHg-treated rats from approximately 19.92 $\pm$ 1.92 – 21.18 $\pm$ 0.21 μl/kg/min to 67.32 $\pm$ 3.31 μl/kg/min and 80.23 $\pm$ 1.92 μl/kg/min after administration of THA at a dose of 300 and 600 μmol/kg BW, respectively ($p<0.05$). Thus, these data indicated that THA caused a transient increase in urine flow rate or induced acute diuresis, which lasted for only 30-min. Furthermore, it was interesting that the urinary excretion of mercury was transiently increased after THA administration (Table 16, Figure 19). However, the amount of mercury excretion in the urine after THA administration were accounted for only 16.45 $\pm$ 2.32 pmol/kg BW and 18.10$\pm$1.45 pmol/kg BW, respectively as compared with the treatment dose of MeHg (40 μmol/kg BW). No significant difference was observed between low and high dose of THA. However, it seems likely that THA might modulate the mercury excretion by shifting into the urine route, which is a minor route of excretion in a normal circumstantial.

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**Table 11.** Effect of 2,4,6 - trihydroxyacetophenone (THA) administration on bile flow rate (µl/kg/min) in the glutathione-depleted rats.

| Treatments | Bile flow rate (µl/kg/min) | | | | |
|---|---|---|---|---|---|
| | 0 | 30 | 60 | 90 | 120 (min) |
| Saline | 62.25 ± 2.77 | 63.59 ± 2.72 | 62.82 ± 2.78 | 62.88 ± 2.71 | 62.36 ± 2.33 |
| Saline + THA 300 µmol/kg BW | 62.02 ± 3.00 | 61.29 ± 2.78 | 61.05 ± 1.53 | 168.83 ± 6.18* | 91.48 ± 11.83* |
| Saline + THA 600 µmol/kg BW | 60.32 ± 1.03 | 60.64 ± 1.54 | 60.51 ± 1.13 | 184.83 ± 5.28* | 88.13 ± 2.61* |
| Saline + MeHg 40 µmol/kg BW + THA 300 µmol/kg BW | 62.96 ± 1.23 | 49.64 ± 1.47*+ | 50.41 ± 1.78*+ | 110.40 ± 2.07*+ | 79.13 ± 2.68*+ |
| Saline + MeHg 40 µmol/kg BW + THA 600 µmol/kg BW | 63.46 ± 1.42 | 47.94 ± 2.99*+ | 49.65 ± 1.85*+ | 120.32 ± 11.08*+ | 80.32 ± 4.56* |
| BSO | 47.34 ± 1.53* | 42.16 ± 1.20* | 47.16 ± 1.02* | 50.56 ± 1.24* | 47.18 ± 3.00* |
| BSO + THA 300 µmol/kg BW | 41.89 ± 2.95 | 42.41 ± 1.01 | 50.63 ± 1.07 | 99.45 ± 3.23* | 60.85 ± 1.44 |
| BSO + THA 600 µmol/kg BW | 46.71 ± 1.22 | 50.28 ± 4.22 | 51.52 ± 3.72 | 119.84 ± 10.87* | 57.27 ± 2.97 |
| BSO + MeHg 40 µmol/kg BW + THA 300 µmol/kg BW | 48.56 ± 3.95 | 37.24 ± 1.01 | 47.69 ± 1.07 | 75.41 ± 3.23*+ | 51.54 ± 1.44 |
| BSO + MeHg 40 µmol/kg BW + THA 600 µmol/kg BW | 50.96 ± 2.62 | 40.12 ± 2.32 | 44.56 ± 2.45 | 90.15 ± 2.79*+ | 55.89 ± 3.45* |
| Acivicin | 44.79 ± 1.83* | 41.36 ± 1.35* | 48.20 ± 2.07* | 48.64 ± 2.77* | 45.70 ± 1.49* |
| Acivicin + THA 300 µmol/kg BW | 47.93 ± 0.93 | 48.65 ± 1.25 | 46.98 ± 0.96 | 98.59 ± 2.07* | 62.85 ± 1.62 |
| Acivicin + THA 600 µmol/kg BW | 41.03 ± 0.86 | 43.56 ± 1.9 | 48.73 ± 3.25 | 109.65 ± 3.06* | 60.40 ± 1.39 |
| Acivicin +MeHg 40 µmol/kg BW + THA 300 µmol/kg BW | 47.93 ± 0.62 | 48.65 ± 4.56 | 46.98 ± 2.10 | 68.42 ± 0.99*+ | 63.95 ± 3.26 |
| Acivicin + MeHg 40 µmol/kg BW + THA 600 µmol/kg BW | 41.03 ± 0.46 | 43.56 ± 3.16 | 48.73 ± 5.00 | 89.65 ± 9.25*+ | 60.46 ± 2.56 |

The animals received BSO, or acivicin before injection of MeHg and/or intraduodenally administration of THA 300 or 600 µmol/kg BW at 90 $^{th}$ min.

Each value is mean ± SEM of 8 rats/group.

* $p < 0.05$ significant difference when compared with control at the corresponding time.

$^{+}$ $p < 0.05$ significant difference when compared with the THA-treated group.

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**Table 12.** Effect of 2,4,6 - trihydroxyacetophenone (THA) administration on biliary secretion of glutathione, GSH (nmol/kg/min) in the glutathione-depleted rats.

| Treatments | GSH (nmol/kg/min) | | | | |
|---|---|---|---|---|---|
| | 0 | 30 | 60 | 90 | 120 (min) |
| Saline | 100.58 ± 4.32 | 111.23 ± 5.42 | 109.77 ± 2.45 | 106.45 ± 4.23 | 112.53 ± 3.26 |
| Saline + THA 300 µmol/kg BW | 105.17 ± 2.14 | 107.86 ± 2.15 | 108.02 ± 3.07 | 88.38 ± 1.28* | 92.46 ± 1.67* |
| Saline + THA 600 µmol/kg BW | 111.95 ± 1.64 | 107.65 ± 2.73 | 113.98 ± 7.75 | 61.16 ± 2.90* | 72.22 ± 2.81* |
| Saline + MeHg 40 µmol/kg BW + THA 300 µmol/kg BW | 117.32 ± 3.21 | 63.35 ± 2.46*+ | 68.02 ± 4.32*+ | 33.46 ± 1.42*+ | 36.56 ± 2.42*+ |
| Saline + MeHg 40 µmol/kg BW + THA 600 µmol/kg BW | 113.46 ± 2.48 | 58.42 ± 1.42*+ | 60.38 ± 2.46*+ | 22.32 ± 2.56*+ | 30.42 ± 2.14*+ |
| BSO | 23.69 ± 2.06* | 23.24 ± 2.60* | 26.20 ± 2.80* | 27.41 ± 1.19* | 25.17 ± 1.59* |
| BSO + THA 300 µmol/kg BW | 20.94 ± 1.42 | 24.39 ± 2.56 | 27.78 ± 3.52 | 19.20 ± 2.18* | 18.56 ± 3.25* |
| BSO + THA 600 µmol/kg BW | 22.82 ± 1.85 | 21.48 ± 1.21 | 21.55 ± 1.47 | 16.21 ± 1.85* | 15.51 ± 1.79* |
| BSO + MeHg 40 µmol/kg BW + THA 300 µmol/kg BW | 17.43 ± 1.41 | 9.94 ± 0.49*+ | 12.32 ± 1.66*+ | 9.11 ± 1.77*+ | 11.00 ± 1.15*+ |
| BSO + MeHg 40 µmol/kg BW + THA 600 µmol/kg BW | 24.47 ± 1.96 | 11.26 ± 2.79*+ | 12.41 ± 2.83*+ | 8.75 ± 2.75*+ | 10.25 ± 2.79*+ |
| Acivicin | 28.15 ± 0.73* | 22.04 ± 1.05* | 24.41 ± 2.14* | 22.53 ± 2.20* | 22.04 ± 2.59* |
| Acivicin + THA 300 µmol/kg BW | 24.41 ± 2.14 | 22.53 ± 2.20 | 22.04 ± 2.59 | 16.12 ± 0.73* | 18.04 ± 1.50 |
| Acivicin + THA 600 µmol/kg BW | 19.84 ± 1.04 | 20.62 ± 2.22 | 20.13 ± 1.19 | 17.36 ± 1.32* | 18.46 ± 0.82 |
| Acivicin +MeHg 40 µmol/kg BW + THA 300 µmol/kg BW | 21.05 ± 1.91 | 15.83 ± 1.37*+ | 18.47 ± 1.68* | 10.28 ± 1.57*+ | 13.63 ± 1.52*+ |
| Acivicin + MeHg 40 µmol/kg BW + THA 600 µmol/kg BW | 21.67 ± 1.54 | 15.81 ± 0.94*+ | 15.79 ± 0.98*+ | 9.89 ± 0.70*+ | 10.66 ± 0.90*+ |

The animals received BSO, or acivicin before injection of MeHg and/or intraduodenally administration of THA 300 or 600 µmol/kg BW at 90 $^{th}$ min.

Each value is mean ± SEM of 8 rats/group.

* $p < 0.05$ significant difference when compared with control group at the corresponding time.

+ $p < 0.05$ significant difference when compared with the THA-treated group at the corresponding time.



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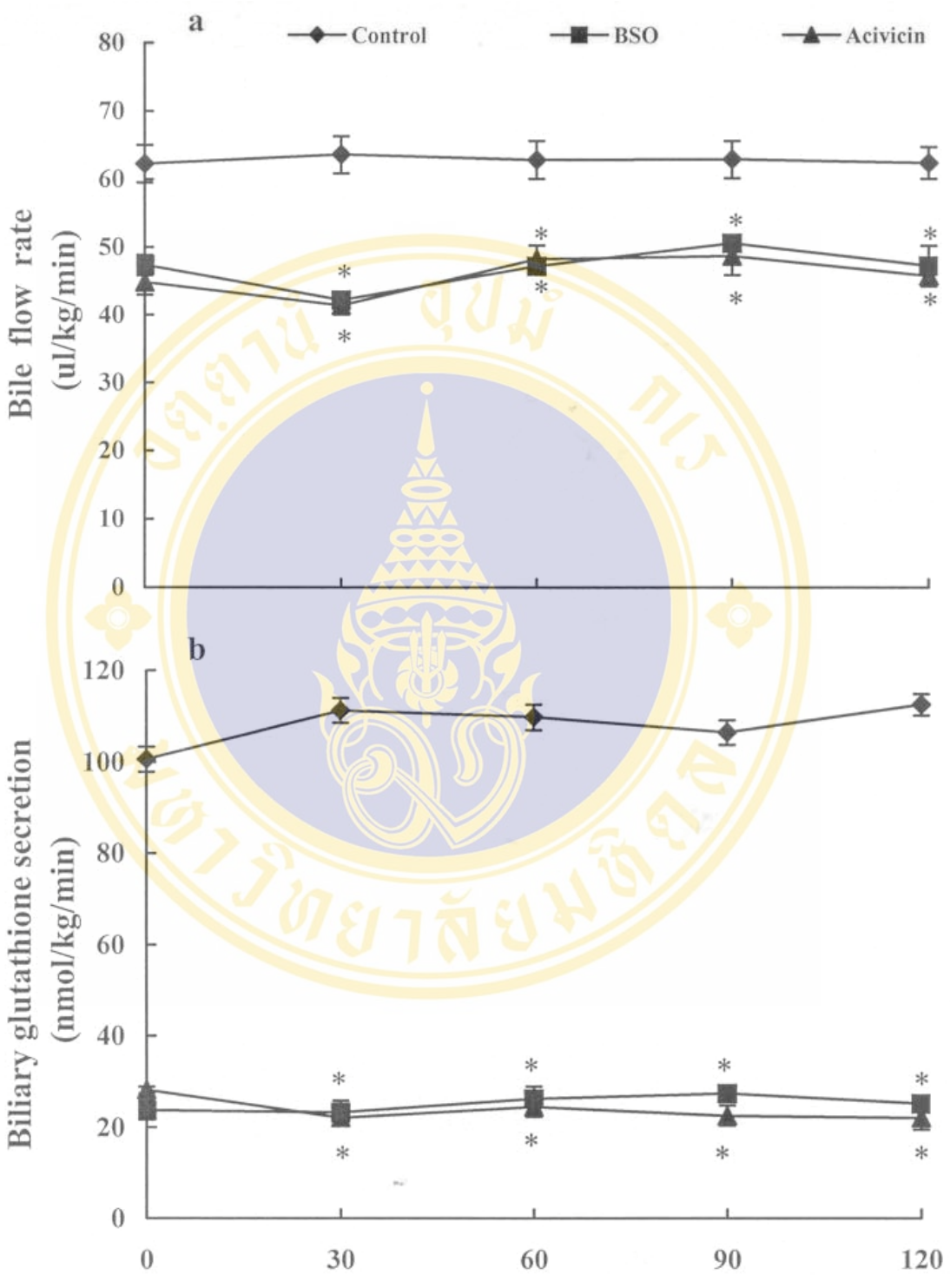


**Figure 10.** The bile flow rate (a) and biliary secretion of glutathione (b) in rats-pretreated with glutathione depletors (BSO or acivicin).
Each point represents mean ± SEM of 8 rats/group.
* $P<0.05$ significant difference when compared with the control group at the corresponding time.

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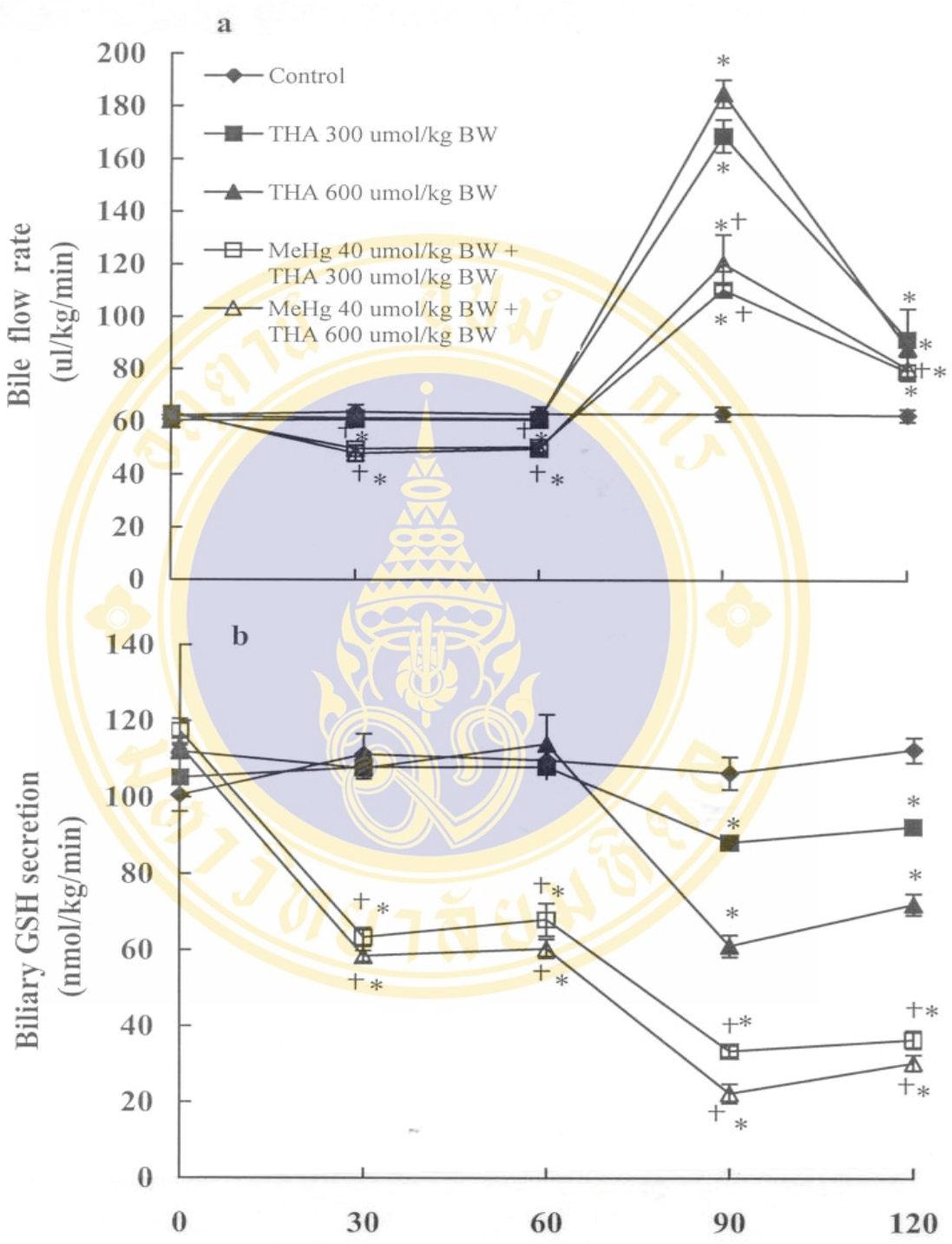


**Figure 11.** The bile flow rate (a) and biliary secretion of glutathione (b) in rats pretreated with saline before injection of MeHg and intraduodenally administration of THA.
Each point represents mean ± SEM of 8 rats/group.
* $p<0.05$ significant difference when compared with control at the corresponding time.
+ $p<0.05$ significant difference when compared with the THA-treated group at the corresponding time.

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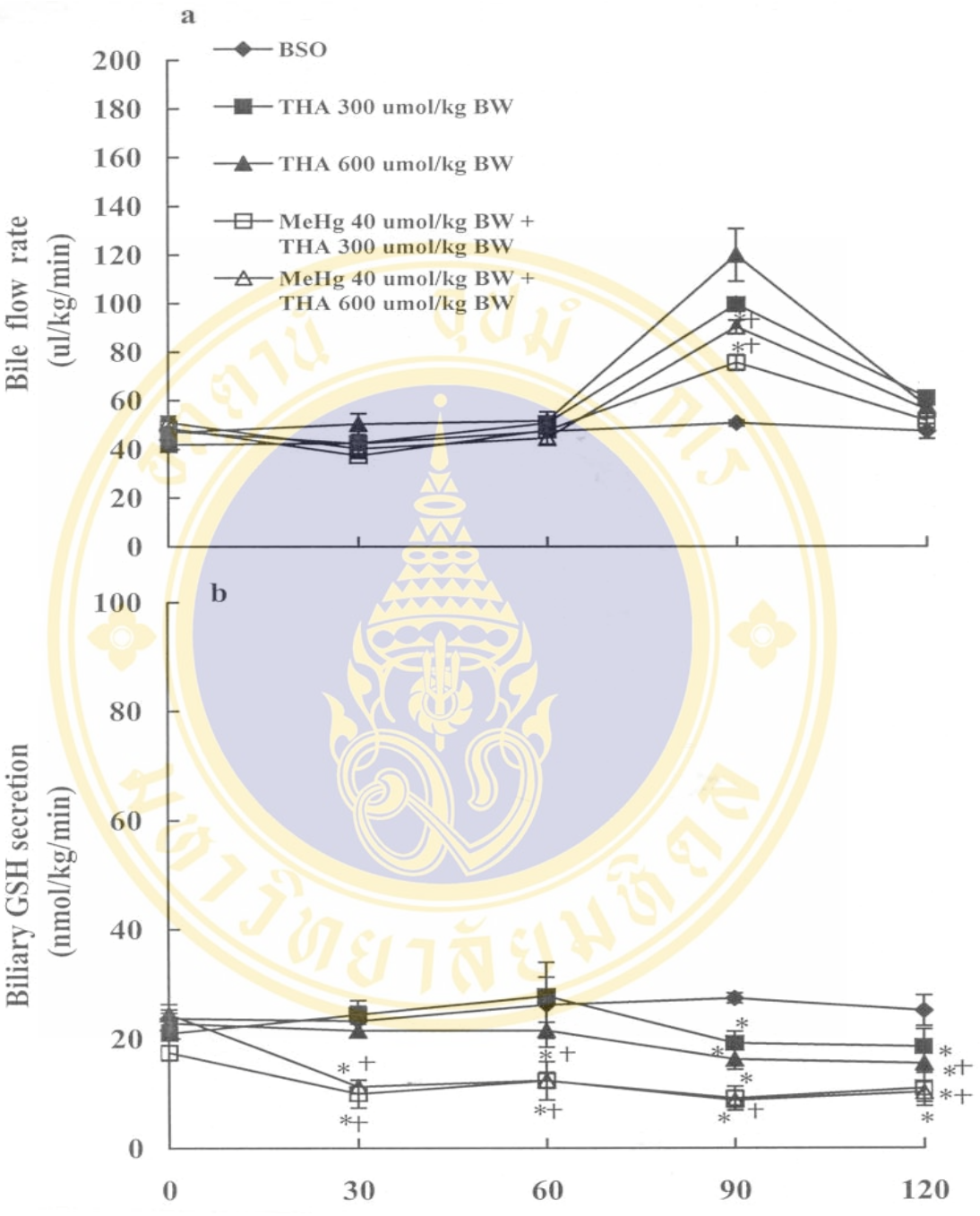


**Figure 12.** The bile flow rate (a) and biliary secretion of glutathione (b) in rats pretreated with BSO before injection of MeHg and intraduodenally administration of THA.
Each point represents mean ± SEM of 8 rats/group.
* $p<0.05$ significant difference when compared with BSO at the corresponding time.
+ $p<0.05$ significant difference when compared with the corresponding THA-treated groups at the corresponding time.

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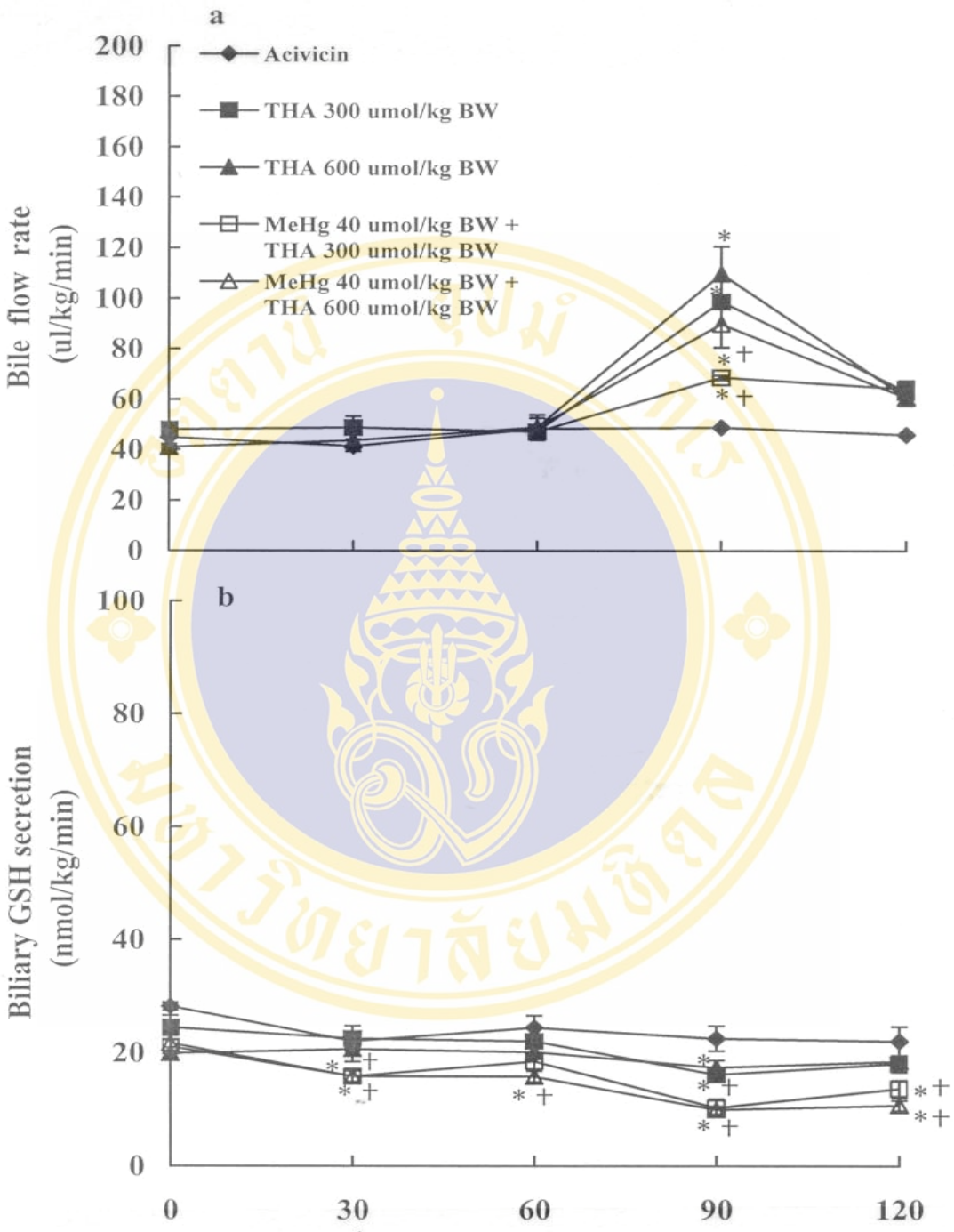


**Figure 13.** The bile flow rate (a) and biliary secretion of glutathione (b) in rats pretreated with acivicin before injection of MeHg and intraduodenally administration of THA.
Each point represents mean ± SEM of 8 rats/group.
* $p<0.05$ significant difference when compared with control at the corresponding time.
+ $p<0.05$ significant difference when compared with the corresponding THA-treated groups at the corresponding time.

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**Table 13.** Effect of 2,4,6 - trihydroxyacetophenone (THA) on cumulative biliary excretion of mercury (μmol/kg BW) in glutathione-depleted rats.

| Treatments | Cumulative biliary excretion of mercury (μmol/kg BW) | | | | |
|---|---|---|---|---|---|
| | 0 | 30 | 60 | 90 | 120 (min) |
| Saline + MeHg 40 μmol/kg BW + THA 300 μmol/kg BW | 0 | 0.30 ± 0.03 | 0.50 ± 0.05 | 0.56 ± 0.03 | 0.60 ± 0.02 |
| Saline + MeHg 40 μmol/kg BW + THA 600 μmol/kg BW | 0 | 0.27 ± 0.01 | 0.46 ± 0.04 | 0.50 ± 0.02 | 0.55 ± 0.04 |
| BSO +MeHg 40 μmol/kg BW + THA 300 μmol/kg BW | 0 | 0.20 ± 0.01* | 0.25 ± 0.01* | 0.30 ± 0.03* | 0.33 ± 0.02* |
| BSO +MeHg 40 μmol/kg BW + THA 600 μmol/kg BW | 0 | 0.15 ± 0.02* | 0.20 ± 0.04* | 0.22 ± 0.04* | 0.25 ± 0.01* |
| Acivicin + MeHg 40 μmol/kg BW + THA 300 μmol/kg BW | 0 | 0.22 ± 0.04* | 0.30 ± 0.02* | 0.35 ± 0.02* | 0.38 ± 0.02* |
| Acivicin + MeHg 40 μmol/kg BW + THA 600 μmol/kg BW | 0 | 0.17 ± 0.05* | 0.23 ± 0.04* | 0.27 ± 0.01* | 0.30 ± 0.03* |

The animals were pretreated with BSO, or acivicin before injection of MeHg at 60 $^{th}$ min and/or intraduodenally administration of THA 300 or 600 μmol/kg BW at 90 $^{th}$ min.

Each value is mean ± SEM of 8 rats/group.

* $p < 0.05$ significant difference when compared with the saline-pretreated group at the corresponding time.

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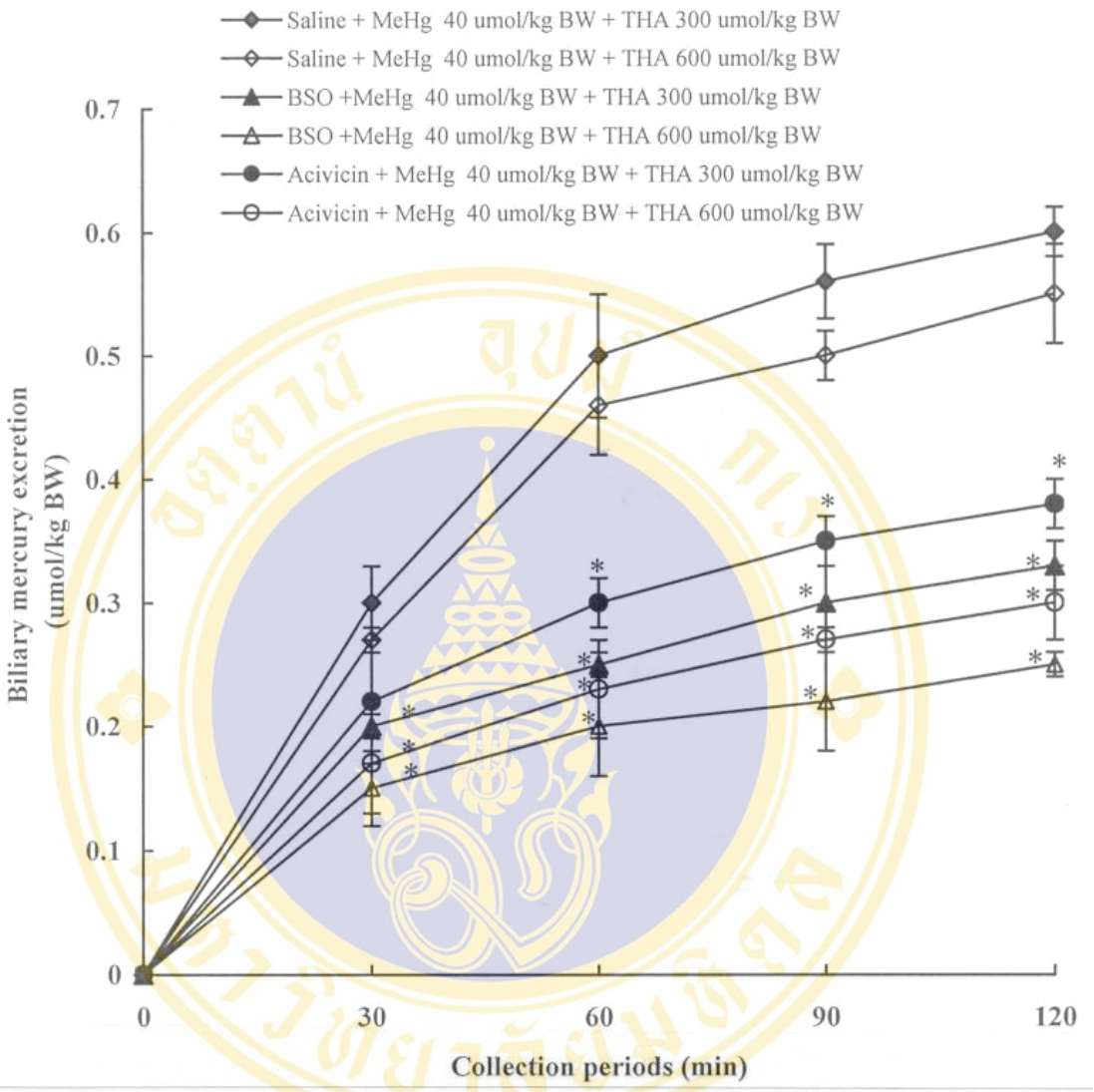


**Figure 14.** The biliary excretion of mercury in rats pretreated with glutathione depletors. The animals were pretreated with BSO or acivicin before portal injection of MeHg and intraduodenal administration of THA.
Each point represent mean ± SEM of 8 rats/group.
* $p<0.05$ significant difference when compared with the saline pretreated group at the corresponding time.

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**Table 14.** Effect of 2,4,6 - trihydroxyacetophenone (THA) on liver glutathione, mercury concentrations and the activity of glutathion *S*-transferase (GST) in the glutathione-depleted rats.

| Treatments | Glutathione (μmol/g liver) | Mercury (μmol/g liver) | GST (μmole/mg protein/min) |
|---|---|---|---|
| Saline | 6.10 ± 0.21 | - | 2.90 ± 0.12 |
| Saline + THA 300 μmol/kg BW | 5.90 ± 0.16 | - | 3.25 ± 0.11 |
| Saline + THA 600 μmol/kg BW | 6.63 ± 0.24 | - | 4.60 ± 0.20+ |
| Saline + MeHg 40 μmol/kg BW + THA 300 μmol/kg BW | 5.05 ± 0.36 | 24.56 ± 3.38 | 1.98 ± 0.06+ |
| Saline + MeHg 40 μmol/kg BW + THA 600 μmol/kg BW | 4.95 ± 0.98 | 20.32 ± 3.49 | 2.02 ± 0.12+ |
| BSO | 4.35 ± 0.36* | - | 2.89 ± 0.12 |
| BSO + THA 300 μmol/kg BW | 4.05 ± 0.34* | - | 2.70 ± 0.18* |
| BSO + THA 600 μmol/kg BW | 3.77 ± 0.29* | - | 2.73 ± 0.15* |
| BSO + MeHg 40 μmol/kg BW + THA 300 μmol/kg BW | 3.58 ± 0.18* | 36.46 ± 2.25* | 1.99 ± 0.14++ |
| BSO + MeHg 40 μmol/kg BW + THA 600 μmol/kg BW | 3.11 ± 0.26* | 32.62 ± 3.21* | 2.00 ± 0.12++ |
| Acivicin | 4.18 ± 0.34* | - | 2.42 ± 0.12 |
| Acivicin + THA 300 μmol/kg BW | 3.40 ± 0.24* | - | 2.56 ± 0.15* |
| Acivicin + THA 600 μmol/kg BW | 3.11 ± 0.26* | - | 2.73 ± 0.19* |
| Acivicin +MeHg 40 μmol/kg BW + THA 300 μmol/kg BW | 3.05 ± 0.13* | 38.94 ± 2.10* | 1.66 ± 0.14+++ |
| Acivicin + MeHg 40 μmol/kg BW + THA 600 μmol/kg BV | 3.17 ± 0.23* | 38.99 ± 4.66* | 1.83 ± 0.16+++ |

The animals were pretreated with BSO, or acivicin before injection of MeHg at 60 $^{th}$ min and/or intraduodenally administration of THA 300 or 600 μmol/kg BW at 90 $^{th}$ min.

Each value is mean ± SEM of 8 rats/group.

* $p < 0.05$ significant difference when compared with corresponding control group.

+ $p < 0.05$, ++ $p<0.05$, +++ $p<0.05$ significant difference when compared with saline, BSO, and acivicin-pretreaed rat (without THA, MeHg), respectively.

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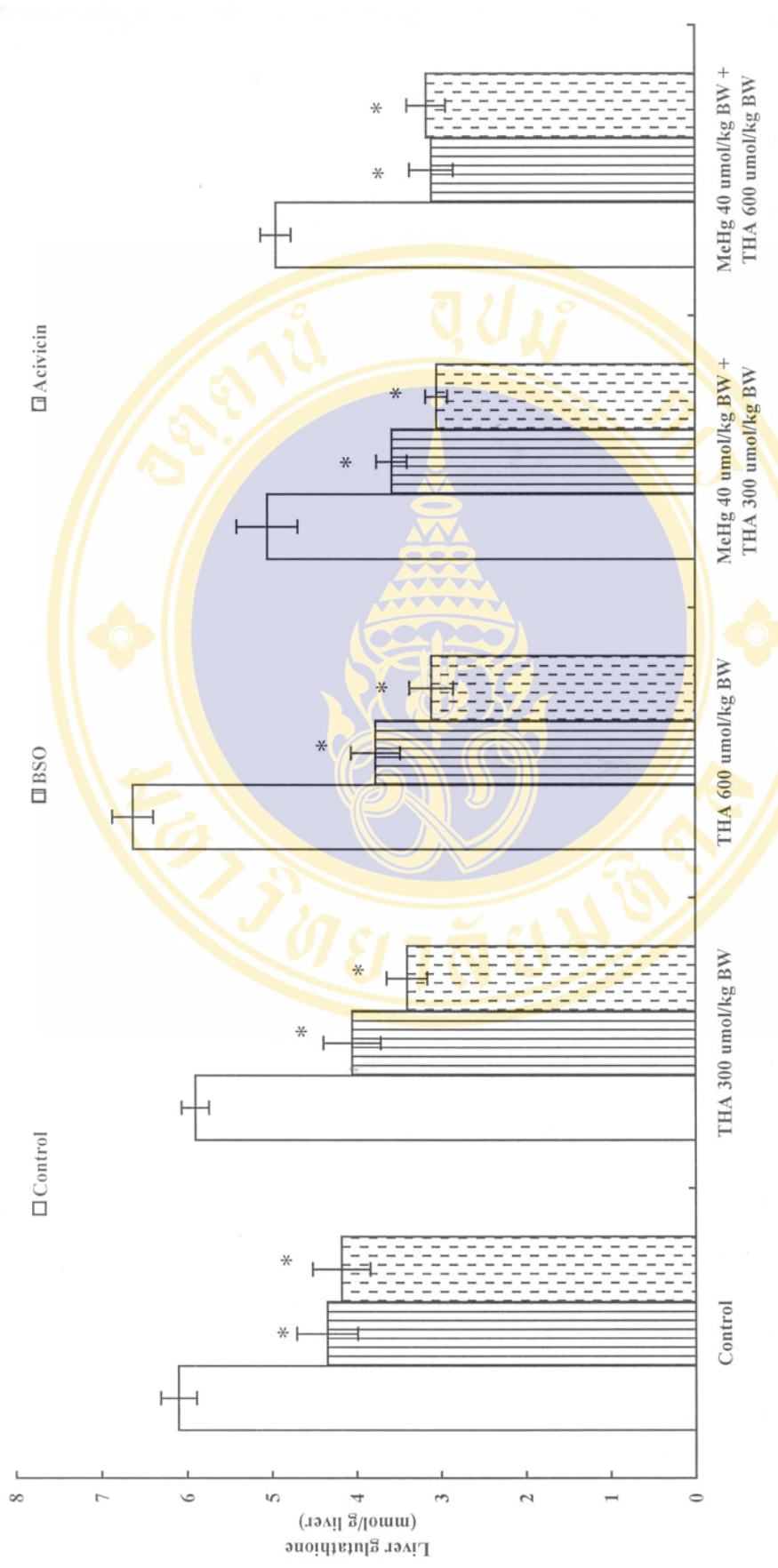


**Figure 15.** Effect of THA on hepatic glutathione concentration in rats pretreated with BSO or acivicin. The animals received BSO or acivicin 90th min before administration of MeHg and/or THA.
Each bar represents mean ± SEM of 8 rats/group.
* $p<0.05$ significant difference when compared with corresponding control groups.

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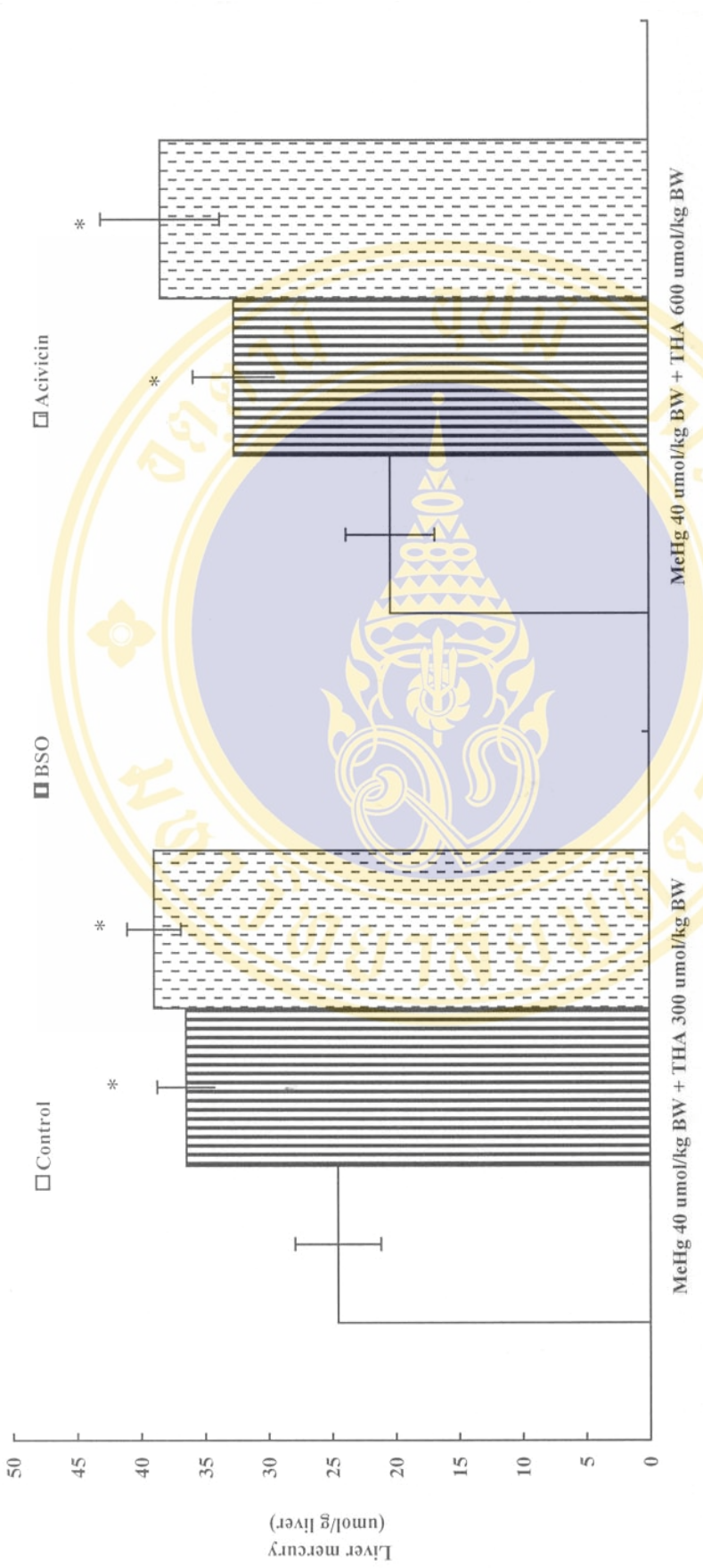


**Figure 16.** Effect of THA on hepatic mercury concentration in rats pretreated with BSO or acivicin. The animals received BSO or acivicin 90$^{th}$ min before administration of MeHg and/or THA .
Each bar represents mean $\pm$ SEM of 8 rats/group.
* $p<0.05$ significant difference when compared with corresponding controls groups.

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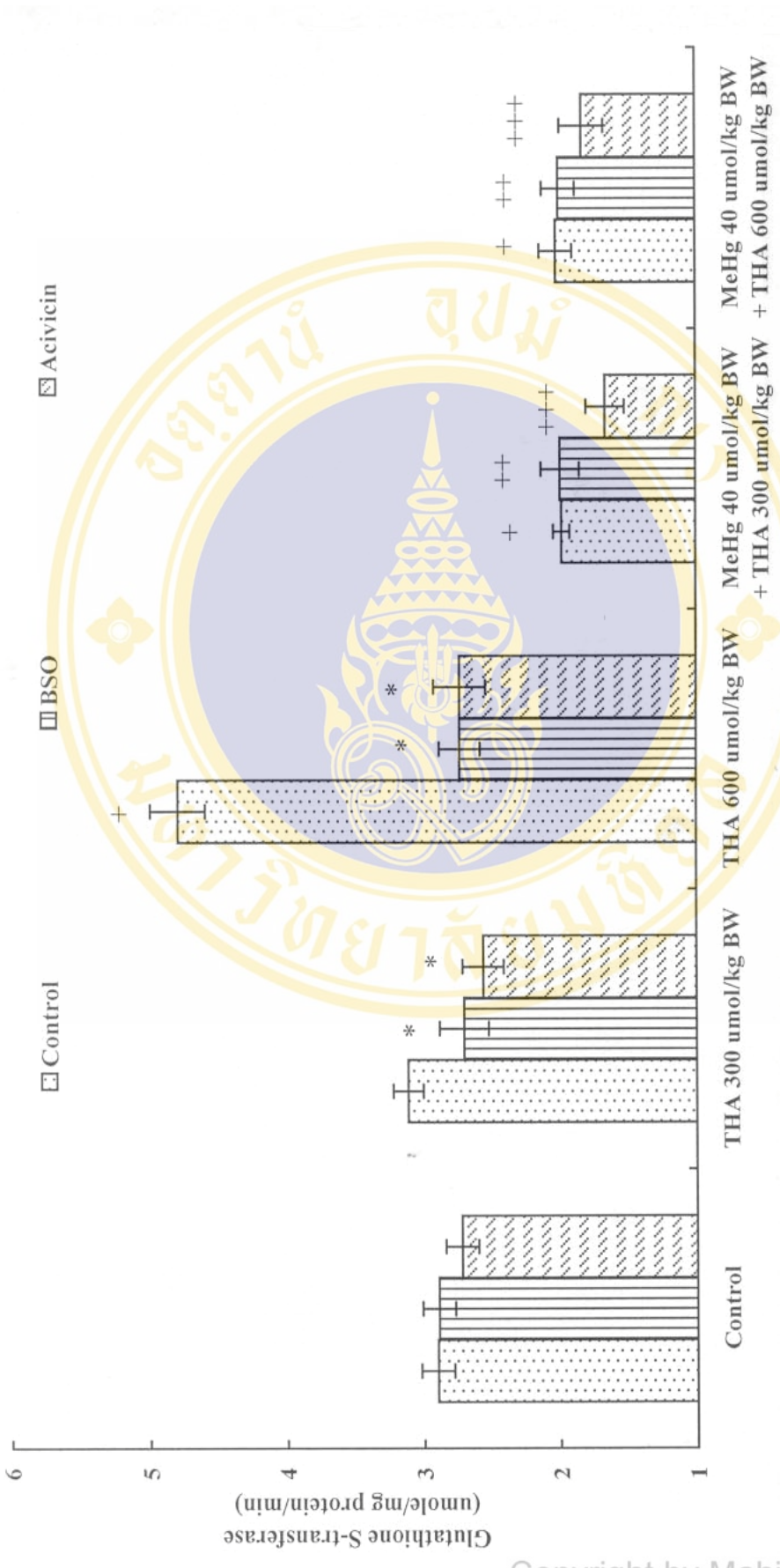


**Figure 17.** Effect of THA on hepatic glutathione *S*-transferase (GST) activity in rats pretreated with BSO or acivicin. GST activity towards CDNB were measured in rats liver homogenates, which were pretreated with BSO or acivicin before administration of MeHg and/or THA at 90$^{th}$ min.
Each bar represents mean $\pm$ SEM of 8 rats.
* $p<0.05$ significant difference when compared with control.

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**Table 15.** Effect of 2,4,6 - trihydroxyacetophenone (THA) on urine flow rate (µl/kg/min) in the MeHg-treated rats.

| Treatments | Urine flow rate (µl/kg/min) | | | | |
|---|---|---|---|---|---|
| | 0 | 30 | 60 | 90 | 120 (min) |
| Control | 14.11 ± 0.25 | 29.01 ± 5.91 | 30.81 ± 4.76 | 45.66 ± 6.71 | 50.66 ± 4.17 |
| THA 300 µmol/kg BW | 13.35 ± 5.48 | 28.20 ± 8.46 | 30.66 ± 6.66 | 61.04 ± 6.94* | 48.16 ± 4.45 |
| THA 600 µmol/kg BW | 20.65 ± 3.02 | 32.60 ± 3.51 | 33.39 ± 5.61 | 80.23 ± 1.13* | 65.67 ± 8.50 |
| MMC 40 µmol/kg BW | 16.63 ± 0.42 | 14.40 ± 0.81* | 15.85 ± 1.13* | 40.75 ± 1.80 | 42.88 ± 2.86 |
| MMC 40 µmol/kg BW + THA 300 µmol/kg BW | 21.70 ± 0.89 | 11.73 ± 1.16* | 21.18 ± 0.21* | 67.32 ± 3.31+ | 38.19 ± 5.18* |
| MMC 40 µmol/kg BW + THA 600 µmol/kg BW | 15.46 ± 2.89 | 16.00 ± 5.34* | 19.92 ± 1.92* | 80.23 ± 1.98*+ | 55.10 ± 3.80 |

Rats were treated with MeHg (40 µmol/kg BW) for 30-min before intraduodenally administration of THA at a dose 300 or 600 µmol/kg.

Urine sample was collected in 30-min periods for 120 min.

Each value is mean ± SEM of 8 rats.

* $p < 0.05$ significant difference when compared with control at the corresponding time.

+ $p < 0.05$ significant difference when compared with the MeHg-treated group at the corresponding time.

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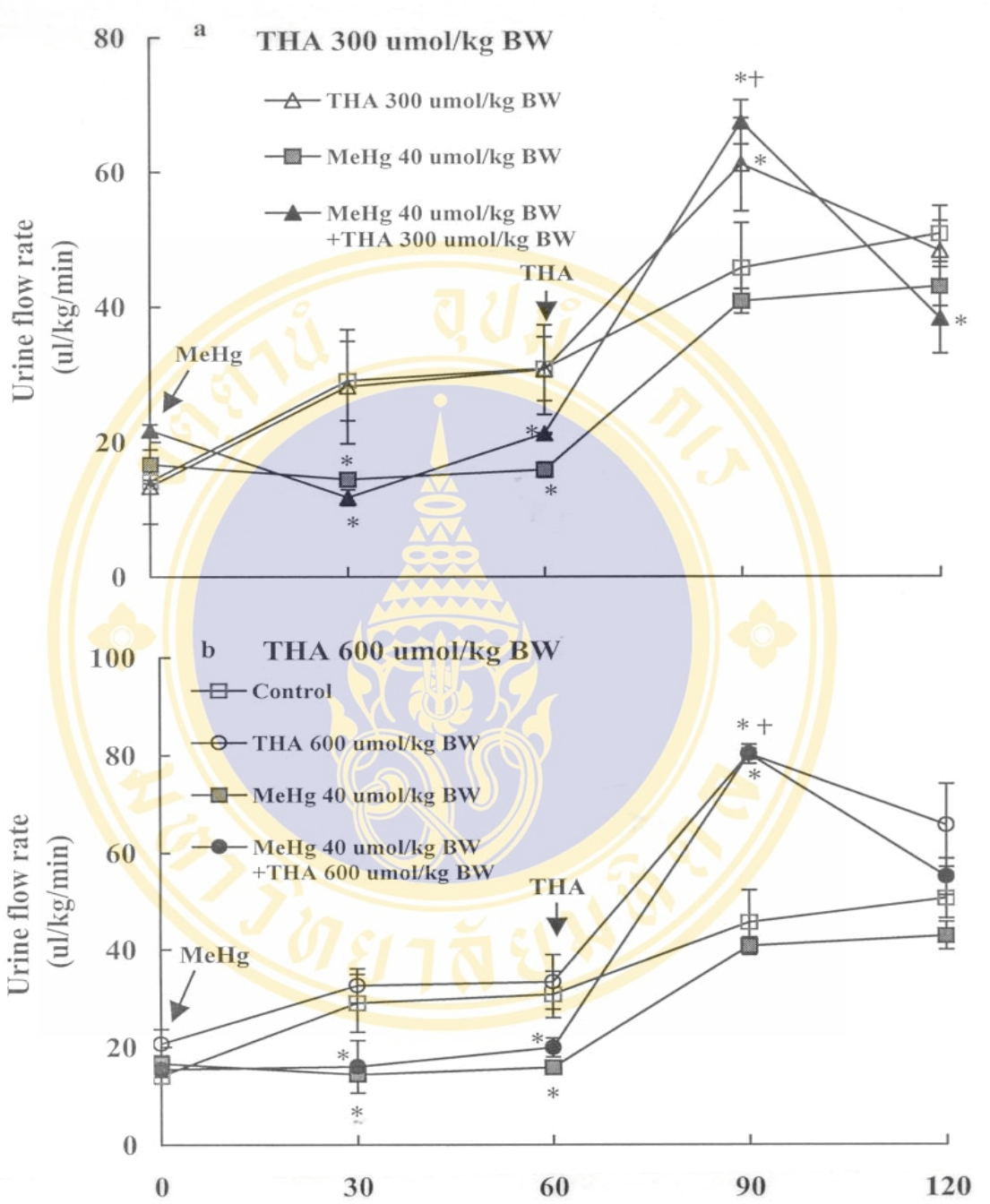


**Figure 18.** Effect of THA administration on the urine flow rate. Rats were pretreated with MeHg (40 μmol/kg BW) at 30th min before intraduodenal administration of THA at a dose of 300 (a) or 600 (b) μmol/kg BW. Urine sample was collected in 30-min periods for 120 min.
Each point represents mean ± SEM of 8 rats/group.
* $p<0.05$ significant difference when compared with control at the corresponding time.
+ $p<0.05$ significant difference when compared with THA-treated group at the corresponding time.

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**Table 16.** Effect of 2,4,6 - trihydroxyacetophenone (THA) on cumulative urinary mercury output (pmol/kg BW) in the MeHg-treated rats.

| Treatments | Cumulative urinary mercury output (pmol/kg BW) | | | | |
|---|---|---|---|---|---|
| | 0 | 30 | 60 | 90 | 120 (min) |
| MMC 40 µmol/kg BW | 0.00 | 2.03 ± 0.90 | 3.64 ± 1.25 | 5.13 ± 1.79 | 7.13 ± 1.55 |
| MMC 40 µmol/kg BW + THA 300 µmol/kg BW | 0.00 | 2.59 ± 1.20 | 5.48 ± 1.55 | 12.01 ± 2.19+ | 16.45 ± 2.32+ |
| MMC 40 µmol/kg BW + THA 600 µmol/kg BW | 0.00 | 2.19 ± 0.95 | 4.79 ± 1.25 | 13.11 ± 1.55+ | 18.10 ± 1.45+ |

Rats received MeHg (40 µmol/kg BW) for 30-min before intraduodenally administration of THA at a dose 300 or 600 µmol/kg. Urine sample was determined in 30-min periods for 120 min.

Each value is mean ± SEM of 8 rats/group.

+ $p < 0.05$ significant difference when compared with the MeHg-treated group at the corresponding time.



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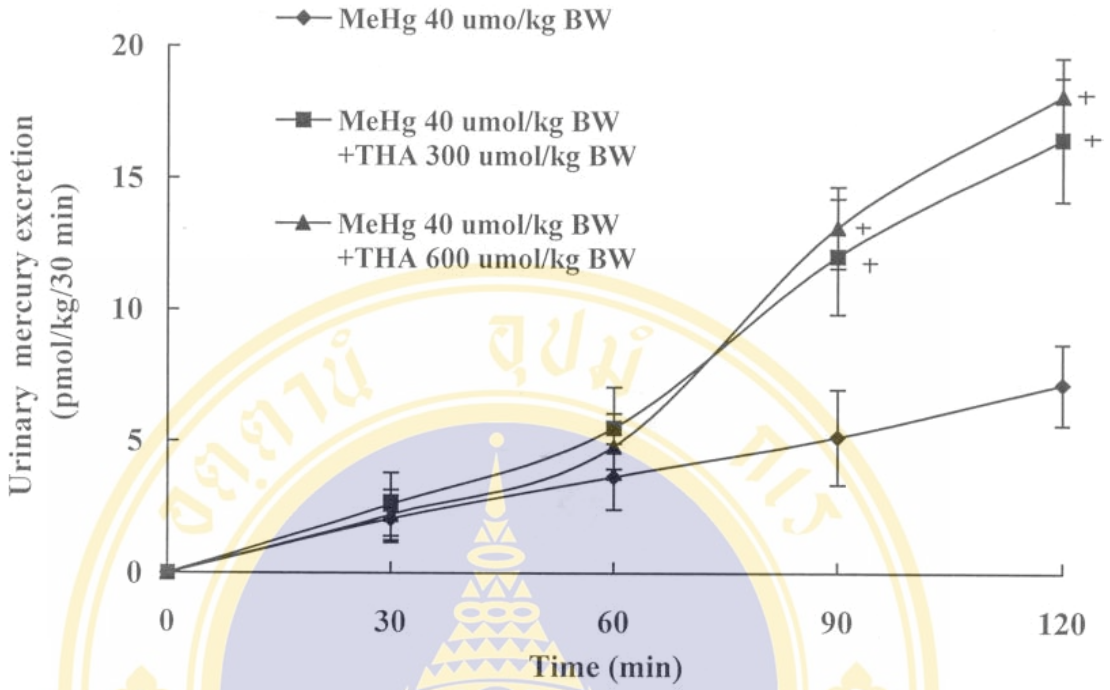


**Figure 19.** Effect of THA administration on the cumulative urinary mercury excretion. Rats were pretreated with MeHg (40 μmol/kg BW) at 30$^{th}$ min before intraduodenal administration of THA (300 or 600 μmol/kg BW). Urine sample was collected in 30-min periods for 120 min.
Each point represents mean ± SEM of 8 rats/group.
+ $p<0.05$ significant difference when compared with the MeHg-treated group at the corresponding time.

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# CHAPTER V
# DISCUSSION

The present study demonstrated a dose-related effect of MeHg in inducing hepatotoxicity and the choleretic effect of THA in modulating the hepatic excretion of MeHg. Bile is the major route of elimination of MeHg. Although BFR was markedly increased by THA, the biliary excretion of MeHg was not accelerated. In contrast, THA inhibited the excretion of MeHg into bile. However, THA did not augment the hepatotoxicity of MeHg. A diversion of the excretion from the biliary to the urinary route was observed. Possible mechanisms of its interferences were discussed.

## I. Effect of MeHg on Hepatotoxicity

Bile is the major route of elimination of MeHg. Although the hepatobiliary transport of this metal has been extensively investigated, there are some unclear on the intoxication regarding the toxic dose level, the biliary elimination and the detoxification. In the present study, MeHg was injected into the portal vein and was directed into the liver. The effect of the first pass to the liver or hepatic presystemic elimination might, at least, was more accurate to determine its elimination than general systemic elimination. A bolus injection of MeHg into the portal vein abruptly decreased BFR, which indicated that MeHg impaired either bile formation or secretion. The inhibition on bile production has often been used as indicator of hepatotoxicity. The decreased bile secretion in the present study was accompanied by increases in plasma AST, ALT, and ALP activities and biliary activity of ALP. The increasing hepatotoxicity and impairment of the bile secretion with increasing dose of MeHg were evident. The dose-related effects of MeHg in inducing cholestasis and hepatotoxicity in the present study were consistent with the earlier studies by Ballatori and Truong (1995c) in which they demonstrated an increase in perfusion pressure in the isolated rat liver by a chloride complex of MeHg.

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Hg has strong electrophilic characteristics, tends to bind to plasma membrane and a variety of enzyme systems inside the hepatocytes, especially those containing free sulfhydryl groups, including those of microsomes and mitochondia, producing cell injury and cell death (Zalups and Lash, 1996). Hepatic injury, which causes a reduction of bile flow, termed cholestasis (Popp and Cattley, 1991) is frequently observed. Disturbance of bile flow by toxic agents may be due to a selective injury or blockade of the mechanism for hepatic uptake, processing, and/or excretion of the components of bile. Interference with the excretory phase may result from functional or structural lesions (Garbiel and Michiel, 1994). The possible causes of cholestasis induced by MeHg in the present study might be related to both direct and indirect action of metals on plasma membrane functions. Imesch et al, (1992) reported the direct effects that Hg *in vitro* caused the release of the α-subunit of $Na^+$,$K^+$,-ATPase from its membrane anchor. Therefore, it is possible that Hg impaired bile flow in the present study by inhibition on $Na^+$,$K^+$,-ATPase activity. Sellinger et al. (1991) also demonstrated the direct and specific toxic action of Hg on sodium-coupled alanine transport on the membrane. In their studies using membrane vesicles from hepatocytes of *Raja erinacea*, Hg strongly inhibited isotopic exchange of amino acids across the membranes. Moreover, Markovich and James (1999) studied in *Xenopus* oocytes and reported that Hg strongly inhibited sat-1 which encoded a sulfate/bicarbonate anion changer expressed in the rat liver and kidney. Grosso and De Sousa (1993) also observed the suppression of the water membrane permeability by Hg in which the effect could be reversed by treatment with various thiol compounds such as dithiotheitol, presumably by removing the metal from its site of action (Prestor et al., 1991). The indirect effect of MeHg on membrane function could be resulted from the interference with intracellular processes including the production of available metabolic energy. Inhibition of the energy supply would then indirectly reduce the active transport capacity of the cell membrane. Recently, Denny et al., 1993 and Shenker et al., 1999 demonstrated that MeHg causes swelling of mitochondria which subsequently depleted the ATP pool of the cytosol. Moreover, MeHg which enhanced the proton permeability in membranes of lysosome was also

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noted in addition to cause leakage of plasma membrane and release hepatic enzymes from cytoplasm into the circulation (Ashour et.al., 1993; Bragadin et.al., 2002).

MeHg is excreted into bile and is classified as a compound, which has slowest excretion rate. It is excreted less than 20 % in the feces and urine within 4 days (Gregus and Klaasen, 1986). In 1986, Gregus and Klaasen reported that 0.8 % of the administered dosages of MeHg was excreted into bile in 2 hr. In the present study, the biliary excretion of Hg was approximately 1.18 – 3.42 % in 2 hr. The discrepancy of results in these two studies might be due to the differences in the dose and route of MeHg administration. In the present study, the toxic dose of MeHg was employed and injected directly into the portal vein whereas in the earlier study, the maximum tolerated dose, which was non-toxic, was used and was given intravenously (Thompsom and Klaassen, 1986). These route-dependent differences have previously been reported the biliary excretion of Hg after portal administration was approximately two folds higher than that after systemic administration.

Although the amount of Hg excreted into bile in the present study was increased with the dose, the fractional biliary excretion of Hg was decreased with the increased dose. The initial rate of Hg excretion into bile in first 30 min was increased with the dose, afterward the excretion rate was found to be steadily maintained and independent on the administered dose. This finding is in agreement with that reported previously by Gregus and Klaassen (1986) in which the fraction rate of dosage excreted into bile was independent of the dosage of MeHg. Two possibilities might be accounted for these observations. The limitation of the biliary Hg excretion might be due to the saturation of Hg transport from liver into bile and/or a toxic depression of the hepatobiliary transport process by toxic dose of MeHg used. The excretion of MeHg into bile is mainly by forming a complex with cystein, GSH or other thiol (Ballatori and Clarkson, 1983, 1985). The increase or decrease in biliary excretion of nonprotein thiols in rats has been reported to induce similar changes in the biliary excretion of the Hg (Klaassen, 1975). Likewise, in the present study the amounts of the hepatic GSH and biliary GSH were decreased with the increased dose of MeHg. Inhibition of the formation of the excretory GSH compound as well as outward movement of the GSH from the hepatocytes across the canalicular and/or sinusoidal

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membrane might be another mechanisms accounting for this observation. Multidrug resistance-associated protein 2 (MRP2), which is highly expressed in the bile canaliculus of hepatocytes, has been reported to mediate hepatobiliary transport of many organic compounds including GSH and GSH *S*-conjugates (Paulusma C, et al., 1999). In addition, recent studies indicated that the hepatic organic solute transporters (Oatp1 and Oatp2) at the basolateral membrane also playe a role in the efflux of GSH derivatives and GSH *S*-conjugates from hepatocytes to plasma, (Li et al., 1998; Li et al., 2000). As the excretory rate of Hg was rather constant, independent on the dose, the toxicity of MeHg on the transport system might not be the major factor determining this limitation. They might depend mainly on the saturation of the transport process for excretion, which was not clear in the present study for the extent of interference on availability of GSH for formation of excretory complex and on the transported system. MeHg at a dose of 40 μmol/kg BW would be employed in the subsequent study to investigate the modulating effect of THA on its excretion.

## II. Effect of THA on MeHg-Induced Hepatotoxicity

Since bile is the major route for MeHg elimination, any methods that lead to an increase in the biliary excretion of MeHg should be considered to have therapeutic significance. It has been demonstrated that the biliary excretion of Hg is enhanced by methionine, 3-methyl-4-dimethyl-azobenzene, D-penicillamine or dimercaptosuccinic acid (DMSA), and trans-stilbene oxide (TSO) (Magos et al, 1978, 1985). These compounds are known to provide GSH by different mechanisms except for TSO, which is a glutathione *S*-transferase (GST) inducer. Moreover, Haddow et al (1972) has shown that phenobarbitone, an inducer of GST in the liver, also increased the biliary excretion of MeHg. This evidence indicates that various microsomal enzyme inducers could increase the biliary excretion of MeHg.

Bile secretion is usually accompanied by the excretion of both endogenous and exogenous compounds. With enhancing rate of bile secretion, the excretion rate of those entire excretory compounds would be accelerated. THA has previously been demonstrated to enhance bile flow rate in a dose-dependent manner (Piyachaturawat et al., 1998). In consistent with the earlier study, THA caused a dose-dependent

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increase in BFR, which peaked at 30 min after administration. The choleretic action of THA involved both bile acid-dependent and bile acid-independent fractions of bile flow. The bile acid-dependent flow is the flow dependent on the osmotic pull of the bile salts after their active transported into the canalicular lumen whereas bile acid-independent flow is the flow apparently dependent on the active transport of non-bile acids. It included $Na^+$, $HCO_3^-$, $Cl^-$, GSH and/or the active metabolites of THA. THA or 2,4,6-trihydroxyacetophenone contains hydroxy groups, which are preferably conjugated with glucuronic acid. Recently, the metabolite of its analog, 4-monohydroxy acetophenone has been identified as a glucuronide conjugate, which was a cholephilic organic compound.

It has been shown in the earlier study that, THA enhance the hepatic clearance of sulfobromophthalein and decrease the elevated of plasma ALP in the EE-induced cholestatic rats (Piyachaturawat et al., 2002). In the present study, THA administration was able to increase BFR in the MeHg-treated rats, indicating that the capability for bile secretion in the animals was not completely abolished. In addition, THA might have ability to alleviate the impairment of BFR in the MeHg-rats.

Surprisingly, THA, which is a choleretic agent and activates GST activity, strongly decreased the secretion of GSH and Hg into bile. It is possible that THA might indirectly interfere Hg excretion by affecting availability of GSH. THA itself, particularly at the high dose might conjugate with GSH in addition to formation of glucuronide conjugation before excreted into bile and caused a decrease in Hg excretion. Another possible interfering mechanism of THA might be due to THA and/or its metabolite was transported into bile by the same transport system of GSH and its conjugate compounds including MeHg-GSH at canalicular membrane. A number of recent findings indicated that MRP2 is responsible for the canalicular transport of GSH or its conjugate and the process is ATP-dependent (Rebbeor et al, 1998; Paulusma et al., 1999; Ballatori et al., 1998; Ballatori et al., 2002). Impaired biliary GSH secretion and reduced bile flow in MRP2 mutation (Ballatori et al., 1995a) supported the suggestion that biliary GSH transport is a major driving force for bile formation (Ballatori and Truong, 1992). The GSH secretion into bile has also been reported to be inhibited by indocyanin green and sulfobromophthalein (BSP)

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(Ballatori and Clarkson, 1983). This inhibition was accompanied by a parallel inhibition of Hg excretion and occurred without any changes in the liver GSH or Hg levels. Similarly, the present study showed that the liver Hg and GSH content were not altered by THA administration.

The GST activity was not altered after THA administration in the MeHg-treated rats as compared with the MeHg-treated rats alone without THA. This was probably due to MeHg intoxication by binding with GST enzyme resulting in inhibition of the GST activity. Based on this view, Dierickx (1985) also reported that organic mercury directly interacts with GST by binding to this protein and causes its function loss.

Although, the biliary excretion of MeHg was suppressed by THA, the hepatotoxicity was not significantly augmented by this compound. Activities of plasma ALT, AST, and ALP in both bile and plasma were not significantly altered after THA administration. These results implicated that THA itself at the experimental dose was not hepatotoxic, which confirmed the earlier report (Piyachaturawat et al., 2002).

## III. Effect of THA in Rat Pretreated with GSH Depletors

The involvement of the choleretic THA with cellular GSH status was examined in the rats pretreatment with GSH depletors; BSO and acivicin by which two different GSH depletion mechanisms are attained. The mechanism of depletion of the intracellular hepatic GSH by BSO is accomplished by inhibition of γ-glutamyl-cysteine synthetase, which is the rate-limiting enzyme involved in the intracellular systhesis of GSH (Griffith and Meister, 1979). Unlike BSO, depletion of hepatic GSH by acivicin is due to its inhibition on the activity of the enzyme γ-glutamyltransferase at the canalicular membrane, resulting in the secreted GSH or its conjugate is not enzymatically broken down to cysteine, which is normally reabsorbed very rapidly and efficiently by the sodium-dependent neutral transport system. By reducing the pool of reabsorbable cysteine, which serves as the rate-limiting substrate involved in the biosynthesis of GSH, intracellular depletion of GSH occurs (Zalups and Lash, 1997). In agreement with its action, the concentration of

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GSH in bile in the present study was also significantly decreased in the acivicin pretreatment. This finding provided evidence that excretion of Hg into bile was dependent on the hepatic GSH level and the activity of the γ-glutamyltransferase on the canalicular membrane.

Pretreatment of normal rats with BSO as well as acivicin in the present study caused significant decreases in hepatic GSH status to approximately 70 – 80 % which were in agreement with those reported previously by Zalups et al., (1999). As GSH have been implicated to provide an osmotic driving force in the hepatic bile formation (Ballatori and Truong, 1992), thus the fall of BFR and biliary GSH secretion in the GSH-depleted rats in the present study supported the significant of GSH in bile secretion and also indicated the depleting level of GSH in the liver.

However, the suppressed levels of BFR in the BSO or acivicin treatment were able to stimulate by THA administration, although they were far below than that of THA in the absence of GSH depletor. These results indicated that the induction of BFR by THA was partly a GSH-dependent. Not only the further reduction of GSH in bile by THA in the GSH-depleted rats, but biliary excretion of Hg was also further decreased. Therefore, it might be possible that the decreased biliary excretion of Hg was due to the decrease of availability of GSH and consequently decreased secretion of GSH into bile and increased Hg accumulation in the liver. In support of this contention, Ballatori and Clarkson (1983, 1985), demonstrated that the change in Hg excretion was parallel to the change in GSH secretion.

Although the biliary excretion of Hg was inhibited by THA, the urinary Hg excretion was substantially increased. It indicated that THA might modulate MeHg excretion by diverting from biliary to other route. The content of Hg in the liver was slightly increased but not significant by THA. In general, drugs and their biotransformation products are eliminated from the body by excretion into the urine or bile. It is assumed that the urinary and the biliary pathways of excretion are complementary to each other. (Hirom et al., 1976). If one of these routes is blocked, excretion by the alternative pathway is increased. This might be the case of the present study in which the urinary excretion was increased after the biliary excretion

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was inhibited. In addition, the high accumulation of Hg in liver after THA administration might be due to the return of the MeHg-GSH complex which left the liver into the systemic circulation. These mercaptide complex has been reported to be less toxic as compared with MeHg it (Ballatori and Troung, 1995c).

In conclusion, THA, the choleretic substance, strongly inhibited biliary excretion of MeHg. The inhibition observed might be associated with the toxicity of MeHg itself and with the interference of THA on at least two steps in the excretory processes of Hg into bile. THA might reduce the availability of intracellular GSH for the formation of a MeHg-GSH complex for excretion. In addition, THA and its metabolites might compete with the excretory MeHg complex at the canalicular transporter. An understanding on the excretory mechanism and the interactions between the therapeutic compounds are important for setting the detoxification strategies. Co-administration of some compounds might lead to the further progressive increase of toxicity.

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# CHAPTER VI
# CONCLUSION

1. A bolus injection of MeHg (20 – 80 μmol/kg BW) into portal vein abruptly decreased BFR in a dose-related manner. The decreased BFR was accompanied by with a decrease in the biliary secretion of GSH.
2. The percent excretion of mercury into the bile was decreased with increasing the dose of MeHg administered. Meanwhile, the accumulation of MeHg in the liver was increased and the rate of biliary Hg excretion was steadily maintained.
3. Activities of plasma AST, ALT, and ALP in both plasma and bile were markedly increased with the MeHg administered in a dose-related manner.
4. The concentration of hepatic GSH at two hours after MeHg injection was decreased while the concentration of hepatic Hg was increased in a dose-related manner.
5. THA (300 and 600 μmol/kg BW) was able to enhance the bile secretion whereas the secretion of GSH into bile was reciprocally affected in the MeHg-treated animals.
6. Although THA has capability to increase BFR in the MeHg-treated animals, it decreased biliary excretion of Hg.
7. After receiving THA, the concentrations of GSH and Hg in the liver were not altered .
8. THA itself increased GST activity in a dose-related manner (300 – 600 μmol/kg BW). However, the administration of THA to the MeHg-treated rats did not alter GST activities, which had been suppressed and maintained at a very low level in the MeHg-treated rats.
9. THA administration to the MeHg-treated rats did not increase the plasma levels of AST, ALT, ALP, and biliary ALP.
10. After giving GSH depletors, BSO and acivicin, the basal bile secretion was reduced. Moreover, the biliary secretion of GSH was also markedly decreased.

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11. THA administration to the BSO- or acivicin-pretreated rats was able to increase BFR but the level was far below that by THA in non-GSH depleted rats.
12. The biliary secretion of GSH was further decreased by THA administration, which indicated that the stimulation bile secretion by THA was partly dependent on GSH.
13. The Hg excretion into bile was decreased after THA administration in GSH-depletion-pretreated rats and followed by the MeHg administration.
14. THA administration did not alter the hepatic GSH concentration and GST activity in all GSH-depleted rats.
15. THA caused a transient increase in UFR (acute diuresis). The urinary excretion of mercury was transiently increased after THA administration. It is likely that THA might modulate the Hg excretion by shifting into the urinary route, which is a minor route of excretion in normal circumstance.
16. The inhibition of biliary Hg excretion by THA might be associated with the reduced availability of intracellular GSH and THA and/or its metabolites might inhibit the excretory MeHg complex at the canalicular transporter.
17. From these results, an understanding on the excretory mechanism and the interaction between the therapeutic intended compounds are important for the detoxification strategies.

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# REFERENCES


1. Aberg B, Ekman L, Falk R, Greitz U, Persson G, Snihs JO. Metabolism of methylmercury (203 Hg) compounds in man. Arch Environ Health 1969; 19 (4): 478-484.
2. Agency for toxic substances and disease registry. Top 20 hazardous substance: ATSDR/EPA priority list for 1999. http://atsdr.cdc.gov:8080/cxcx3.html.
3. Akerboom PM and Sies H. Assay of glutathione, glutathione disulfide and glutathione mixed disulfides in biological samples. Method in Enzymology, Jakoby WB Ed. Academic Press, New York, 1981; 77: 373-398.
4. Alexander J, Aaseth J. Organ distribution and cellular uptake of methyl mercury in the rat as influenced by the intra and extracellular glutathione concentration. Biochem Pharmacol 1982; 31: 685-690.
5. Al-Majed NB, Preston MR. Factors influence the total mercury and methylmercury in the hair of the fishermen of Kuwait. Environ Pollu 2000; 909:239-250.
6. Andersen HR and Andersen O. Pharmacol. Toxicol. 1993; 73: 192-201.
7. Ashour H, Abdel-Rahman M and Khodair A. The mechanism of methylmercury toxicity in isolated rat hepatocytes. Toxicol Lett 1993; 69: 87-96.
8. Ballatori N and Dutuzak. Identification and characterization of high and low-affinity transport systems for reduced glutathiane in liver cell canalicular membrane. J Biol Chem 1994; 269: 19731-19737.
9. Ballatori N, Clarkson TW. Biliary secretion of glutathione and of glutathione-metal complexes. Fundam Appl Toxicol 1985; 5(5): 816-31.
10. Ballatori N, Clarkson TW. Biliary transport of glutathione and methylmercury. Am J Physiol 1983; 244: G435-G441.
11. Ballatori N, Clarkson TW. Dependence of biliary secretion of inorganic mercury on the biliary transport of glutathione. Biochem Pharmacol 1984; 33: 1093-1098.


Copyright by Mahidol University

12. Ballatori N, Clarkson TW. Sulfobromophthalein inhibition of glutathione and methylmercury secretion into bile. Am J Physiol 1985: 248: G238-G245.
13. Ballatori N, Jacob R, and Boyer JL. Intrabiliary glutathione hydrolysis. A source of glutamate in bile. Biol Chem 1986; 261: 7860-7865.
14. Ballatori N, Jacob R, Barrett C and Boyer JL. Biliary catabolism of glutathione and differential reabsorption of its amino acid constituents. Am J Physiol 1988; 254: G1-G7.
15. Ballatori N, Lieberman MW and Wang Wei. N-Acetylcysteine as an antidote in methylmercury poisoning. Environ Health Perspect 1998; 106: 267-271.
16. Ballatori N, Gatmaitan Z, Truong AT. Impaired biliary excretion and whole body elimination of methylmercury in rats with congenital defect biliary glutathione excretion. Hepatology 1995a; 22: 1469-1473.
17. Ballatori N, Truong AT. Multiple canalicular transport mechanisms for glutathione S-conjugates: transport on both ATP-and voltage-dependent carriers. J Biol Chem 1995b; 269: 3594-3601.
18. Ballatori N, Truong AT. Mechanisms of hepatic methylmercury uptake. J Toxicol Environ 1995c; 46; 343-353.
19. Balllatori Nazzareno, Clarkson TW and Wang Wei. N-Acetylcysteine as an antidote in methylmercury poisoning. Drug Metab Rev 1991; 23, 83-132.
20. Balllatori Nazzareno, Lieberman, MW and Wang Wei. N-Acetylcysteine as an antidote in methylmercury poisoning. Environ Health Perspect 1998, 106, 267-271.
21. Berndt WO, Baggett J McC, Blacker A and Houser M. Renal glutathione and mercury uptake by kidney. Fund Appl Toxicol 1985; 5: 832-839.
22. Bizily SP, Rugh CL, Summers AO, Meagher RB. Phytoremediation of methylmercury pollution: mer B expression in Arabidopsis thaliana confers resistance to organomercurials. Proc Natl Acad Sci USA 1999; 96(12): 6808-6813.
23. Bragadin M, Marton D, Manente S, Grasso M, Toninello A. Methylmercury induces the opening of the permeability transition pone in rat liver mitochondria. J Inorg Biochem 2002; 89(1): 159-162.

Copyright by Mahidol University

24. Bragadin M, Scutari G, Manente S, Toninello A. The interaction of methylmercury with lysosomes from rat liver. Inorganica Chimica Acta 2002; 336; 163-167.
25. Change, Louis W. Magos, L. Suzuki, Tsuguyoshi. Toxicology of metal. CRC Press. Inc. 1996.
26. Clampitt RB. Young Scientists Award Fecture 1977: An investigation into the value of some clinical biochemical tests in the detection of minimal changes in liver morphology and function in the rats. Arch Toxicol Suppl 1987; 1: 1-13.
27. Denny MP, Hare MF, Atchison W. Toxicol Appl Pharmacol 1993; 122; 222.
28. Dierickx PJ. *In vitro* interaction of organic mercury compounds with soluble glutathione S-transferases from rat liver. Pharmacol Res Commun 1985; 17 (5): 489-500
29. Dutczak WJ, Ballatori N. gamma-Glutamyltransterase-dependent biliary-hepatic recycling of methyl mercury in the guinea pig. J Pharmacol Exp Ther 1992; 262: 619-623.
30. Dutczak WJ, Clarkson TW, Ballatori N. Biliary-hepatic recycling of xenobiotics: gallbladder absorption of methyl mercury. Gastroentero 1991; G873-G880.
31. Fang SC. Comparative study of uptake and tissue distribution of methylmercury in female rats by inhalation and oral routes of administration. Bull Environ Contam Toxicol 1980; 24(1): 65-72.
32. Friberg L, Kullman L, Lind B, Nylander M. Mercury in the central nervous system in relation to amalgam fillings. Lakartidningen 1986; 83(7): 519-22.
33. Gerhardsson L and Skerfving S. Toxicoloty of metals: Concepts on biological markers and biomonitoring for metal toxicity. Chang WL Ed, Magos L and Suzuki T Assoc Ed, CRC Press, Florida, 1996, 95-96.
34. Gregus Z and Klaassen DC. Disposition of metals in rats: A comparative study of fecal, urinary, and biliary excretion and tissue distribution of eighteen metals. Toxicol Appl Pharmacol 1986 85: 24-38.

Copyright by Mahidol University

35. Griffith OW and Meister A. Potent and specific inhibition of glutathione synthesis by buthionine sulfoximine (S-n-butyl homocysteine sulfoximine). J Biol Chem 1979; 254; 1205-1210.
36. Grosso A and DeSousa BC. Mercury blockage of apical water channels in toad skin (Bufo marinus). J Physiol 1993; 468; 741-752.
37. Haddow JE, Fish CA, Marshell PC, Lester R. Biliary excretion of mercury enhanced by spironolactone. Gastroentero 1972; 63: 1053-1058.
38. Hirom PC, Millburn P and Smith RL. Bile and urine as complementary pathways for the excretion of foreign organic compounds. Xenobiotica 1976; 6; 55-64.
39. Imesch E, Moosmayer M and Anner BM. Mercury weakens membrane anchoring of Na-K-ATPase. Am J Physiol 1992; 262; F837-F842.
40. Junghans RP. A review of the toxicity of methylmercury compounds with application to occupational exposures associated with laboratory uses. Environ Res 1983; 31:1-31.
41. Klaassen CD. Biliary excretion of mercury compounds. Toxicol Appl Pharmacol 1975; 33; 356-365.
42. Li L, Lee TK, Meier PJ, Ballatori N. Identification of glutathione as a driving force and leukotriene C4 as a substrate for Oatp1, the hepatic sinusoidal organic solute transporter. J Biol Chem 1998; 273: 16184-16191.
43. Li L, Meier PJ, Ballatori N. Oatp 2 mediates bidirectional organic solute transport: a role for intracellular glutathione. Molel Pharmacol 2000; 58: 335-340.
44. Lipfert T, Moskowitz P, Fthenakis V, Dehllips M, Viren J, Saroff L. Assessment of adults risk of paresthesia due to mercury from coal combustion. Water Air and Soil Pollu 1995; 80: 1139-1148.
45. Madhu C, Mitchell DY, Klaassen CD. Effect of P-450 inducers o biliary excretion of glutathione and its hydrolysis product. Correlation between hepatic gamma-glutamyltranspeptidase activity and the proportion of glutathione hydrolysis products in bile. Drug Metab Dispos 1993; 21(2): 342-350.

Copyright by Mahidol University

46. Magos L, Cikrt M, Snowden R. The dependence of biliary methylmercury secretion on liver GSH and ligandin. Biol Pharmacol 1985; 34(3); 301-305.
47. Magos L, Clarkson TW and Allen J. The interrelationship between non-protein bound thiols and the biliary excretion of methylmercury. Biochem. Pharmacol 1978; 27; 2203-2208.
48. Markovich D and James KM. Heavy metals mercury, cadmium, and chromium inhibit the activity of the mammalian liver and kidney sulfate transporter sat-1, Toxicol Appl Pharmacol 1999; 154(2): 181-187.
49. Meredith MJ and Reed DJ. Status of the mitochondria pool of glutathione in the isolated hepatocytes. J Biol Chem 1982; 257: 3747-3753.
50. Micttinen JK. Absorption and elimination of dietary mercury ($Hg^{2+}$) and methylmercury in man. Environ Res 1973; 32:233-246.
51. Miura K, Ikeda K, Naganuma A. Important role of glutathione in susepitibility of mammalian cells to methymercury. *In vitro* Toxicol 1994; 7: 59-63.
52. Naganuma A, Oda-Urano N, Tanaka T and Imura N. Possible role of hepatic glutathione in transport of methyl mercury into mouse kidney. Biochem Pharmacol 1988; 37: 291-296.
53. Nickle RA, Mercury. Top of the hit parade for eight years. Drug Chem Toxicol 1999; 22(1): 129-142.
54. Nielsen JB and Anderson O. Methymercuric chloride toxicokinetics in mice. I: effect of strain, sex and route of administration and dose. Pharmacol Toxicol 1991; 68(3): 201-207.
55. Nielsse JB. Toxicokinetics of mercuric chloride and methylmercuric chloride in mice. J Toxicol Environ Health 1992; 37(1): 85-122.
56. Nieminen AL, Gores GJ, Dawson TL, Herman B and Lemasters JJ. Toxic injury from mercuric chloride in rat hepatocytes. J Biol Chem 1990; 265: 2399-2408.
57. Norseth T and Brendeford M. Intracellular distribution of inorganic and organic mercury in rat liver after exposure to methylmercury salts. Biochem Pharmacol 1971; 20(6): 1101-1107.

Copyright by Mahidol University

58. Norseth T and Clarkson TW. Biotransformation of methylmercury salts in the rat studied by specific determination of inorganic mercury. Biochem Pharmacol 1970; 19: 2775-2783.

59. Ostlund K. Studies on the metabolism of methymercury and dimethylmercury in mice. Acta Pharmacol Toxicol (Copenh) 1969: 27(Suppl 1): 1-132.

60. Paulusma CC, van Geer MA, Evers R, Ottenhoff R, Borst PO, Elferink RP. Canalicular multispecific organic anion transporter/multidrug resistance protein 2 mediates low-affinity transport of reduced glutathione. Biochem J 1999; 338; 393-401.

61. Piyachaturawat P, Chai-ngam N, Komaratat P, Chuncharunee A, Sukamrarn A. Choleretic activity of phloracetophenone in rats: tructure-function studies using acetophenone analogues. Eur J Pharmacol 2000; 387: 221-227.

62. Piyachaturawat P, Ercharuporn S, Suksamrarn A. Estrogenic activity of Curcuma comosa extract in rats. Asia Pacific J Pharmacol 1995; 10:121-126.

63. Piyachaturawat P, Srivoraphan P, Chuncharunee A. Komaratat P, Sukamrarn A. Cholesterol lowering effects of a choleretic phloracetophenone in hypercholesterolemic hamsters. Eur J Pharmacol 2002b; 439: 141-147.

64. Piyachaturawat P, Suwanampai P, Komaratat P, Chuncharunee A, Sukamrarn A. Effect of phloraacetophenone on bile flow and biliary lipids in rats. Hepatology Research 1998;12: 198-206.

65. Piyachaturawat P, Tubtim C, Chuncharunee A, Komaratat P, Sukamrarn A. Evaluation of the acute and subacute toxicity of a choleretic phloracetophenone in experimental animals. Toxicol Lett 2002a; 129: 123-132.

66. Pop JA and Cattley RC. Handbook of toxicologic pathology: Hepatobiliary system. Haschek WM and Rousseaux CG, Eds. Harcourt Brace Jovanorich, New York, 1991: 293.

67. Rabenstein KL, Glutathione: Chemical, Biochemical and Medical aspects, Vol 3, Dolphin D, Auramovic O and Poulson R. Eds. John Wiley and Sons, New York 1989; 147.

Copyright by Mahidol University

68. Rebbeor JF, Gregory CC, Ballatori N. Inhibition of Mrp2- and Ycf1p-mediated transport by reducing agents: evidence for GSH transport on rat Mrp2. Biochim et Biophysic Acta 2002; 1559: 171-178.

69. Rebbeor JF, Gregory CC, Henson JH, Boyer JL, Ballatori N. ATP-dependent GSH and glutathione S-conjugate transport in skate liver: role of an Mrp functional homologue. Am J Physiol 2000; 279: G417-G425.

70. Refsvik T and Norseth T. Methyl mercuric compounds in rat bile. Acta Pharmacol Toxicol (Copenh) 1975; 36(1): 67-78.

71. Refsvik T. Excretion of methyl mercury in rat bile: the effect of diethylmaleate, cyclohexene oxide and acrylamide. Acta Pharmacol Toxicol (Copenh) 1987; 42(2); 135-141.

72. Rice DC, Gilbert SG. Effects of developmental exposure to methyl mercury on spatial and temporal visual function in monkeys. Toxicol Appl Pharmacol 1990; 102: 151-163.

73. Schutz A, Skarping G and Skerfving S. Trace element analysis in biological specimens, techniques and instrumentation in analytical chemistry. Herber, R.F.M. and Stoeppler,, M., Eds., Elsevier, Amsterdam, 1994;15:403

74. Sellinger M, Ballatori N and Boyer JL. Mechanism of mercurial inhibition of sodium-coupled alanine uptake in liver plasma membrane vescicles form Raja erinacea. Toxicol App Pharmacol 1991; 107; 369-376.

75. Shenker BJ, Gue TL, Shapiro JM. Induction of apoptosis in human T-cells by methylmercury: temporal relationship between mitochondrial dysfunction and loss of reductive reserve. Toxicol Appl Pharmacol 1999; 157;23.

76. Silbernagl S. Handbook of Physiology, Vol. II, Sect. 8, Windhager, E.E., Ed., Oxford University Press, New York, 1992: 1938.

77. Stacey NH and Klaassen CD. Comparison of the effects of metals on cellular injury and lipid peroxidation in isolated rat hepatocytes. J Toxicol Environ Health 1981; 7: 139-147.

78. Suksamrarn A, Eiamong S, Piyachaturawat P, Byrne LT. A phloracetophenone glucoside with choleretic activity form *Curcuma comosa*. Phytochemistry 1997; 45: 103.105.

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79. Tanaka T, Naganuma A and Imura N. Role of gamma-glutamyltranspeptidase in renal uptake and toxicity of inorganic mercury in mice. Toxicology 1990; 60: 187-198.

80. Tanaka-Kagawa T, Naganuma A and Imura N. Tubular secretion and reabsorption of mercury compounds in mouse kidney. J Pharmacol Exp Ther 1993;264; 776-782.

81. The merch index: An encyclopedia of chemicals, drugs, and biological. Budavari S, O' Neil MJ, Smith A, Heckelman PE, Eds, Eleventh Edition, Merck and Co., Inc. Rahway, N.J. U.S.A., 1989;3238.

82. Thompson TN and Klaassen CD. Disposition of metals after portal and systemic administration to rats. Toxicol Appl Pharmacol 1986; 68; 442-450.

83. Urano T, Iwasaki A, Himeno S, Naganuma A. Methylmercury absorption from rat intestine. Toxicol Lett 1990; 50: 159-164.

84. Walsh CT. The influence of age on the gastrointestinal absorption of mercuric chloride and methylmercury chloride in the rat. Environ Res 1982; 27(2): 412-420.

85. Wood JM and Wang HK. Microbial resistance to heavy metals. Environ Sci Technol 1983; 17: 82a-90a.

86. World Health Organization. Conference on intoxication due to alkylmercury-treated seed, Baghdad, Iraq. 9-13 September. Bull. World Health Organ 1974; 53(Suppl.): 105-112.

87. World Health Organization. *IPCS* Environmental health criteria 101:methyl mercury. Geneva, 1990; 293: 18-52.

88. Wu MF, Ison JR, Wecker JR, Lapham LW. Cutaneous and auditory function in rats following methyl mercury poisoning. Toxicol Appl Pharmacol 1985; 79: 377-388.

89. Zalups RK and Barfuss DW. Accumulation of inorganic mercury along the renal proximal tubule of the rabbit. Toxicol Appl Pharmacol 1990; 106: 245-253.

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90. Zalups RK and Cherian MG. Renal metallothionein metabolism after a reduction of renal mass. II. Effect of zinc pretreatment on the renal toxicity and intrarenal accumulation of inorganic mercury. Toxicol 1992; 71: 103-117.

91. Zalups RK and Lash LH. Depletion of glutathione in the kidney and the renal disposition of administered inorganic mercury. J Pharmacol Expes Therapeu 1997; 25(4);516-523.

92. Zalups RK, Barfuss DW and Lash LH. Disposition of inorganic mercury following biliary obstruction and chemically induced glutathione depletion: Dispositional changes one hour after the intravenous administration of mercuric chloride. Toxicol Appl Pharmacol 1999; 157; 135-144.

93. Zalupz RK and Lash LH. Toxicology of metals: Interactions between glutathione and mercury in the kidney, liver, and blood. Chang WL Ed, Magos L and Suzuki T Assoc Ed, CRC Press Florida, 1996; 145-160.

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# APPENDIX A

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# APPENDIX A

# DETERMINATION OF TRANSAMINASE (ALT)

## (Reitman and Frankel method)

**Principle** L-alanine + ∝-ketoglutarate $\xrightleftharpoons{ALT}$ glutamate + pyruvate

Hydrazone which forms a orange color in alkaline solution occurs from the reaction between oxaloacetate and 2,4-dinitrophenylhydrazine.

**Reagents** (Prod. 1501, 1601: Bio-Medical Laboratory)

1. ALT substrate (∝-ketoglutarate-alanine substrate)
   Consists of ∝-ketoglutarate and L-alanine at pH 7.5.
2. Phenylhydrazine (2,4-dinitrophenylhydrazine in HCl)
3. 0.4 N NaOH
4. Standard pyruvate solution: Store all reagents at 2 – 8 $^{o}$C

## Test Procedure

1. Add the solution into each tube as follows:

| Tube No. | Test (ml) |
|---|---|
| 1. ALT substrate<br>Incubate at 37 $^{o}$C min | 0.25 |
| 2. Serum<br>Mix, incubate at 60 $^{o}$C min exactly | 0.05 |
| 3. Phenylhydrazine<br>Mix, stand at room temperature for 20 min | 0.25 |
| 4. NaOH | 2.5 |

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2. Mix well and stand at room temperature for 5 min.
3. Read optical density at 505 nm by MILTON ROY SPECTRONIC GYNESYS 5 Spectrophotometer against distilled water blank.
4. The activity of ALT is determined from the calibration curve.

| **Tube NO.** | **Blank** | **1** | **2** | **3** | **4** |
|---|---|---|---|---|---|
| **(ALT; IU/ml)** | **(0)** | **(23)** | **(50)** | **(83)** | **(125)** |
| 1. Standard pyruvate (ml) | - | 0.025 | 0.050 | 0.075 | 0.100 |
| 2. ALT substrate (ml) | 0.250 | 0.225 | 0.200 | 0.175 | 0.150 |
| 3. Distilled water (ml) | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 |
| 4. Phenylhydrazine (ml) | 0.25 | 0.25 | 0.25 | 0.25 | 0.25 |
| Mix and allow to stand for 20 min at room temperature | | | | | |
| 5. 0.4 N NaOH (ml) | 2.5 | 2.5 | 2.5 | 2.5 | 2.5 |

5. Mix well and exactly 5 min later, read absorbance at 505 nm by Spectrophotometer, using water at the blank.
6. Plot a calibration curve of optical density corresponding units of ALT.

**Note:** If the value of ALT is higher than 125 IU/ml, dilute the sample by distilled water.

## Reference

Reitman S, Frankel S. A calorimetric method for the determination or serum glutamine oxaloacetic and glutamic pyruvic transaminase. Am J Clin Path 1957; 28: 56-63.

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# APPENDIX B

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# APPENDIX B

# DETERMINATION OF TRANSAMINASE (AST)

## (Reitman and Frankel method)

**Principle** L-aspatate + ∝-ketoglutarate $\overset{\text{AST}}{\rightleftharpoons}$ glutamate + oxaloacetate

Hydrazone which forms a orange color in alkaline solution occurs from the reaction between oxaloacetate and 2,4-dinitrophenylhydrazine.

**Reagents** (Prod. 1501, 1601: Bio-Medical Laboratory)

1. ALT substrate (∝-ketoglutarate-aspatate substrate)
   Consists of ∝-ketoglutarate and L- aspatate at pH 7.5.
2. Phenylhydrazine (2,4-dinitrophenylhydrazine in HCl)
3. 0.4 N NaOH
4. Standard pyruvate solution: Store all reagents at 2 – 8 $^{o}$C

## Test Procedure

1. Add the solution into each tube as follows:

| Tube No. | Test (ml) |
|---|---|
| 1. ALT substrate<br>Incubate at 37 $^{o}$C min | 0.25 |
| 2. Serum<br>Mix, incubate at 60 $^{o}$C min exactly | 0.05 |
| 3. Phenylhydrazine<br>Mix, stand at room temperature for 20 min | 0.25 |
| 4. NaOH | 2.5 |

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2. Mix well and stand at room temperature for 5 min.
3. Read optical density at 505 nm by MILTON ROY SPECTRONIC GYNESYS 5 Spectrophotometer against distilled water blank.
4. The activity of ALT is determined from the calibration curve.

## Calibration curve

| **Tube NO.** | **Blank** | **1** | **2** | **3** | **4** | **5** |
|---|---|---|---|---|---|---|
| **(ALT; IU/ml)** | **(0)** | **(20)** | **(55)** | **(95)** | **(148)** | **(216)** |
| 1. Standard pyruvate (ml) | - | 0.025 | 0.050 | 0.075 | 0.100 | 0.125 |
| 2. ALT substrate (ml) | 0.250 | 0.225 | 0.200 | 0.175 | 0.150 | 0.125 |
| 3. Distilled water (ml) | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 |
| 4. Phenylhydrazine (ml) | 0.25 | 0.25 | 0.25 | 0.25 | 0.25 | 0.25 |
| Mix and allow to stand for 20 min at room temperature | | | | | | |
| 5. 0.4 N NaOH (ml) | 2.5 | 2.5 | 2.5 | 2.5 | 2.5 | 2.5 |

5. Mix well and exactly 5 min later, read absorbance at 505 nm by Spectrophotometer, using water at the blank.
6. Plot a calibration curve of OD corresponding units of ALT.

**Note:** If the value of ALT is higher than 216 IU/ml, dilute the sample by distilled water.

## Reference

Reitman S, Frankel S. A calorimetric method for the determination or serum glutamine oxaloacetic and glutamic pyruvic transaminase. Am J Clin Path 1957; 28: 56-63.

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# APPENDIX C

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# APPENDIX C

# DETERMINATION OF ALKALINE PHOSPHATASE (ALP)

# (Phenolpthalein Monophosphate Method)

## Principle

Alkaline phosphatase hydrolyses phenophthalein monophosphate in alkaline solution to produce phenophtalein (a pink color solution).

## Reagents

1. Alkaline phosphate substrate: phenophthalein monophosphate dissolves in AMP buffer, which is the most specific for alkaline phosphatase. Stores at 2 – 8 $^{o}$C.
2. Alkaline phosphate buffer: the solution consists of 0.1 M phosphate pH 11.2 in order to get the sharpest pink. Store at room temperature.
3. Standard phenopthalein: store at room temperature and do not contact with light.

## Procedure

1. Add the solution into each tube as below:

| | Test | Blank |
|---|---|---|
| ALP substrate (drop) | 1.0 | 1.0 |
| Distilled water (ml) | 1.0 | 1.1 |
| Incubated at 37 $^{o}$C, 5 min | | |
| Serum or plasma (ml) | 0.1 | - |
| Incubate at 37 $^{o}$C, 20 min exactly | | |
| ALP buffer (ml) | 10 | 10 |

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2. Mix and read O.D. (optical density) at 550 nm by MILTON ROY SPECTORNIC GENESYS 5 spectrophotometer.

## Standard Curve

| Tube NO. | 1 | 2 | 3 | 4 |
|---|---|---|---|---|
| Units of ALP | (25) | (50) | (75) | (100) |
| ALP buffer (ml) | 0.025 | 0.050 | 0.075 | 0.100 |
| Standard (ml) | 0.225 | 0.200 | 0.175 | 0.150 |

Mix and read O.D. at 550 nm. Using buffer as a blank.

*If the value of AP is higher than 100 IU, dilute the sample by 0.9 % NaCl.

## References

1. Authur LB, Sharon JG, Charles MC and George EP. Clin Chem 1996; 12: 482.
2. Fubr J and Stary EA. Lab 1969; 15: 55.

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# APPENDIX D

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# APPENDIX D
# PROTEIN DETERMINATION WITH FOLIN PHENOL REAGENT
# (Lowry et al., 1951)

## Reagents

Reagent A 2 % $Na_2CO_3$ in 0.1 N NaOH: Dissolve 4.0 g NaOH in distilled water. While stirring, add 20 g $Na_2CO_3$ distilled water to a volume of 1,000 ml.

Reagent B 4 % $Na^+ – K^+$ tartrate: Dissolve 4 g of $Na^+ – K^+$ tartrate in 100 ml distilled water.

Reagent C 2 % $CuSO_4.5\ H_2O$: Dissolve 2 g of $CuSO_4.5\ H_2O$ in water make up 100 ml.

Reagent D Alkaline Copper solution: Freshly mix 25 ml of reagent A with 125 µl of 4 % tartrate solution and 125 µl of 2 % copper sulfate solution.

Reagent E Folin Phenol in $H_2O$: Folin Ciocalteu phenol reagent (Sigma Co.) was immediately diluted with $H_2O$ in a 1:1 ratio before use.

## Standard

Protein standard solution (BSA) was prepared by dissolving 0.05 g of bovine serum albumin from Sigma Co. (BSA) in 100 ml of triple distilled water.

## Procedures

1. The samples were diluted to appropriate concentrations and added with reagent A to obtain a total volume of 200 µl.
2. Then added 1 ml of reagent D.

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3. Added 100 µl of reagent E and mixed by vortex immediately. This rapid mixing is important since the reagent decomposes rapidly.

As schematic procedure:

| Solution | Standard | | | | | | Unknown tube |
|---|---|---|---|---|---|---|---|
| | 0 | 10 | 20 | 30 | 40 | 50 | (ml) |
| BSA solution | 0 | 0.02 | 0.04 | 0.06 | 0.08 | 0.1 | - |
| reagent A | 0.2 | 0.18 | 0.16 | 0.14 | 0.12 | 0.1 | 0.15 |
| sample | - | - | - | - | - | - | 0.05 |
| reagent E | ← | ← | 1 ml | | → | → | 1.0 |
| Folin | → | → | 0.1 ml | | ← | ← | 0.1 |

4. Incubated for at least 30 min at room temperature.
5. Read optical density at 770 nm by spectrophotometer.

## Calculation

The concentration of standard BSA is plotted versus absorbance at 770 nm and concentration of the unknowns are determined from the standard curve.

## Reference:

Lowry OH, Roseburge NJ, Farr AL and Randall RT. Protein measurement with Folin.

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# APPENDIX E

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# APPENDIX E

# DETERMINATION OF GLUTATHIONE *S*-TRANSFERASE

# (Habig and Jacobsky, 1981)

## Principle

The glutathione thiolate formation is relied on a direct change in the absorbance of the substrate 1,2-Dichloro-4-nitrobenzene (DCNB), when it is conjugated with glutathione (GSH). This reaction is catalyzed by glutathione-s-transferase, and the change in absorbance at OD 345 was linear function of enzyme concentration.

## Reagents

1. Solution A: 50 mM Glutathione (GSH) (Freshly prepared)

Dissolved 1.5366 g glutathione (reduced form) in distilled water and make up to 100 ml, and use immediately.

2. Solution B: 10 mM 1,2-Dichloro-4-nitrobenzene (DCNB) (Freshly prepared)

Dissolved 0.192 g DCNB in distilled ethanol and make up to 100 ml, and use immediately.

3. Solution C: 0.1 M Phosphate buffer, pH 6.5

Mix 0.1 M $Na_2HPO_4$ solution and 0.1 M $KH_2PO_4$ until pH 6.5.

## Enzymatic Procedure

1. Incubate medium are composed of:

| | | |
|---|---|---|
| Solution A | 0.1 | ml |
| Solution B | 0.1 | ml |
| Solution C | 0.78 | ml |

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2. Pipette each solution into the microcuvette and mix, then use this solution set zero absorbance at optical density 345 nm.

3. Add 0.02 ml of cytosolic fraction into the microcuvette and mix well.

4. The reaction mixture is incubated in the mixcrocuvette at room temperature for 5 min and measure O.D. at 1, 2, 3, 4 and 5 min.

5. The activity of glutathione *S*-transferase is calculated from the optical density at 345 nm and the molar extinction coefficient of 8.5 $mM^{-1}$, $cm^{-1}$. The activity is expressed as μmoles/mg protein/min.

6. The change in absorbance is a linear function of enzyme concentration and of the time for at least 3 min.

## Calculation

$$\text{Activity } (\mu\text{mole/mg protein/min}) = \frac{\text{O.D. change (1 min) x total volume}}{\text{Em x mg protein used}}$$

## Reference

Habig WH, Jacobsky WB. Assay for differentiation of glutathione *S*-transferase. Method Enzymol 1981; 77, 398-405.

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# APPENDIX F

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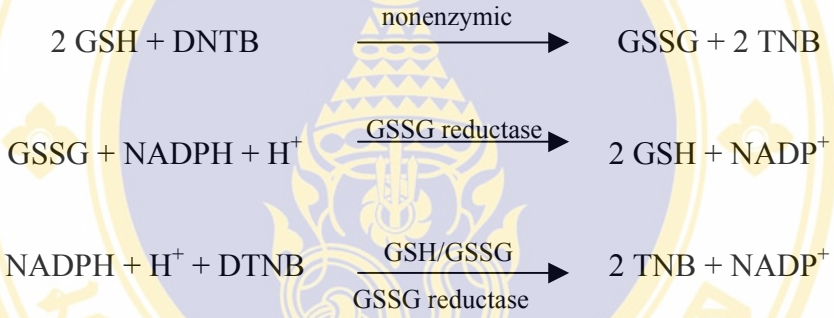
# APPENDIX F
# DETERMINATION OF LIVER AND BILIARY GLUTATHIONE
# (Theodorus and Helmut, 1981)

## Principle

The sum of the reduced and oxidized forms of glutathione can be determined using a kinetic assay in which catalytic amounts of GSH or GSSG and glutathione reductase bring about the continuous reduction of 5,5'-dithiobis (2-nitrobenzoic acid) (abbreviated DTNB) by NADPH according to the following reactions:




The reaction rate is proportional to the concentration of glutathione at values up to about 2 μM. The formation of 5-thio-2-nitorbenzonate (TNB) is followed spectrophotometrically at 412 nm (or at 405 nm with Hg-line photometers). The sensitivity of the assay may be enhanced by measuring NADPH fluorometrically.

## Reagents

1. 0.1 M potassium phosphate and 1 mM EDTA at pH 7.0; prepare with from stock solutions of 0.1 M $KH_2PO_4$ and 0.1 M $K_2HPO_4$, each containing 1 mM EDTA.
2. 5,5'-Dithiobis (2-nitrobenzoic acid) (DTNB), 1.5 mg/ml (Freshly prepared): Dissolve DTNB in 0.5 % $NaHCO_3$ and store in the dark (light sensitive).
3. NADPH, 4 mg/ml: Dissolve NADPH in 0.5 % $NaHCO_3$ and store at 4 $^oC$.

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4. Glutathione reductase, 6 units/ml: Dilute commerical enzyme in buffer each day.
5. Glutathione disulfide (GSSG), 10 μM, Prepare standard daily from stock solution (1 mM).

## Enzymatic Procedure

1. The cytosolic supernatant fraction prepared as described in subcellular fraction is assayed for GSH by DTNB method.
2. Add solution into each tube as follow:

| Solution | Standard Tube (ml) | | | | | Unknown tube |
|---|---|---|---|---|---|---|
| | 0.02 | 0.05 | 0.01 | 0.2 | 0.3 | |
| | (nmole) | | | | | |
| Phosphate buffer | 1.098 | 1.095 | 1.09 | 1.08 | 1.07 | 1.0 |
| Cytosolic fraction | - | - | - | - | - | 0.1 |
| GSH | 0.002 | 0.005 | 0.01 | 0.02 | 0.03 | - |
| DTNB | → | → | 0.02 | ← | ← | 0.02 |
| NADPH | → | → | 0.05 | ← | ← | 0.05 |
| GSH reductase | → | → | 0.02 | ← | ← | 0.02 |

3. Mix rapidly and then allow incubation at room temperature for 60 min.
4. Record the linear increase in absorbance at 412 nm.
5. The amount of glutathione level is expressed as nmole/g liver.

## Calibration Curve

1. Prepare series of tubes containing GSH in the following concentrations: 0.02 nmole, 0.05 nmole, 0.1 nmole, 0.2 nmole, and 0.3 nmole.
2. Perform the procedure as in step 2.
3. Determine the optical density at 412 nm.

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4. Plot a calibration curve of optical density versus nmole of GSH.

## Reference

Theodorus PM and Helmut S. Assay of Glutathione, Glutathione Disulfide, and Glutathione Mixed Disulfides in Biological Samples. Methods of Enzymol 1981; 77: 373-82.

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# APPENDIX G

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# APPENDIX G
# DETERMINATION OF LIVER AND BILIARY MERCURY

## Instrument

A Perkin-Elmer Model 372 atomic absorption spectrometer, equipped with a deuterium background corrector, a HGA-500 graphite furnace, and a mercury hollow-cathode lamp operated at 6 mA (spectral band-pass = 0.2) and at a resonance wavelength of 253.6 nm. The optimized experimental furnace conditions were as follows: drying, 110 $^{o}$C for 15 s, ramp time 5 s; ashing, 150 $^{o}$C for 10 s, ramp time 5 s; atomization, 1100 $^{o}$C for 5 s, ramp time 1 s; cleaning, 2000 $^{o}$C for 5 s, ramp time 1 s.

## Reagents

1. Distilled, deionized water (DDW)
2. 0.04 M methanolic tetramethyltin solution (freshly prepare by dissolving 544 μl of tetramethyltin in 10 ml of methanol)
3. 0.01 M solution of sodium thiosulfate ($Na_2S_2O_3.5H_2O$) (prepare by dissolving 0.2482 g in 100 ml of DDW)

## Standards

Stock standard methylmercury (II) chloride 0.1251 g was dissolved in 100 ml of ethanol and store at –10 $^{o}$C. Standard mercury working solutions 10 μg of Hg/ml of methylmercury compound was prepared by appropriate dilutions of the stock solution with ethanol and was store at 5 $^{o}$C

## Sample preparation of homogenates

1. A 5 g portion of liver were finely chopped.
2. Add 5 ml of deionized distilled water and homogenized.
3. Bile sample were promptly used.

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## Procedure

1. 0.5 g of the homogenate and 50 µl of bile sample in a 10-ml glass vial, 5 ml of 1% NaCl (w/v) in 1 N hydrochloric acid and 1 ml of benzene were added.
2. The vial was stoppered, shaken in a horizontal shaker for 30 min, and then centrifuged for 5 min at 6000 *g*.
3. The benzene layer was transferred into a 10-ml vial. The operation was repeated three times with additional 1-ml aliquots of benzene.
4. The mixture was then shaken for 5 min and centrifuged as before.
5. The organic layers were combined and stored in one glass vial for determination of total organic mercury.
6. The aqueous layer was separated and added 0.25 ml of methanolic 0.4 M tetramethyltin.
7. The vial was stoppered and placed in a beaker of boiling water for 5 min.
8. Thereafter the vial was shaken in a horizontal shaker for 5 min and cooled.
9. 1 ml of benzene was added to the vial and it was shaken again for 10 min.
10. The mixture was centrifuged for 5 min at 6000*g*, and the organic layer was transferred into a glass vial.
11. The aqueous layer was extracted three times with new 1 ml aliquots of benzene, shaken for 5 min, and centrifuged as before.
12. The benzene layers were collected in a glass vial for determination of inorganic mercury.
13. 1-ml of 0.01 M $Na_2S_2O_3$ solution was added to the benzene layers of organic and inorganic mercury and vortexed for 30 s.
14. The benzene layer was discarded and the aqueous layer stored at 5 $^{o}$C until the time of GFAAS measurement

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## References

Filippellli, M., 1987. Determination of trace amounts of organic and inorganic mercury in biological materials by graphite furnace atomic absorption spectrometry and organic mercury speciation by gas chromatography. *Anal. Chem* 1987; 59: 116-118.



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