STUDY OF ACUTE AND SUBACUTE TOXICITY OF 2,4,6-TRIHYDROXYACETOPHENONE AND ITS EFFECT ON HEPATIC EXCRETORY FUNCTION

CHATSUDA TUBTIM

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CHATSUDA TUBTIM : STUDY OF ACUTE AND SUBACUTE TOXICITY OF 2,4,6-TRIHYDROXYACETOPHENONE AND ITS EFFECT ON HEPATIC EXCRETORY FUNCTION. THESIS ADVISORS: PRAYAD KOMARATAT, Ph.D., PAWINEE PIYACHATURAWAT, Ph.D., CHAIVAT TOSKULKAO, D.V.M., Ph.D. 167 p. ISBN 974-664-788-1.

Toxicity of phloracetophenone (2,4,6-trihydroxyacetophenone, THA), the aglycone part of phloracetophenone glucoside from *Curcuma comosa* (family Zingiberaceae), which has previously been reported to stimulate bile secretion and lower plasma lipids, was evaluated. Acute toxicity of a single dose of THA was studied in mice (weanlings and adults), hamsters, and rats of both sexes, by oral and intraperitoneal administration. LD_{50} values were dependent on species, sex of animals and routes of administration, but was not age dependent. THA was practically classified as a non-toxic compound when it was given to rats by an oral route (i.g.). The LD_{50} value in adult male and female rats was higher than 6 g/kg BW. Hamsters and mice appeared to be the more sensitive to THA than rats. LD_{50} value via intraperitoneal (i.p.) route in adult males were 338 and 365 mg/kg BW, respectively. At lethal doses, the animals became excited, with ataxia and hypersensitivity to noise, followed by convulsions and death with respiratory paralysis. Subacute toxicity was studied in adult male mice, by giving a daily sublethal dose of THA (37-300 mg/kg BW, i.g.) for 30 consecutive days. A high dose of THA (150 mg/kg BW) induced marked changes of hepatocytes at periportal area including vacuolization of hepatocyte and nuclear degeneration. In severe cases, the affected area was expanded from the periportal area into the central area. Plasma concentrations of liver enzymes, alanine and aspartate aminotransferases and other biochemical parameters including bilirubin, BUN and hepatic triglyceride content were slightly increased. The content of liver glutathione varied but remained within the normal range. It is suggested that THA, particularly at high dose, partially induced subacute toxicities in mice and probably, those toxic effects can be attributed to THA itself, not its metabolite.

Furthermore, hepatic excretory function, after THA treatment, was evaluated by using the sulfobromophthalein (BSP) clearance method. Plasma disappearance and biliary excretion of an intravenous BSP injection were assessed in bile duct-cannulated controls and 17α-ethinylestradiol (EE)-induced cholestatic rats. A single intraduodenal administration of THA at a dose of 50 or 100 mg/kg BW immediately increased bile flow rate, which peaked at 15 min. The acute increase of bile flow rate by THA enhanced hepatic clearance of BSP and decreased plasma alkaline phosphatase in EE-cholestatic rats to normal levels, but did not affect bilirubin. Prolonged treatment with THA for 5 days increased basal bile flow rate and BSP clearance in EE-cholestatic rats. These results suggest that THA at a biologically active dose had low toxicity and might be safe for further development as a therapeutic agent for a short treatment in some hepatic disorders.

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ฉัตรสุดา ทับทิม: การศึกษาถึงพิษเฉียบพลันและกึ่งเฉียบพลันของสาร 2,4,6-ไตรไฮดรอกซีอะเซโตฟีโนนและผลต่อหน้าที่การขับทิ้งของตับ (STUDY OF ACUTE AND SUBACUTE TOXICITY OF 2,4,6-TRIHYDROXYACETOPHENONE AND ITS EFFECT ON HEPATIC EXCRETORY FUNCTION) คณะกรรมการควบคุมวิทยานิพนธ์: ประหยัด โกมารทัต, ปร.ด., ภาวิณี ปิยะจตุรวัฒน์, ปร.ด., ชัยวัฒน์ ต่อสกุลแก้ว, ปร.ด. , 167 หน้า ISBN 974-664-788-1

การวิจัยนี้เป็นการศึกษาถึงความเป็นพิษของสารฟลออะเซโตฟีโนนหรือ 2,4,6-ไตรไฮดรอกซีอะเซโตฟีโนน (2,4,6-trihydroxyacetophenone, THA) ซึ่งเป็นส่วนที่ไม่ใช่น้ำตาลของฟลออะเซโตฟีโนนกลูโคไซด์ที่เกิดขึ้นตามธรรมชาติในว่านชักมดลูก (พืชในตระกูล Zingiberaceae) เนื่องจากมีรายงานถึงประสิทธิภาพของสารนี้ว่ามีฤทธิ์กระตุ้นการหลั่งน้ำดี และส่งผลให้ระดับโคเลสเตอรอลในเลือดลดต่ำลง ดังนั้นสาร THA จึงเป็นสารที่น่าสนใจและอาจจะพัฒนาไปเป็นยาลดไขมันในเลือดได้ต่อไป แต่เนื่องจากยังไม่มีการศึกษาถึงความเป็นพิษของสารนี้มาก่อน การวิจัยครั้งนี้จึงมีวัตถุประสงค์เพื่อจะศึกษาถึงความเป็นพิษของสารฟลออะเซโตฟีโนนทั้งในแง่ของพิษเฉียบพลัน พิษกึ่งเฉียบพลัน และผลต่อหน้าที่การขับทิ้งของตับ การศึกษาพิษเฉียบพลันของ THA ทำโดยการป้อน THA ให้ทางปากหรือฉีดเข้าทางช่องท้องในหนูถีบจักร หนูพุกขาว และหนูแฮมเตอร์ ทั้งในเพศผู้และเพศเมีย จากการทดลองพบว่า ค่า LD_{50} (ขนาดของสารที่ทำให้สัตว์ทดลองตายไปครึ่งหนึ่ง) ซึ่งเป็นค่าที่ชี้บ่งขนาดความเป็นพิษของสารเคมี ขึ้นอยู่กับสายพันธุ์ เพศของสัตว์ทดลอง และวิธีการให้สาร THA แต่ค่า LD_{50} ของ THA นี้จะไม่ขึ้นอยู่กับอายุของสัตว์ทดลองที่ใช้ จากผลการทดลองสามารถจัดสาร THA ให้อยู่ในกลุ่มของสารที่ไม่มีพิษต่อหนูพุกขาว โดยเมื่อป้อนให้ทางปาก ค่า LD_{50} มากกว่า 6 กรัมต่อกิโลกรัมน้ำหนักตัวของหนู หนูพุกขาวนับว่ามีความไวต่อการเกิดพิษจาก THA ต่ำสุด ในขณะที่หนูแฮมเตอร์และหนูถีบจักรมีความไวต่อการเกิดพิษจาก THA ได้มากกว่าหนูพุกขาว หลังจากที่สัตว์ทดลองได้รับ THA ในขนาดที่ทำให้ตายโดยการฉีดเข้าทางหน้าท้อง สัตว์ทดลองจะแสดงอาการตื่นตัว มีอาการเดินเซ ชัก และจะตายจากภาวะการหายใจล้มเหลว ได้ทำการศึกษาพิษกึ่งเฉียบพลันของ THA ในหนูถีบจักรตัวผู้ โดยป้อน THA (37-300 mg/kg BW)ให้ทางปากติดต่อกันเป็นเวลา 30 วัน พบว่า THA ขนาดสูง (150 mg/kg BW) สามารถเหนี่ยวนำให้เซลล์ตับเกิดการเปลี่ยนแปลงอย่างชัดเจน โดยในเซลล์ตับจะมี vacuole เกิดขึ้นจำนวนมาก โดยเฉพาะอย่างยิ่งเซลล์ตับบริเวณรอบนอก (periportal area) และการเปลี่ยนแปลงของเซลล์ตับอาจจะขยายลุกลามไปสู่บริเวณส่วนกลาง (central area) ได้นอกจากนี้ยังพบว่าค่าต่างๆทางชีวเคมีของเลือด เช่น เอนไซม์จากตับและค่าอื่นๆมีการเปลี่ยนแปลงโดยเพิ่มขึ้นเล็กน้อย

นอกเหนือจากนี้ยังได้ศึกษาถึงผลของ THA ต่อหน้าที่การขับทิ้งของตับ โดยใช้วิธี BSP clearance โดยฉีด BSP เข้าทางเส้นเลือดและเปรียบเทียบผลที่ได้ระหว่างหนูพุกขาวกลุ่มปกติและกลุ่มที่เหนี่ยวนำให้เกิดการคั่งของน้ำดีด้วยสารเอทธินิลเอสตราไดออล (EE) เข้าทางใต้ผิวหนังเป็นเวลา 5 วันติดต่อกัน พบว่า THA มีฤทธิ์กระตุ้นการหลั่งของน้ำดีได้ทันทีหลังจากฉีดและออกฤทธิ์สูงสุดที่เวลา 15 นาทีหลังจากฉีด การหลั่งน้ำดีที่เพิ่มขึ้นจะเร่งให้มีการขับทิ้งของ BSP ได้เร็วขึ้นด้วย นอกจากนี้ THA ยังสามารถช่วยลดระดับของ alkaline phosphatase เอนไซม์ในหนูกลุ่มที่เหนี่ยวนำให้เกิดการคั่งของน้ำดี THA ไม่มีผลต่อการขับ bilirubin อย่างไรก็ตามจากการทดลองสามารถสรุปได้ว่า THA ในขนาดที่มีฤทธิ์ทางชีวภาพและใช้ในระยะเวลาสั้นจะมีความปลอดภัย ซึ่งอาจจะนำไปสู่การพัฒนาเป็นยารักษาโรคตับบางโรคที่ใช้ระยะเวลาสั้นได้

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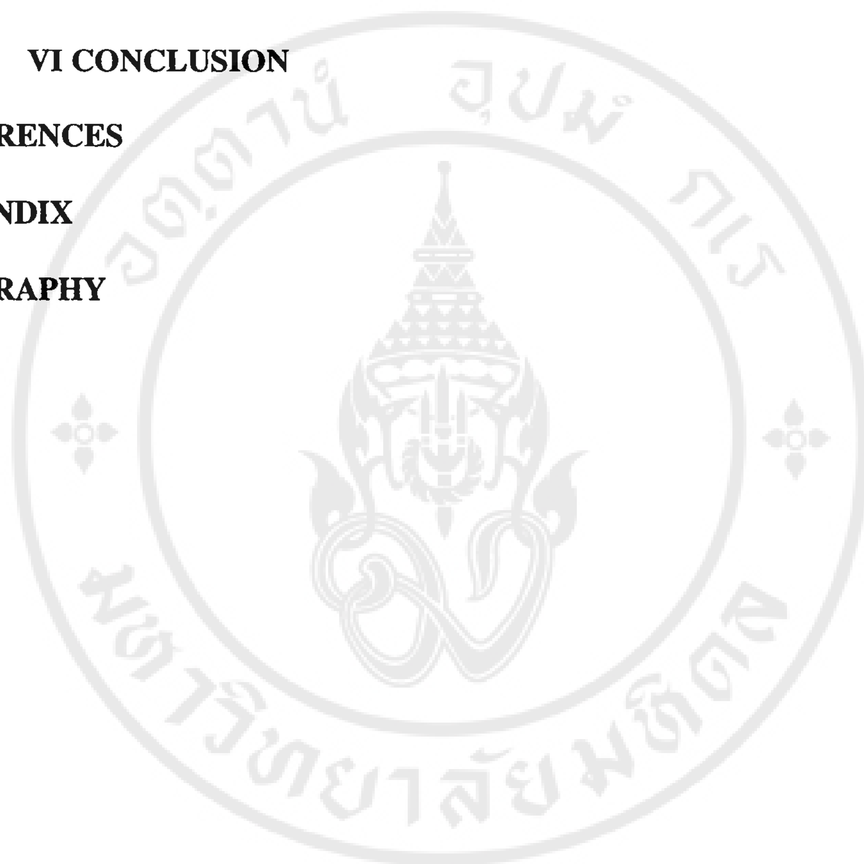

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LIST OF ABBREVIATIONS

A	afferent arteriole
ALT	alanine aminotransferase
ANOVA	analysis of variance
AP	alkaline phosphatase
AST	aspartate aminotransferase
ATP	adenosine 5′-triphosphate
ATPase	adenosine triphosphate
bp	boiling point
BD	bile duct
BFR	bile flow rate
BSDF	bile salt-dependent flow
BSIF	bile salt-independent flow
BSP	sulfobromophthalein
BW	body weight
BUN	blood urea nitrogen
C	Bowman's capsule
C. comosa	*Curcuma comosa*
dl	decilitre
°C	degree Celsius
CV	central vein

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LIST OF ABBREVIATIONS (CONT.)

DHA	2,6-dihydroxyacetophenone
DMSO	dimethylsulfoxide
D	distal convoluted tubule
DW	distilled water
$E_2$3G	estradiol-3-glucuronide
$E_2$17G	estradiol-17β (β-D-glucuronide)
et al.,	with others
etc.	et cetera
EE	ethynylestradiol
EtOH	ethanol
G	glomerulus
GSH	glutathione
g	gram
g/kg BW/d	gram per kilogram body weight per day
H	hepatocyte
h	hour
IU/ml	international unit per millilitre
i.d.	intraduodenum
i.g.	intragastric
i.p.	intraperitoneal
i.v.	intravenous

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LIST OF ABBREVIATIONS (CONT.)

K^+	ion potassium
kg	kilogram
l	litre
LD_{50}	medial lethal dose, lethal dose for 50% of population
µl	microlitre
µM	micromolar, micromole
M	molar
mg	milligram
mg%	milligram percent
min	minute
ml	millilitre
mM	millimolar
mmole	millimole
MW	molecular weight
Na^+	ion sodium
NaCl	sodium chloride
NAD	nicotinamide adenine dinucleotide
NADH	nicotinamide adenine dinucleotide, reduced form
nm	nanometer
P	proximal convoluted tubule
PV	portal vein

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LIST OF ABBREVIATIONS (CONT.)

pH	log concentration of H^+
rpm	round per minute
O.D.	optical density
%	percent
S	sinusoid
SEM	standard error of mean
TG	triglyceride
THA	2,4,6-trihydroxyacetophenone
UDCA	ursodeoxycholic acid
vol	volume
$\overline{X}$	mean

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CHAPTER I

INTRODUCTION

During the past 50 years, hundreds of new drugs have been introduced into market for treatment as well as management of many diseases, which have been considered as non-treatable and life-threatening diseases. These remarkable changes in developing of new drugs have been accelerated by rapid growing of new scientific technology for investigation, testing for biological and chemical activities. One approach to obtain new drugs is by screening for biological activity of natural products according to its believe or its previously discovered chemical entities, besides chemical modification of a known molecule and rational drug design based on biological mechanism (1). Currently, an interesting in screening new biological active compound from indigenous plants is increasing. However, regardless of the approaches in searching and the sources of a new drug, basic and clinical studies of its pharmacology, toxicity and safety must be conducted to ensure of further development and promotion as a candidate new drug. Today, natural products including their derivatives and analogs represent over 50% of all drugs in clinical use, with higher plant-derived natural products representing 25% of the total (2). Plant-derived natural products have long been and will continue to be extremely important as sources of medicinal agents and models for the design, synthesis, and semisynthesis of novel substances for treating humankind's diseases (2).

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Recently, our laboratory have screened biological activities of an indigenous plant, *Curcuma comosa* Roxb. family Zingiberaceae which has traditionally been used for treatment of postpartum uterine inflammation, and as a stomachic and cholagogue in Thailand. A hexane extract of the plant was found to exhibit estrogenic-like activity (3, 4) whereas an ethyl acetate extract exhibited choleretic (5) and hypolipidemic activities (5, 6). Active ingredients of the plant have been examined and one of the choleretic principles of *C. comosa* has been identified as phloracetophenone glucoside (6). The aglycone part of the compound, phloracetophenone, or 2,4,6-trihydroxyacetophenone, THA, (7) has further been demonstrated to posses the choleretic activity. This compound, THA, effectively stimulates bile flow, enhances bile acid secretion and decreases lithogenicity of bile. A recent study on the relationship between chemical structure and choleretic activity of compounds by using different phloracetophenone analogs and acetophenone derivatives has indicated that THA is the most effective in stimulating bile flow and bile acids excretion (7). As the biliary excretion of bile acid is the major route for removal of cholesterol from the body, the choleretic activity of phloracetophenone has been demonstrated to accompany with its ability in lowering plasma cholesterol (5, 6, 7). From this view, it is of interest that THA, aglycone of phloracetophenone glucoside, which is a biologically active naturally occurring compound, may have potential for development as a therapeutic agent for dissolving gallstone and lowering of plasma lipid, particularly, plasma cholesterol. From our earlier study, the doses of THA in exerting biological activities have been established (7), however, there have been no information available regarding the toxicity of those levels of the compound.

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It is important to evaluate whether those levels were toxic to animals. Therefore, the present study was undertaken to evaluate toxicity of a new potential therapeutic agent, 2,4,6-trihydroxyacetophenone, to assess whether THA has therapeutic effects in preventing the hepatocellular cholestasis.

The objectives of the present study are as follows:

1. To investigate the acute toxicity of 2,4,6-trihydroxyacetophenone, THA, in various animal species via various routes of administration and evaluate the possible causes of death in animals.

2. To investigate the subacute toxicity of 2,4,6-trihydroxyacetophenone, THA, after daily administration for 30 consecutive days.

3. To evaluate the effect of 2,4,6-trihydroxyacetophenone, THA, treatment on the excretory function of liver by using BSP dye-clearance method. In addition, the therapeutic potential of the compound in preventing the hepatocellular cholestasis effect of estrogen in ethinylestradiol (EE)-induced cholestatic rats was also evaluated.

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CHAPTER II

LITERATURE REVIEWS

I. *Curcuma Comosa (C. Comosa)* (Roxb.)

Curcuma comosa is a plant in the *Curcuma* species of the Zingiberaceae family. Members of this species such as *C. longa* Linn., *C. aromatica* Salisb., *C. zedoaria* Roxb., and *C. xanthorrhiza* Roxb. have been widely used as an indigenous medicine in many Asian countries including China, India, Indonesia and Thailand. Medicinally, *Curcuma* species are reputed to have value as antihepatotoxic (8), anti-inflammatory (9), emmenagogue (10), aromatic, stomachic, and bile-expelling agents (4). As far as modern drugs are concerned the pharmacological studies of these plants have been extensively conducted.

C. comosa Roxb. is commonly known as Wan chak mod look, which has been widely used in folk medicine for treatment of inflammation in Thailand. From the previous study in our laboratory on the biological activities of the crude extracts of *C. comosa*, the uterotrophic, choleretic, and hypolipidemic activities have been reported (4). The hexane extract was the most effective in inducing uterotrophic and estrogenic activities, whereas the butanol extract as well as the ethyl acetate extract exhibited the stimulating effect on bile secretion (3, 4, 5). In addition, the ethyl acetate extract was further demonstrated to decrease plasma triglyceride and cholesterol in animals (6, 7). By using biological activity-guide, one of choleretic principles of *C. comosa* has been identified as phloracetophenone glucoside (6).

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Evaluation on the choleretic activity of the glucoside indicated that choleretic activity derived from the aglycone part of the compound, phloracetophenone (2,4,6-trihydroxyacetophenone, THA) (7). The choleretic activity of THA essentially showed a dose-dependent pattern. Both secretory volume and bile acid output were increased with the dose of THA, which subsequently led to lower plasma cholesterol (7).

II. Acetophenone and Acetophenone Analogs (2,6-Dihydroxy-acetophenone and 2,4,6-Trihydroxyacetophenone)

ACETOPHENONE

1. Physical properties

The chemical formula for acetophenone is C_8H_8O, and its molecular weight is 120.15 (11, 12). Acetophenone occurs as a colorless liquid that is slightly soluble in water (11, 12, 13).

2. Health Hazard Information

Acute Effects

Acute (short-term) exposure of humans to acetophenone vapor may produce skin irritation and transient corneal injury. A decrease in light sensitivity in exposed humans has been reported (11, 13). Acute oral exposure has been observed to cause hypnotic or sedative effects, hematological effects, and a weakened pulse in humans (13, 14). Congestion of the lungs, kidneys, and liver are also reported in rats acutely exposed to high levels of acetophenone via inhalation (13). Tests involving acute exposure of animals, such as the LD_{50} test in rats, mice, and rabbits, have

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demonstrated that acetophenone has moderate acute toxicity from oral and dermal exposure (15).

Chronic Effects (Non-cancer)

No information is available on the chronic (long-term) effects of acetophenone in humans. Degeneration of olfactory bulbs cells has been reported in rats chronically exposed via inhalation. In another study, chronic inhalation exposure of rats produce hematological effects and at high doses, congestion of cardiac vessels and pronounced dystrophy of the liver are observed (11, 13). And in these two independent studies, no effects were observed in rats chronically exposed to acetophenone in their diet (11, 13, 16).

Reproductive Development Effects

No information is available on the reproductive or developmental effects of acetophenone in humans. In one study of pregnant rats exposed dermally, no effects on reproduction or development are noted (11, 13).

Cancer Risk

No information is available on the carcinogenic effects of acetophenone in humans or animals.

ACETOPHENONE ANALOGS

Several acetophenone analogs are found to occur naturally in both plants and animals (17-20). In 1988, Calixto et al (17) reported the action of 2-hydroxy 4,6-dimethoxy acetophenone (2-OH-4,6-OCH_3-acetophenone) isolated from *Sebastiania schottiana*. The compound has a direct and non-selective inhibition of contraction of smooth and cardiac muscles. In 1989, Jurenka et al (18) isolated three acetogenins

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which are 2,6-dihydroxyacetophenone (2,6-$(OH)_2$) ; 2,4-dihydroxyacetophenone (2,4-$(OH)_2$) and (2,4,6-$(OH)_3$-acetophenone) from exocrine secretion of *Stephanitis spp.* These compounds have been demonstrated to be *in vitro* inhibitor of mammalian and insect-derived prostaglandin H synthases. The naturally occurring acetophenones, 4-OH and 4-OH-3-OCH_3-acetophenone, were also isolated from a shrub *Salsola tuberculatiformis* and the active substances from this shrub have been reported to interfere with adrenal 11β-hydroxylase, the terminal enzyme in glucocorticoid biosynthesis. Its interaction with the cytochrome $P450_{11\beta}$-dependent hydroxylase, as well as the inhibition of the conversion of deoxycorticosterone to corticosterone (19) has been noted. Recently, two acetophenone glucosides, 4-OH and 3,4-$(OH)_2$-acetophenone, were isolated from the root of *Cynanchum taiwanianum* (20). Species of plants in genus *Cynanchum* (Aselepiadaceae) have been used in folk medicine of China for the treatment of chronic tracheitis and as antifebrile, diuretic, antitussive, expectorant anodyne, and tonic drugs (20). In addition of having those biological activities, our laboratory have recently demonstrated choleretic and hypolipidemic activities of the hydroxylated acetophenone analogs including 2,6-dihydroxyacetophenone, DHA, and 2,4,6-trihydroxyacetophneone, THA. The chemical structures of the two compounds are shown in Figure A. Acetophenone consists of a benzene ring nucleus and a functional ketone group. Both DHA and THA have a similar core structure, but they contain differences in positions and number of OH-groups.

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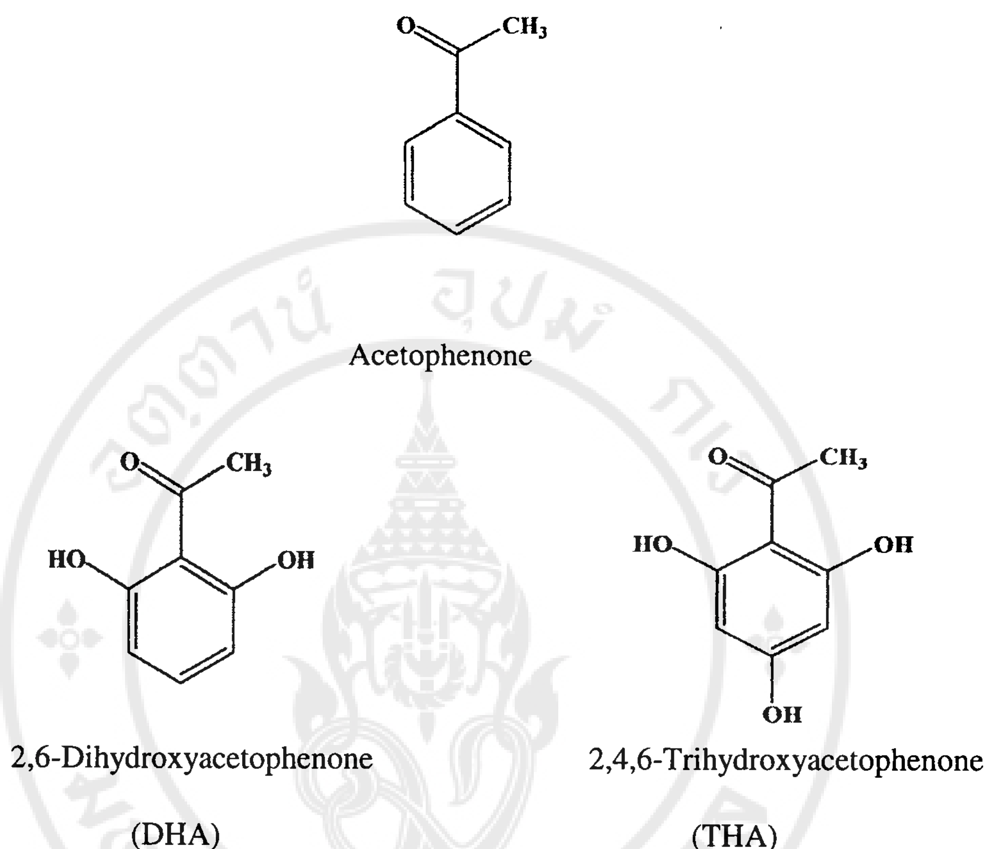

Figure A. Chemical structure of acetophenone and two hydroxylated acetophenone.

There has been no reported on the metabolic fate of DHA and THA. Generally the metabolism of xenobiotics by the liver occur via a wide variety of pathways, which is not only due to the great diversity in the structure of xenobiotics but also to the large number of different types of drug modifying reactions. In addition, the list of reactions is still growing as increasing numbers of drugs are studied. Xenobiotics are metabolized by enzymes those exist for these specialized purposes, and by enzymes catalyzing normal reactions of cellular metabolism (21). The processes that most xenobiotics undergo in the body can be divided into the two categories of Phase I reactions and Phase II reactions (21). A Phase I reaction

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introduces reactive, polar functional groups onto lipophilic ("fat-seeking") toxicant molecules. In their unmodified forms, such toxicant molecules tend to pass through lipid-containing cell membranes and may be bound to, and transported through the body by, lipoproteins. Because of the functional group attached, the product of a Phase I reaction is usually more water-soluble than the parent xenobiotics species, and more importantly, possesses a "chemical handle" to which a substrate material in the body may become attached so that the toxicant can be eliminated from the body. The binding of such a substrate is a Phase II reaction, and it produces a conjugation product that is amenable to excretion from the body (21).

2,6-Dihydroxyacetophenone (DHA)

Earlier study on the evaluation in pyridine nucleotide kinetics for both aminopyrine and ethylmorphine-*N*-demethylases in the presence of 2,6-dihydroxyacetophenone, (DHA) has demonstrated that DHA inhibits certain microsomal mixed function oxidase reactions. The nature of the inhibition in the presence of varying NADPH concentrations was shown to be slope-linear, intercept-linear non-competitive (22). DHA was shown to combine preferentially with the ferric cytochrome P450-substrate complex in the presence of aminopyrine, but had a greater affinity for the ferrous cytochrome P450-substrate complex when ethylmorphine was present. DHA would be expected to inhibit the reduction of the cytochrome P450-substrate complex that is generally accepted as the rate-limiting step of mixed function oxidation reactions. In the previous results, a marked inhibition of NADPH cytochrome P450 reductase activity in the presence of DHA was observed (22).

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Phloracetophenone (2,4,6-Trihydroxyacetophenone, THA) the aglycone part of phloracetophenone glucoside, a naturally occurring compound from *C. comosa* family Zingiberaceae, has previously been reported to stimulate bile flow, increase bile acid excretion and lower lithogenicity of the secreting bile. As the excretion of bile acids is a major route for removal of cholesterol from the body, the increased bile acid excretion was observed to accompany with an ability to lower plasma cholesterol and triglyceride (5, 6, 7). This information is interesting in terms of potential development of this compound as a new lipid lowering drug.

III. Drug Discovery and Development

Currently, many new drugs are introduced into the market. They are originated from many different sources. In fact, the natural products are served as the source of all drugs (2). Most new drugs have been reported to be discovered by testing (screening) large numbers of natural products as well as synthetic compounds for a variety of biologic activities. Once a compound is identified to have an effect, numerous chemical modifications are made and tested until one is found to be suitable as a candidate for further evaluation.

Most new drug candidates are identified through one of three approaches: chemical modification of a known molecule, screening of natural products or previously discovered chemical entities for biological activity, or rational drug designs based on an understanding of biological mechanisms (1). Candidate drugs that survive the initial screening and profiling procedures must be carefully evaluated for potential risk before clinical testing is begun. Depending on the proposed use of the drug, preclinical toxicity testing includes most or all of the procedures shown in Table

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A. While no chemical can be certified as completely "safe" (free of risk), since every chemical is toxic at some level of dosage, it is possible to estimate the risk associated with exposure to the chemical under specified conditions if appropriate tests are carried out (1).

The major kinds of information needed from the preclinical toxicity study are (1) acute toxicity-effects of large single doses up to the lethal level; (2) subacute and chronic toxicity-effects of multiple doses, which are especially important if the drug is intended for chronic use in humans; (3) effects on reproductive functions, including teratogenicity; (4) carcinogenicity; (5) mutagenicity; and (6) investigative toxicology (1). In addition to the studies shown in Table A, several quantitative estimates are desirable. These include the "no-effect" dose, the maximum dose at which the specified toxic effect is not seen; the minimum lethal dose, the smallest dose that is observed to kill any animals; and, if necessary, the median lethal dose (LD_{50}), the dose that kills approximately 50% of animals. Historically the latter value (LD_{50}) was calculated with a high degree of precision and was used to compare toxicity of compounds relative to their therapeutic doses. It is now realized that a high degree of precision may not be necessary to compare toxicity (23). Therefore, the median lethal dose is now an approximate value estimated from the smallest number of animals possible. These doses are used to calculate the initial dose to be tried in humans, usually taken as 1/100-1/10 of the no-effect dose.

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Table A. Safety tests (1).

	Approach	Comment
Acute toxicity	Acute dose that is lethal in approximately 50% of animals. Determine maximum tolerated dose. Usually 2 species, 2 routes, single dose.	Compare with therapeutic dose.
Subacute toxicity	Three doses, 2 species. Up to 6 months may be necessary prior to clinical trial. The longer the duration of expected clinical use. The longer the subacute test.	Clinical chemistry, physiologic signs, autopsy studies, hematology, histology. Identify target organs of toxicity.
Chronic toxicity	One to 2 years. Required when drug is intended to be used in humans for prolonged periods. Usually run concurrently with clinical trial.	Goals of subacute and chronic tests are to show which organs are susceptible to drug toxicity. Tests as noted above for subacute.
Effect on reproductive performance	Effects on animal mating behavior, reproduction, parturition, progeny, birth defects.	Examines fertility, teratology, perinatal and postnatal effects, lactation.
Carcinogenicity potential	Two years, 2 species. Required when drug is intended to be used in humans for prolonged periods.	Hematology, histology, autopsy studies.
Mutagenicity potential	Effects on genetic stability of bacteria (Ames test) or mammalian cells in culture; dominant lethal test in mice.	Increasing interest in this problem.
Investigative toxicology	Determine sequence and mechanisms of toxic action. Develop new methods for assessing toxicity.	May allow rational and earlier design of safer drugs.

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A promising drug that seems safe enough in preliminary testing is then carried through the following series of steps:

Animal studies

a. Acute and subacute toxicity

b. Therapeutic index

c. Pharmacokinetics and metabolic pathways

ACUTE TOXICITY

Toxicity is defined as any harmful effect of a chemical or a drug on a target organism. The Organization for Economic Cooperation and Development (OECD) (24), defined acute toxicity as " the adverse effects occurring within a short time of administration of a single dose of a substance or multiple doses given within 24 hours. Acute toxicity studies are conducted in animals to ascertain the total adverse biological effects caused during a finite period of time following administration of single, frequently large doses of an agent (or several doses repeated over a short interval of time). The effects observed in the animals are usually directly related to the amount of the poisonous substance administered orally, dermally, or via inhalation. The effects are often spectacular since high doses of the agent are administered and death, sudden or otherwise, is frequently the most important endpoint measured (25).

The objectives of acute toxicity testing are to define the intrinsic toxicity of the chemical, predict hazard to non target species or toxicity to target species, determine the most susceptible species, identify target organs. It provides information for risk assessment of acute exposure to the chemical provide information for the

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design and selection of dose levels for prolonged studies. The most important and practical of all, provide valuable information for clinicians to predict, diagnose, and preserve treatment for acute overexposure (poisoning) to chemicals. Acute toxicity studies are designed to express the potency of the toxicant in terms of the median lethal dose (LD_{50}), a value representing the estimated dose causing death of 50% the universal population of the species exposed under the defined condition of the test (24).

Many acute toxicity studies have been conducted solely for the purpose of determining the LD_{50} of a chemical. However, acute toxicity is not equivalent to the LD_{50}, and that the LD_{50} is not an absolute biologic constant to be equated. The LD_{50} is only one of many indices used in defining acute toxicity. A well-designed acute toxicity study should include consideration of the dose-response relationship of both lethal and nonlethal parameters. Sometimes, biochemical measurements in an acute test can aid including the mechanism of toxic actions. Histopathology of organs may also be helpful in finding the cause of death and identifying the target organ (24).

SUBACUTE TOXICITY

Subacute or subchronic studies are designed to examine the adverse effects resulting from repeated exposure over a portion of the average life span of an experimental animal. Properly designed subacute or subchronic studies give valuable information on the cumulative toxicity of a substance on target organs and on physiologic and metabolic tolerance of a compound at low-dose (relative to acute toxicity testing doses) prolonged exposure. By monitoring many different parameters, including histopathologic evaluations, a wide variety of adverse effects can be

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detected. The results from such studies can provide information that will aid in selecting doses for chronic, reproductive, and carcinogenicity studies. Subacute or subchronic studies are also valuable in establishing doses at which no toxicological effects are evident, a critical factor in risk assessment. It has been suggested that subacute or subchronic data may be sufficient to predict the hazard of long-term, low-dose exposure of a particular compound (26). Even though acute toxicity studies data indicate that a compound is practically nontoxic, prolonged exposure studies cannot be automatically precluded from the process of safety evaluation. Acutely nontoxic compounds may be toxic after prolonged exposure, even at low doses, due to accumulation, changes in enzymes levels, and disruption of physiologic and biochemical homeostasis. Therefore, subacute testing is considered essential for all new chemicals before their specific hazard can be assessed and legitimate safety assessment made.

The exposure period in subacute or subchronic studies may vary, depending on the objective of the study, the species selected for the study, and the route of administration employed. A generalization which is often made is that subacute or subchronic studies do not exceed 10% of the animals' lifespan (subacute or subchronic usually conducted over a 21- to 90 days period) (27). The most common routes of administration employed in subacute or subchronic toxicity studies are oral, dermal, and inhalation. Subacute or subchronic toxicity studies should always attempt to expose the animals by the same route that man is most likely to be exposed.

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The aims of the subacute studies are as follows:

1. To ascertain the biological effects of reported administration of the test agent on potential target organs of the body at dosages that do not elicit acute toxicity.

2. To establish a dose-effect relationship between biochemical, physiological, and morphological effects over the dosage range and the duration of administration (or exposure) of the agent.

3. To ascertain the maximum dosage level that produces no discernible ill effects following repeated exposure.

4. To explore the possible mechanisms by which the toxicant elicits its effects.

THERAPEUTIC INDEX

The LD_{50} of a drug is not nearly so important as the difference between the dose required for toxicity and the therapeutic index. Nevertheless, in animal experiments the therapeutic index, defined as the ratio of the LD_{50} to the median effective dose (ED_{50}), has been widely used as an initial approximation of drug toxicity.

$$\text{Therapeutic index} = \frac{LD_{50}}{ED_{50}}$$

Clinically, of course, the concept of effectiveness in relation to nonlethal toxicity (margin of safety) is more important than any specific ratio. A physician is most interested in knowing how far the usual therapeutic dose can be exceeded before adverse effects are encountered (28).

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IV. Fate of Xenobiotics

When laboratory animals are exposed to xenobiotics, toxic responses occur relatively frequently in the liver compared with other organs. The reasons for this frequency are probably numerous but certainly include the high metabolic capability and the portal blood supply of the liver. The liver is a major site of metabolism resulting in the activation of exogenous chemicals or xenobiotics toxic metabolites. Organs that lack such metabolic capabilities are less susceptible to toxins requiring metabolic activation. The liver is also the first organ to be exposed to ingested toxins due to its portal blood supply. Therefore, toxins may be at least partially removed from the circulation during the first pass, providing protection to other organs while increasing the likelihood of hepatic injury.

A toxicant can cause injury only after it is absorbed by the organism. In addition, the nature and intensity of the effects of a chemical on an organism depend on its concentration in the target organs. The concentration depends not only on the administered dose but also on other factors, including absorption, distribution, and excretion (29). The pattern of entry and fate of chemicals in the body showed in (Figure B).

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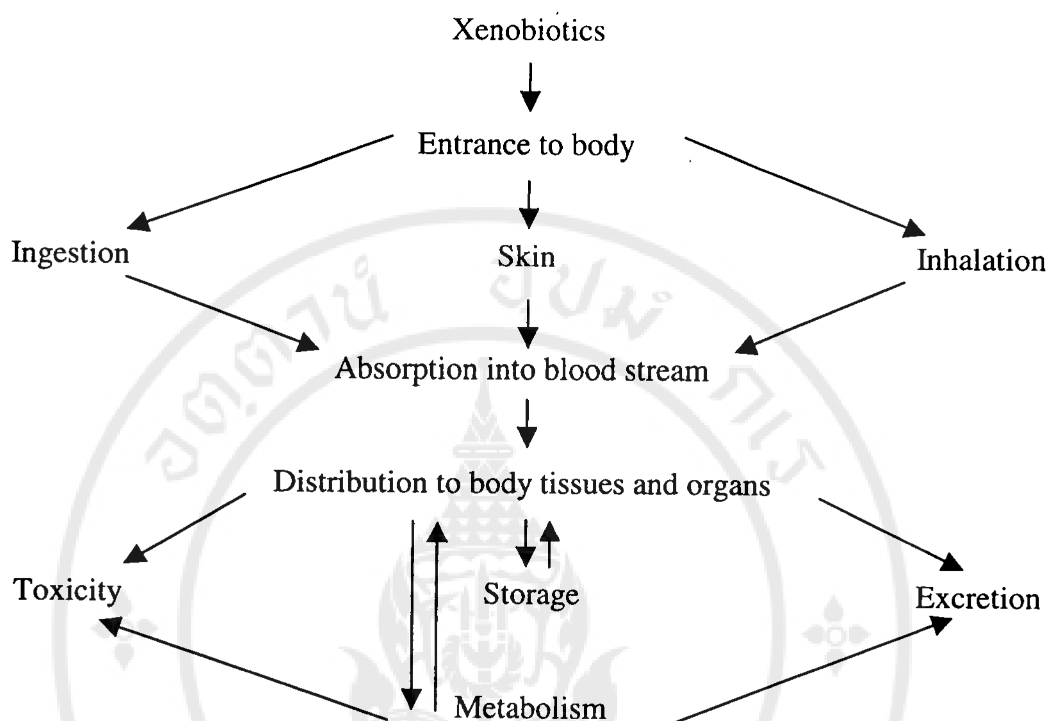

Figure B. Entry and fate of chemicals in the body (21).

1. Absorption of xenobiotics

Absorption is the process by which a compound enters the body from the environment. Before a chemical can exert systemic toxicity, it must first enter the body and be absorbed into the blood, distribute through the organism and reach its site of action at a high enough concentration for a sufficient period of time to interact with the target tissue. The main routes by which xenobiotics are absorbed are the gastrointestinal (GI) tract, lungs, and skin. However, in toxicological studies; such special routes as intraperitoneal, intramascular, and subcutaneous injections are also used (30).

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2. Distribution of xenobiotics

After the chemical enters the blood, it is distributed rapidly throughout the body. The rate of distribution to each organ is related to the blood flow through the organ, the ease with which the chemical crosses the local capillary wall and the cell membrane, and the affinity of components of the organ for the chemical (30).

3. Metabolism of xenobiotics

The role of the liver in activation and detoxification of xenobiotics is of central importance. Metabolite reactions of xenobiotics in liver are generally grouped into two categories: Phase I and Phase II. Most xenobiotics acquire the polar groups in Phase I reactions, which consist of oxidation and reductions, which usually result in a more polar metabolite. Phase II reactions consist of conjugations of molecules containing unstable polar groups with endogenous groups including with glucuronide, sulfate, glutathione, acetyl, amino acid, or methyl groups. Some already contain polar groups and can enter the Phase II reactions without Phase I metabolic transformation. Detoxification may be considered the optimal goal of these reactions in which potentially toxic xenobiotics is rendered in active and/or excreted. However, sometimes the same enzymatic machinery for detoxification may result in a metabolite that is more toxic than the parent xenobiotics, thereby resulting in activation (21).

4. Excretion of xenobiotics

After absorption and distribution in the organism, xenobiotics are excreted, which can be rapidly or slowly. A generally accepted indicator of the rate of elimination of a toxicant is its "half-life" (t ½), which is the time required for 50% of

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it to be removal from the bloodstream. The toxicants are excreted as the parent chemicals, as their metabolites, and/or as conjugated of them. Toxicants are eliminated from the body by several routes. The kidney is perhaps the most important organ for excretion of xenobiotics, as more chemicals are eliminated from the body by this than by any other route. Many xenobiotics, though, have to be biotransformed first to more water-soluble products before they can be excreted into urine. The second important route of elimination of numerous xenobiotics is via feces, and the third, primarily for gases, is via the lungs. Biliary excretion of xenobiotics and/or their metabolites is most often the major source of fecal excretion, but a number of other sources can be significant for some compounds (30).

5. Biliary excretion

The liver is an important organ for the excretion of xenobiotics, especially for compounds with high polarity (anionic and cationic), conjugates greater than 300. In general, once these compounds are in the bile, they are not reabsorbed into the blood but are excreted via the feces. This route of elimination is perhaps the most important contributing source to fecal excretion of xenobiotics and even more important for the excretion of their metabolites. The liver is in a very advantageous position for removing toxic agents from blood after absorption from the gastrointestinal, because blood form the gastrointestinal passes through the liver before reaching the general circulation. Thus, liver extracts compounds from blood and thereby prevent their distribution to other parts of the body. Furthermore, the liver is the main site for biotransformations of toxicants, and the metabolites may be excreted directly into bile. Xenobiotics and/or their metabolites entering the intestine with bile may be excreted

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with feces, or when the physicochemical properties are favorable for reabsoption, an enterohepatic circulation may ensure (30).

V. Hepatic Excretory Function

Chemicals entering the systemic circulation may be excreted by the liver unchanged or after modification within the hepatocyte. Compounds that undergo biliary excretion have been divided into three classes based on the bile/plasma concentration ratios obtained during their excretion (31, 32). Examples of class A substances include sodium, potassium, and chloride ions as well as glucose; these compounds have a bile/plasma ratio of about 1.0. Class B substances, for example, bile salts, bilirubin, BSP, and many xenobiotics, achieve a bile/plasma ratio of more than 1.0, usually between 10 and 1000. Among class C substances, which have a bile/ plasma ratio of less than 1.0, are macromolecules such as inulin, phospholipids, mucoproteins, and albumin. In terms of detecting and quantifying hepatic damage, the compounds of class B are of particular interest. Their biliary excretion is mediated by several multicomponent transport systems (33). For example, most organic acids (e.g., bilirubin and BSP) are believed to be excreted by a common transport system in the liver.

The liver has the ability to remove lipophilic molecules from plasma and to excrete them into bile. Some of these molecules can be excreted unchanged or after conversion in the liver to more polar forms. The liver has selective uptake and carrier-mediated excretory mechanisms for several major classes of molecules. These are (1) organic anions, including exogenous dyes such as sulfobromophthalein and indocyanine green, and endogenous molecules such as bilirubin and bile salts; (2)

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organic cations; and (3) neutral organic compounds. The organic anion dyes seem to share a series of transport steps in hepatocytes that are separated from the transport mechanism for bile acids, two major organic anion transport systems exist. A number of clinical tests of this liver function have been developed.

VI. Sulfobromophthalein (BSP) in liver excretory function test

The most common class B chemical used in the detection of liver injury is BSP. BSP is anionic phthalein dye for the investigation of hepatic function was first suggested by Roundtree et al., in 1913 (34). Subsequently, Rosenthal and White (35) showed that intravenously administered sulfobromophthalein (BSP) was removed from the blood primarily by the liver, and that the rate of clearance of this dye from plasma was useful in evaluating liver function. After intravenous injection, BSP is present in the cardiovascular compartment. Its disappearance from the circulatory system depends on its uptake by the liver. The removal of BSP from the plasma is dependent on the simultaneous operation of a number of hepatic processes, for example, active transport across the plasma membrane into a storage compartment, metabolic transformation, and adenosine triphosphate (ATP)-dependent transport across the canalicular membrane (36, 37, 38). The use of BSP to assess liver function is based on the observation that dye removal from blood is delayed by hepatic dysfunction (39). For many years the standard test involved determination of the percentage of dye remaining in plasma (percent retention) at a stated time after intravenous injection have been established. This bolus-injection and single-blood sample test are useful in detecting abnormalities in BSP removal, but it provides little insight into mechanisms that after the disappearance of dye from the plasma.

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Attempts to gain more precise information about liver function led to the use of increased frequencies of sampling with determination of BSP fractional disappearance (40), plasma half-life, and clearance (41).

VII. Cholestasis

Hepatic injury, which causes an arrested bile flow, is termed cholestatic or intrahepatic cholestasis (42). Cholestasis is a term initially used by pathologists to describe the microscopic finding of bile stasis in the liver. For the clinician it refers to a spectrum of diseases in which there is interference with intrahepatic or extrahepatic bile flow (43). Cholestasis leads to two major consequences. First, there is defective excretion of substances that are normally eliminated in the bile. These substances then accumulate in the serum and tissues. Second, there is defective secretion of bile salts needed for fat absorption (30). Toxic agents can interfere with bile flow by selective injury or blockade of the mechanism for hepatic uptake, processing, or excretion of the components of bile. Interference with the excretory phase may result from functional or structural lesions (39). In order to understand the mechanisms by which xenobiotics can cause cholestasis, it is important to understand a basis of normal bile flow.

Bile flow depends on (1) transport of constituents of bile from sinusoidal blood into the hepatocytes; (2) metabolic alteration of some of the constituents by the hepatocytes; (3) transport into the canalicular lumen; (4) passage along the bile ductules and ducts, with varying degrees of modification of the bile during this flow (44).

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Bile flow involves two main components: canalicular bile and ductular bile. However, most alteration has been move on the formation of canalicular bile. Bile acids are the main osmotic active compound in bile. Transport of bile acids from sinusoidal blood into the hepatocyte, it appears to be facilitated by binding proteins (ligandin and Z protein) which have differential affinity for various anions (45, 46). Metabolic transformation of the molecule within the hepatocyte is required for many but not all of the anions to be excreted in bile (47). Bilirubin, for example, must be conjugated with glucuronate or sulfate and BSP conjugated with glutathione in order to be excreted out into bile, while indocyanine green is excreted without conjugation or other biotransformations. Transport of bile acids across the canalicular membrane is active and, therefore, energy dependent (48, 49). It has two main components: bile-salt-dependent flow (BSDF), the flow dependent on the osmotic pull of the bile salts after their active transport into the canalicular lumen; and bile-salt-independent flow (BSIF), the flow apparently dependent on the active transport of Na^+ into the canalicular under the influence of the Na^+-K^+- ATPase (50, 51). There is evidence of interaction between BSDF and BSIF (52).

Flow along the bile ductules and ducts may be affected by addition or resorption of water and electrolytes by or between ductular epithelium (53). Destructive or inflammatory lesions of portions of the ductal system may lead or contribute to cholestasis (53). Most views of cholestasis focus on the excretory aspect of bile formation, ie, on defects at or distal to the canaliculus. Interference with hepatic uptake from the blood of substances to be excreted defects in metabolic transformation within the hepatocyte, selective interference with excretion into the

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canaliculus, and injury to the ductal system beyond the canaliculus, all may be variety as forms of intrahepatic cholestasis (50). A number of agents lead to cholestasis by selective injury to one or more of the components of the excretory process (53, 62).

Possible causes of cholestasis:

1. Interference with bile flow can be a result from: (1) lack of ATP (51); (2) injury to the membrane (52, 53); (3) formation of insoluble precipitates in or around the canaliculus (52, 53); (4) impairment of BSDF by blockade of synthesis or of transcanaliculur transport of bile acids (47); (5) impairment of BSIF by inhibition of $Na^+ - K^+$- ATPase (47, 54); (6) interference with formation of normal micelles in the bile (53); (7) abnormal reabsorbtion or secretion of water and electrolytes along the course of the ductules ; and (8) injury to the bile ducts (53).

2. Injury to the canalicular membrane has become an interesting subject since the demonstration that the cholestatic effect of C-17 alkylated anabolic steroids is accompanied by EM evidence of structural abnormalities of the canaliculus (55). Subsequent recognition that swollen, stunted, or lost microvilli could be seen in extrahepatic obstruction as well as in intrahepatic cholestasis led to the assumption that the canalicular abnormalities were the result rather than cause of cholestasis (53). It is now clear that canalicular injury plays a key role in the cholestasis produced by some agents and contributes to the cholestatic effects of others (52, 53, 56).

3. Precipitation of the cholestatic agent in or around the canaliculus may contribute to cholestasis (52, 53). This phenomenon has been seen with LCA and has been suggested as a factor in chlorpromazine-induced cholestasis (53).

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4. Abnormal micelle formation has been suggested as one possible mechanism for the cholestatic effects of the alkylated steroids, perhaps as a result of the competitive exclusion of bile salts from the micelles by the structurally similar steroids (57). The possibility that LCA, by a similar mechanism, can inhibit formation of the normal micelle also has been suggested (53).

5. Depletion of the ATP pool of the cytosol has been demonstrated by Slater and his associates (51, 58) to explain the cholestatic effects of some agents. An example of the cholestatic effects of ATP depletion was provided by ethionine-induced hepatic injury, but the cholestatic effects of ethionine are of limited relevance to the mainly hepatocellular toxicity of that agent.

6. Interference with BSDF is also assumed to be an important factor in the jaundice in the hepatic injury as well as of pure cholestasis. There is convincing evidence that bile flow is dependent on bile salt excretion (49). Furthermore, bile salts not only provide osmotic "pull" for the BSDF but also affect on the "sodium pump", and consequently on BSIF (59). Nevertheless, among agents that cause cholestasis, there are some which may inhibit bile salt synthesis (60) as well as excretion (47).

7. Interference with bile acid transport (47) into the canalicular or with synthesis (60) are found to contribute to cholestatic effects of anabolic and contraceptive steroids is a hypothesis suggested by the structural similarity between the bile acids and these steroids (61). A related hypothesis (57) which implicates displacement, by the steroid of bile acids in the micelles of the bile and consequent

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alteration of bile viscosity as the cholestasis-producing event has been cited previously.

8. Suppression of BSIF by the steroid-induced inhibition of Na^{+}-K^{+}-ATPase and damage to the canalicular membrane also have been implicated in the cholestatic effect of these agents (47, 53). The evidence that steroids induce cholestasis by interference with both BSDF and BSIF is consistent with other observations that suggest an intimate interplay of BSIF with BSDF in the formation of bile (47, 53).

9. Alterations in ductal secretion or reabsorption of fluids and electrolytes may be implicated in cholestasis (47). The evidence that bile duct activity can affect the composition and flow of bile has been summarized by Wheeler (47). Early observations on chlorpromazine (CPZ) jaundice in humans (62) and on the effects of CPZ in experimental animals (63) suggested that dehydration could increase the viscosity of bile and thereby contribute to cholestasis. If this suggestion is valid, the effect of hydration might be mediated by the role of ductal function in bile flow.

10. Lesions of bile ducts can lead to cholestasis. The prototypic experimental model is α–naphthylisothiocyanate-induced cholestasis (64).

Many endogenous compounds and xenobiotics decrease bile flow and referred to as cholestatic agents. Cholestasis induced by anabolic and contraceptive steroids has been observed in humans and laboratory animals (65). In man, the functional impairment, which has been most often demonstrated, is the plasma retention of BSP. Estradiol and estiol were shown to provoke BSP retention in almost all patients receiving the steroids for periods ranging from 4 to 41 days (66, 67). In some patients, BSP retention was evident within 24 hr of initiating steroid treatment,

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however in all cases, the hepatic disposal of BSP reverted to normal within 1 to 4 weeks after ceasing the estrogen therapy. Oral contraceptives have been reported to cause similar effects on BSP clearance (68, 69). Development of animal models to examine the mechanism by which estrogens decreased hepatic excretory function soon followed. Gallagher et al., (70) characterized the structure-activity relationship of the estrogen-induced retention of BSP in rats and showed that both synthetic and natural estrogens were effective, although modification of the phenolic A-ring decreased or abolished activity. The nonsteroidal estrogen diethylstilbestrol was active, whereas C19, C21, and C24 steroids were inactive. Numerous subsequent studies have shown that treatment of rats with ethinylestradiol at dosages of 0.5-1 mg/day for 5-10 days decreased basal bile flow primarily by decreasing the so-called bile acid-independent component of bile flow (71-76). Effects on basal bile salt secretion are varied (73, 77); however, the maximum capacity to secrete taurocholate is consistently inhibited (73, 74). No effects on serum bile acids or bilirubin have been observed (78).

Several hypotheses have been proposed to explain the effects of estrogens seen in the rat model.

1. Increased Permeability of the Biliary Tree

Forker (73) observed an increased clearance of sucrose and mannitol, inert fluid-phase markers that diffuse passively into bile in rats pretreated with estrone (2.5 mg/day for 7 days) relative to controls, indicative of an increased permeability of the biliary tree. The decreased biliary excretion of BSP and its transport maximum have, therefore, been interpreted to reflect not a decreased active transport of BSP into bile, but an increased back-diffusion of BSP from bile to plasma, most likely via the

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paracellular route. The paper by Jaeschke et al. (79) reported that the characterizes the time-course of changes in bile flow, taurocholate secretion, and sucrose and inulin clearance in perfused livers from rats treated with estradiol-17β–valerate, a depot estrogen, at a dosage of 1 mg/kg/wk. These authors were able to dissociate the decreased bile flow and increased biliary taurocholate concentration that was apparent at 7 days from the increased sucrose permeability and clearance that did not appear until week 3 of treatment. The difference between these data and those of Forker (73) is likely due to the much lower dose of estrogen used in the study of Jaeschke et al. (79). These data clearly indicate that an increased permeability of the biliary tree is not the cause of the decrease in bile flow. Whether the decrease in flow is causally related to the subsequent increased permeability or not remains to be determined.

2. Decreased sodium-potassium-stimulated adenosine triphosphatase activity

Transport of bile salts across the hepatocyte requires their uptake across the basolateral membrane via a sodium-dependent, carrier-mediated cotransport process (80-83), translocation across the cell and excretion across the canalicular membrane via a sodium-independent carrier-mediated process (84, 85). Sodium-potassium-stimulated adenosine triphosphatase activity in hepatic plasma membranes is decreased after treatment with estrogens (86, 87) such that a decreased driving force could result in a decreased bile acid influx. Several lines of evidence against this as a major factor in estrogen cholestasis. (a) The excretion of bile acids across the canalicular membrane is independent of sodium (84, 85) and the bile salt uptake capacity of the hepatocyte exceeds its secretory capacity by 6- to 10-fold (88). (b) The

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transport of bilirubin and BSP is independent of sodium, and yet is decreased by the estrogens (89). (c) Phenobarbital treatment of rats reverses the ethinylestradiol-induced depression of bile flow and sodium-potassium-stimulated adenosine triphosphatase activity without altering the transport maximum for taurocholate (90, 91).

3. Decreased Membrane Fluidity

Pharmacological doses of ethinylestradiol have long been known to decrease plasma cholesterol levels in rats (92). This has subsequently been attributed to a marked increase in the number of hepatic low density lipoprotein receptors (93) and a concomitant increased hepatic clearance of plasma lipoproteins (93, 94). The increased cholesterol uptake by the liver as a result of ethinylestradiol treatment reflects in an increased cholesterol ester content of liver homogenate and plasma membranes (86, 94). Fluidity of these membranes is decreased as measured by electron spin resonance probes (86) and fluorescence polarization (95). Simon and coworkers have proposed that the decreased membrane fluidity is responsible for the decrease in bile flow, sodium-potassium-stimulated adenosine triphosphatase activity, and the transport maximum for taurocholate and BSP (91). Boelsterli et al. (96) demonstrated that rats treated with ethinylestradiol for 3 days, and found that sodium-potassium-stimulated adenosine triphosphatase activity and membrane fluidity were not decreased even though bile flow was markedly decreased, indicating that decreased membrane fluidity is not the cause of the decrease in bile flow.

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4. Hepatic Estrogen Receptors

Hepatic estrogen receptors have been well-characterized (97, 98) and are thought to mediate a variety of estrogen effects, such as the enhanced synthesis of rennin substrate (99), sex-steroid binding globulin (100), thyroxin-binding globulin (101), and ceruloplasmin (102). The role of estrogen receptors in mediating the changes associated with estrogens-induced cholestasis, however, has not been systematically investigated. Although admittedly speculative, it is reasonable to suggest that estrogen receptors mediated an increased synthesis of hepatic plasma membrane low-density lipoprotein receptors and alanine carriers and concomitant decreased synthesis of organic anion and bile acid carriers.

In summary, the precise mechanisms by which estrogens induce cholestasis are not known. Nevertheless, the models developed for studying the problem have provided valuable insights into the factors regulating the transport of organic anions and bile acids and the generation of bile flow. Application of the methodologies have recently been developed for study of transport processes in canalicular versus basolateral membrane (103), the identification of proteins in these membranes thought to be responsible for the transport of organic anions (104, 105), and the techniques of molecular biology should soon provide new insight into the mechanisms of, and appropriate treatment modalities for estrogen–induced cholestasis.

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CHAPTER III

MATERIALS AND METHODS

I. Animals

Animals used in this study were Swiss albino mice of both sexes including adult (25-30 g) and weanling (12-15 g), and adult Wistar rats (150-250 g) of both sexes. They were supplied by the National Animal Center, Salaya, Mahidol University, Nakornprathom, Thailand. Adult Syrian golden hamsters (100-120 g) of both sexes were supplied by the Animal Production Center, Faculty of Science, Mahidol University, Bangkok, Thailand. All animals were kept in the room where the temperature was approximately 25±2 °C, the relative humidity was at approximately 65% and they were maintained in 12-h light-dark cycle. Rats were kept in hanging cages while mice and hamsters were in the cages with bedding. They were fed with regular rat chow (Pokphand Animals Fed Co., Ltd.; Bangkok, Thailand) and tap water *ad libitum*. Prior to each experiment, animals were fasted overnight (about 16-18 hours) and allowed free access to water.

II. Chemicals

Phloracetophenone or 2,4,6-trihydroxyacetophenone ,THA, was purchased from Fluka Chemie AG (Buchs, Switzerland) and its purity is of over 99 %. 17α-Ethinylestradiol (EE), NAD, glutathione, and sulfobromophthalein (BSP) were purchased from Sigma Chemicals Co., (St. Louis, MO, and U.S.A). Enzymatic

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reagent kits for blood urea nitrogen, plasma aspartate aminotransferase (AST or GOT), alanine aminotransferase (ALT or GPT), bilirubin and triglyceride determinations were purchased from Bio-Medical Laboratory (BM-Lab, Bangkok, Thailand). All other chemicals and solvents used throughout this investigation were of analytical grade.

III. Experiment

Experiment I: Acute toxicity of 2,4,6-trihydroxyacetophenone in mice, rats and hamsters

Acute toxicity of a single dose of phloracetophenone or 2,4,6-trihydroxyacetophenone, THA, was investigated by giving the compound either intraperitoneally (i.p.) or intragastrically (i.g.) to various animal species including mice, rats, and hamsters. Prior to experiment, animals were fasted overnight with free access to water, various doses of THA ranging from 0.1-6 g/kg BW (dissolved in DMSO: EtOH: distilled water, 25:15:60) were given via either i.p. or i.g. routes. After administration, the animals were closely observed during the first 3 hrs, and occasionally thereafter for 14 days, for the onset of convulsions, toxic signs and symptoms, and death. Tissues of various organs of dead animals and survivors sacrificed at the end of 14-day period were examined for gross changes. LD_{50} values for each route of administration and each species of animals were calculated according to the method of Litchfield and Wilcoxon (106).

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Possible cause of death induced by acute toxicity of THA was investigated in the adult male Wistar rats. The animals were anesthetized with sodium pentobarbital (50 mg/kg BW, i.p.) and tracheostomized. The femoral artery was cannulated to

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record changes of blood pressure after administration of tested compound. Respiratory rate and EKG were also recorded using dynograph recorder (MAC-LAB^{R} instrument).

Experiment II: Subacute toxicity of 2,4,6-trihydroxyacetophenone in adult male mice

Subacute toxicity of 2,4,6-trihydroxyacetophenone, THA, was investigated in adult male mice weighing between 25-30 g. Animals with free access to food and water were intragastrically (i.g.) given various sublethal doses of THA ranging from 37-300 mg/kg BW once a day for 30 consecutive days. The compound was dissolved in 10% ethanol and further suspended in corn oil. At the end of experimental period, overnight fasted animals were anesthetized with ether. Blood samples were collected from abdominal vein, and plasma samples were separated by centrifugation at 3,000 rpm for 10 min, 4 °C in IEC centrifuge (International Equipment Company, U.S.A.) and were kept at –20 °C for further analysis of plasma levels of AST, ALT, BUN and bilirubin. Livers were kept at –70 °C for determinations of triglyceride and glutathione contents. Tissues of dead animals during the experimental period and survivors sacrificed at the end of the experiment including liver, lung, heart, kidney, spleen, stomach, bladder, seminal vesicles, and testis were weighed and fixed in 10% buffered neutral formalin. Paraffin sections were prepared for routine histopathological examination

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Experiment III: Effect of 2,4,6-trihydroxyacetophenone treatment on hepatic excretory function in normal rats and in ethinylestradiol-induced cholestatic rats.

This study is designed to investigate the effect of 2,4,6-trihydroxyacetophenone, THA, treatment on hepatic excretory function in rats by using sulfobromophthalein, BSP, dye-clearance principle. After intravenous administration, BSP retention in blood and its excretion into bile were determined and used as indices for evaluation the liver excretory function. Adult male Wistar rats (8-10 animals per group) weighing between 200-250 g were used. They were divided into control group and THA-treated groups. The latter were subsequently treated with THA at a dose of 50 or 100 mg/kg BW, intragastrically, for 5 days and a single intraduodenal administration of THA at a dose of 50 or 100 mg/kg BW thereafter they were subjected to the excretory function test. In addition to investigate the effect of THA on the hepatic excretory function in normal rats, its effect was also investigated in ethinylestradiol-induced cholestasis. Animals were induced cholestasis by subcutaneous injection with 17α–ethinylestradiol, EE, at a dose of 5 mg/kg BW for 5 days. These cholestatic animals were randomly divided into EE-control groups, EE-received low and high dose of THA treatment (50 and 100 mg/kg BW, respectively). THA was given along with EE for 5 days by intragastric administration, while the control group received a similar volume of the solvent vehicle. After treatment, the animals were subjected to determine hepatic clearance of BSP. They were fasted overnight with free access to water. Each rat was anesthetized with sodium pentobarbital (50 mg/kg BW), intraperitoneally and a tracheostomy was performed

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(Figure C). The left femoral vein and the left femoral artery were catheterized with PE-50 tubing (Clay Adams, Dickinson Co., NJ, U.S.A.) for infusion of saline (0.9% NaCl) at a rate of 1.2 ml/h using infusion pump (Harvard compact infusion pump model 975, Ohio, U.S.A) and blood sample collection, respectively. A midline laparotomy was performed and the common bile duct was cannulated with PE-10 tubing (Clay Adams, Dickinson Co., NJ, U.S.A.) nearly to its bifurcation to prevent contamination with pancreatic juice. Body temperature was maintained at 37±0.5 °C by overhead heating lamp which was connected to the temperature regulator and monitored with a rectal probe (YSI model 73A, Ohio, U.S.A) in order to prevent hypothermic alteration of bile secretion. All tested animals were intravenous injected with a single dose of BSP (6.25 mg/kg BW) for evaluating hepatobiliary excretory function.

Bile sample collection

After allowing bile flow to stabilize for 30 min, bile sample was collected in a preweighed tube (W1) for 15 min-period for 1 h and after then for 30 min-period the first hour for 2 periods. The tube containing bile sample was weighed again (W2) and the differences between the weights was used to express the collected bile volume by assuming a density for bile 1.0 g/ml. A schedule for bile collection is shown in Figure D. Bile samples were kept at -20 °C for further analysis.

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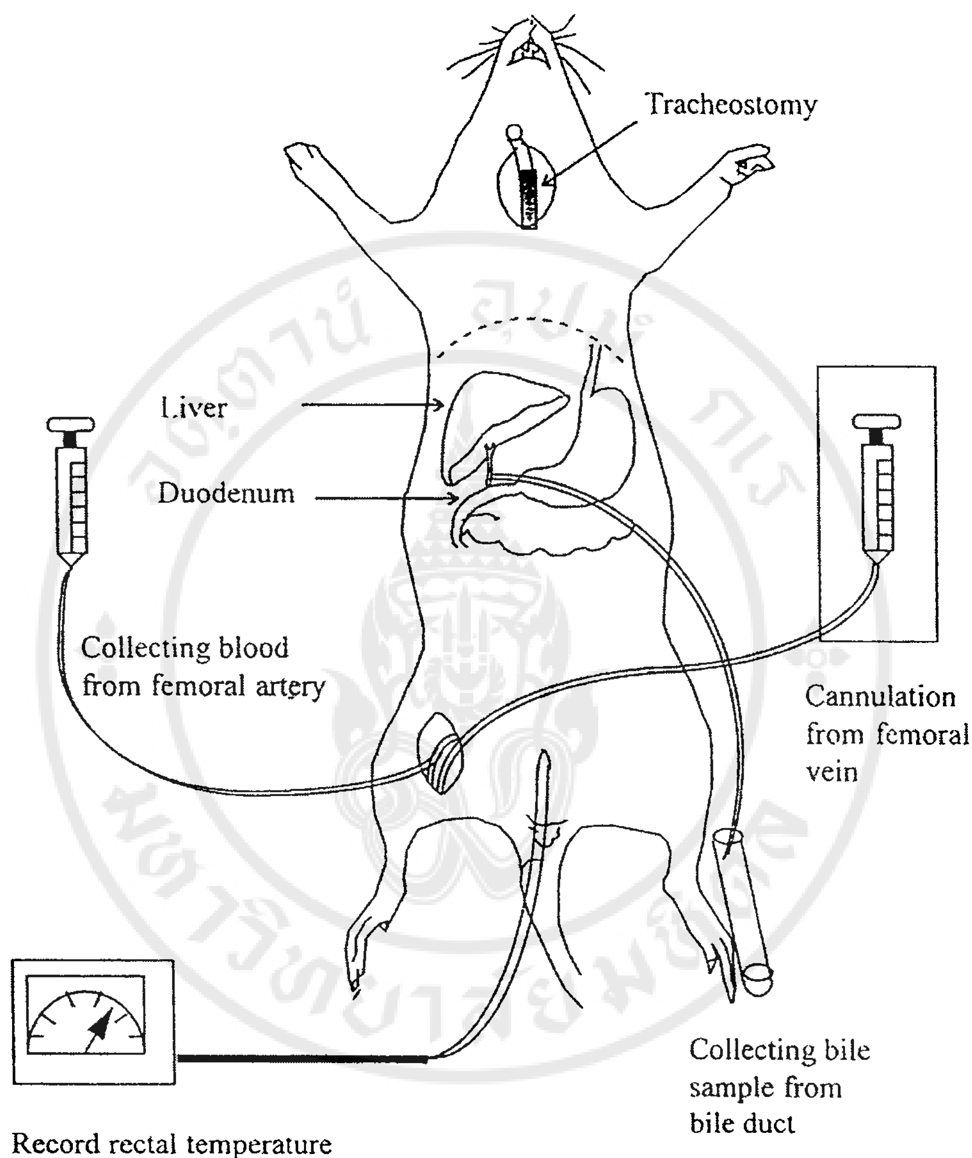

Figure C. Experimental scheme showing a general animal preparation. Animal was anesthetized and tracheostomy was performed. Both the right femoral vein and femoral artery were cannulated for infusion of saline and blood sample collection, respectively. Common bile duct was cannulated for bile collection. Body temperature was maintained by temperature regulator and overhead heating lamp.

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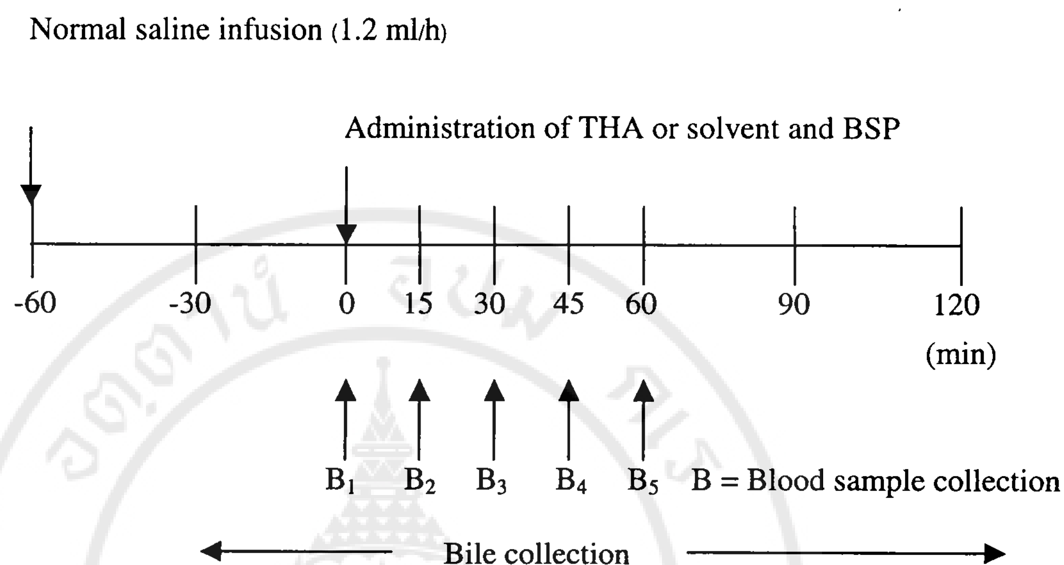

Figure D. A schematic diagram of bile and blood samples collection.

Blood sample collection

A control blood sample was initially collected before administration of BSP, THA or solvent. After administration of BSP, an initial blood sample (B1) was immediately collected, and four blood samples (B2, B3, B4, and B5) were subsequently collected at 15 min-period for 1 h. For THA or solvent administration, it was injected intraduodenally simultaneous with intravenous BSP administration. At the end of experiment each blood sample approximately 0.5 ml was collected in heparinized tube. Plasma sample was separated by centrifugation at 3,000 rpm for 10 min, 4 °C in IEC centrifuge (International Equipment Company, U.S.A.) and it was kept at –20 °C for further analysis of BSP. During blood sample collection, an equal volume of plasma would be replaced with 0.9% NaCl. The concentration of BSP in the plasma was determined by alkalinizing an aliquot (100 µl) of the plasma with an appropriate amount of 0.01 M NaOH and measured absorbance at 580 nm. The

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concentration of BSP in bile (50 μl aliquots) was also determined after the bile volume was measured for each collection period.

IV. Biochemical Analyses

1) Determination of plasma aspartate aminotransferase (AST or GOT)

Plasma AST is a liver enzyme which catalyzes the transfer of amino and keto groups between alpha amino acids and alpha keto acids. Determination of this enzyme was performed according to the method described by Reitman and Frankel in 1957 (Appendix XIV), using aspartic acid and ketoglutaric acid as substrates. The reaction was catalyzed by the transaminase activity which resulted in the formation of glutamic acid and oxaloacetic acid. The oxaloacetic acid was then reacted with 2,4-dinitrophenyl hydrazine giving a product of hydrazone which was yellow-brown in color and could be measured spectrophotometrically.

2) Determination of plasma alanine aminotransferase (ALT or GPT)

Plasma ALT activity was determined by the method similar to that described for AST except that the substrate used was DL alanine instead of aspartic acid and the product formed in this reaction was pyruvic acid rather than oxaloacetic acid. Pyruvic acid further reacted with 2,4-dinitrophenyl hydrazine and yielded the product of hydrazone (yellow-brown color) which could be measured by spectrophotometer (Appendix XV).

3) Determination of blood urea nitrogen (BUN)

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BUN was determined according to the method described by Wybenga in 1971 (Appendix XVI). The reaction was based on the interaction of urea with diacetyl monoxime in the presence of thiosemicarbazide to produce a chromogen. Intensity of

the developing color was directly proportional to the concentration of urea nitrogen in the reaction mixture and could be measured spectrophotometrically.

4) Determination of total protein

Protein concentration was determined according to the method described by Lowry in 1951 (Appendix XVII). The first step of protein assay is the reaction of protein with cupric ions (Cu^{++}) in alkaline medium, lead to reduce cupric ions (Cu^{++}) to cuprous ions (Cu^{+}). The second step is a reduction of Folin ciocateu's phenol reagent with the alkaline copper-protein complex, giving a characteristic of blue color and its absorbance can be measured at 650 nm.

5) Determination of liver triglyceride

The concentration of liver triglyceride was determined by a modified method as previously described by Mendez in 1975 (Appendix XVIII). Triglyceride content in sample was extracted by a extracting solvent consisting of heptane, isopropanol, and sulfuric acid. Triglyceride in the heptane layer was separated and then was mixed with reacting solutions containing KOH, periodate, ammonium acetate buffer and acetylacetone. The end product was diacetyldihydrolutidine with yellow color which was measured absorbance at 415 nm.

6) Determination of liver glutathione (GSH)

Total glutathione contents in liver were determined by using an enzymatic recycling method described by Theodurus in 1981 (Appendix XIX) in which it was sequentially oxidized by 5,5'-dithiobis-(2-nitro-5-thiobenzoic acid (DTNB)) and was reduced by NADPH in the presence of glutathione reductase. The rate of 2-nitro-5-

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thiobenzoic acid formation was monitored at 412 nm and the amount of glutathione was evaluated from a standard curve.

7) Determination of total bilirubin

The total bilirubin was determined according to the method of Jendrassik in 1938 (Appendix XX). Plasma was diluted with activator, which was a mixture of sodium nitrite, caffeine, sodium acetate and alkaline tartate buffer and then they were allowed to further react with sulfanilic acid. The absorbance of condensation product of total bilirubin with sulfanilic acid was measured at 600 nm. The concentration was expressed as mg/dl.

8) Determination of alkaline phosphatase (AP)

Plasma AP was determined according to the method described by Authur in 1966 (Appendix XXI). Alkaline phosphatase hydrolysed phenolphthalein monophosphate in alkali solution and then produced a phenolphthalein that has a pink color and could be measured at 550 nm.

V. Histopathological Studies

Small pieces of tissue samples were cut and fixed with Bouin 's fixative solution (40% formaldehyde, 5% acetic acid, and 10% picric acid). They were subjected to routine histology technique, which included dehydration, embedding in paraffin and section into 5 μm thickness. The sections were then stained with haematoxylin and eosin (H&E). Histopathology in the stained sections was then examined under a bright field light microscope (Appendix XXII).

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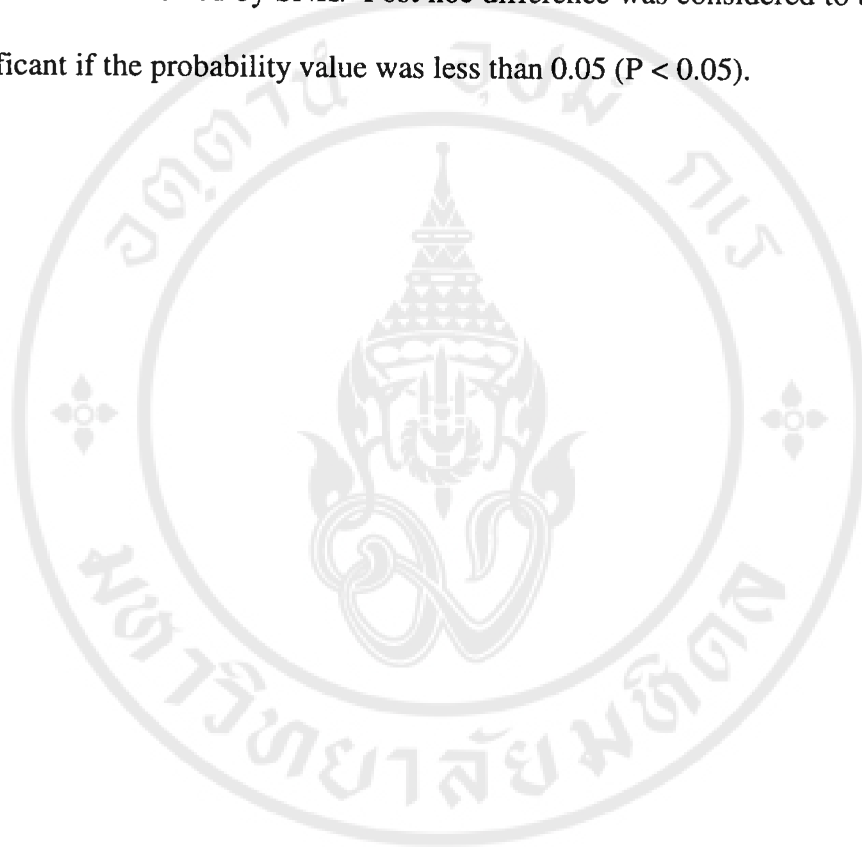
VI. Statistical Methods

All results were expressed as mean ± standard error of mean ($\bar{X}$±SEM). The significance of the difference among groups was analyzed by using ONE-WAY ANOVA and followed by SNK. Post hoc difference was considered to be statistically significant if the probability value was less than 0.05 ($P < 0.05$).



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CHAPTER IV

RESULTS

Experiment I: Acute Toxicity of 2,4,6-Trihydroxy-Acetophenone in Mice, Rats and Hamsters.

Acute toxicity of a single-dose of 2,4,6-trihydroxyacetophenone, THA, was investigated in three different animal species which were mice, rats and hamsters of both sexes by two routes of administration, either intraperitoneally (i.p.) or intragastrically (i.g.). General signs and symptoms were closely observed in 24 hours and the numbers of dead animals were recorded within a period of 14-day after administration of THA for estimation of the median lethal dose (LD_{50}) value. In addition, after the end of the experiment, necropsy findings in survival animals were explored.

The LD_{50} values of THA in three different animal species after either i.p. or i.g. administration are summarized in Table 1. The LD_{50} value of THA was varied according to species and sexes of animals used. Regardless to the route of administration, males appeared to be more sensitive to females. LD_{50} value in males was lower than that in females in all cases. For example; after intraperitoneal administration, LD_{50} value in weanling male mice was 370 mg/kg BW whereas it was approximately 430 mg/kg BW in female mice. However, there was no age differences in response to toxic effect of THA. LD_{50} value in weanling male mice was approximately 370 mg/kg BW, which was comparable to that in adult male mice.

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Likewise, LD_{50} value in weanling females was comparable to that in adult females. Among three animal species studied; hamsters and mice appeared to be more sensitive to THA as compared to those in rats. LD_{50} values were lower whereas rats showed the least sensitivity to THA. LD_{50} value in adult male and female rats, were 620 and 660 mg/kg BW, respectively, which were much higher than those in mice and hamsters.

By intragastric administration of THA, the pattern of response in three animals species was similar to that by intraperitoneal administration. The hamsters and mice were more sensitive to THA and the rat was the least sensitive. THA at a dose of 6 g/kg BW did not induce any lethal to rats.

Regardless of age, sex, and route of administration of THA, general clinical signs and symptoms were found to be similar among these animal species after being treated with THA. At lethal levels, after i.p. administration, animals became excited and exhibited an increase in locomotion activity for about 1-2 min, followed by ataxia and hypersensitive to noise. These responses progressed to decrease of locomotion activity and subsequently lost of strength of the limbs. Some animals became drowsy, lay down in the cages, and finally started the convulsion within 30-60 min. The animals apparently died of respiratory failure within 1-3 hrs. However, all animals, which were received THA intragastrically, could survive. In rats, both adult males and females which intragastrically received THA at a dose of 6 g/kg BW lived up over to 14 days. The survival animals, which received high dose, would pass the stage of hyperactivity, ataxia, and convulsion. They further, fell into the stage of drowsiness, which appeared in the first day. All clinical signs and symptoms were more

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pronounced in animals by i.p. route than those by i.g. route of administration. At autopsy of dead animal in the course of experiment and necropsy finding in surviving animals at the end of experimental period (14 days), no apparent changes by THA treatment in any organs was observed.

To further examination the cause of death in acute toxicity test, vital signs which included EKG and respiratory rate were recorded in animals receiving a single large dose of the compound. After administration of the lethal dose of THA via i.p. route in anesthetized rats, it caused a slight transient reduction of mean arterial blood pressure, while the pattern of the EKG, and heart rate did not change during the first 5-10 min. However, thereafter the respiratory rate and tidal volume were gradually decreased, and progressed to dyspnea and apnea, at about 15-20 min. The respiratory paralysis appeared to be the cause of death as changes of mean arterial blood pressure was shortly returned to control value within 5 min, whereas the respiratory function was progressively deteriorated, it was reduced and finally ceased. At this time, EKG record persisted for the next period of 3 min. Therefore, animals died of respiratory paralysis after administration of lethal dose THA.

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Table 1. Lethality following a single intraperitoneal (i.p.) or intragastric (i.g.) administration of 2,4,6-trihydroxyacetophenone in mice, rats and hamsters.

Animals[a]	Mean BW(g)	Number of animals	Route	LD_{50}[b] (mg/kg BW)	Slope function	Onset of[c] convulsion (min)	Survival time (min)
Mice (M weanling)	12.6±2.6	36	i.p.	370 (293.1-466.4)	1.262	5-25	40 min-3 days
Mice (F weanling)	11.5±2.3	30	i.p.	430 (313.4-589.9)	1.372	5-25	40 min-3 days
Mice (M adult)	26.5±4.9	48	i.p.	365 (328.8-405.1)	1.235	5-25	40 min-3 days
Mice (F adult)	25.1±4.9	20	i.p.	400 (371.7-430.4)	1.076	5-25	40 min-3 days
Hamsters (M adult)	110.0±6.4	48	i.p.	338 (299.1-381.9)	1.245	5-30	1-3h
Hamsters (F adult)	115.0±6.3	48	i.p.	371 (332.7-413.6)	1.251	5-30	1-3h
Rats (M adult)	182.0±8.4	20	i.p.	620 (591.6-649.8)	1.048	45-60	>3h
Rats (F adult)	175.0±7.3	30	i.p.	660 (622.6-699.6)	1.095	45-60	>3h
Mice (M adult)	26.5±4.2	48	i.g.	3200 (2380-4280)	1.339	30-60	1h-5days
Mice (F adult)	25.1±3.9	48	i.g.	3400 (2470-4670)	1.585	30-60	1h-5days
Hamsters (M adult)	110.0±6.1	48	i.g.	2950 (2590-3350)	1.203	45-60	1h-7days
Hamsters (F adult)	115.0±6.1	48	i.g.	3100 (2570-3720)	1.46	45-60	1h-7days
Rats (M adult)	182.0±8.3	48	i.g.	>6000	0	>60	> 14days
Rats (F adult)	175.0±7.7	48	i.g.	>6000	0	>60	> 14days

[a]M and F are male and female animals, respectively.

[b]Calculated by a method of Litchfield and Wilcoxon (106). Figures in parentheses are 95 % confidence limits.

[c]Time range was recorded immediately after 2,4,6-trihydroxyacetophenone administration.

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Experiment II: Subacute Toxicity of 2,4,6-Trihydroxyacetophenone in Adult Male Mice.

Subacute toxicity of 2,4,6-trihydroxyacetophenone, THA, was studied in adult male mice. Animals were intragastrically (i.g.) given various sublethal doses of THA ranging from 37-300 mg/kg BW once a day for 30 consecutive days. Doses used here were based on its pharmacological effectiveness in stimulating bile secretion and lowering plasma lipid (107). During treatment, body weights were recorded weekly. At the end of the experimental period, biochemical parameters in plasma which are indices of liver and kidney functions including AST, ALT, bilirubin and BUN were determined. Tissues of dead animals during the course of experiment and survivors sacrificed at the end of the experiment were weighed and prepared for routine histopathological examination. A portion of liver tissues was removed and kept for determination of triglyceride and glutathione contents.

Table 2 shows changes of body weights, liver weights, and some biochemical parameters in plasma and liver after treatment with THA for 30 consecutive days. All doses of THA used for treatment did not affect body weight gain. The weights of animals in all experiment groups were comparable (Figure 1a). Likewise, all doses of THA did not affect liver weight (Figure 1b). In addition, no mortality was detected throughout the course of experiment. Activities of plasma AST and ALT which are used as diagnostic markers of liver membrane damage, appeared to be increased after THA treatment and showed a dose-related to THA. However, the significant increase was observed only at the last two high doses of THA (150 and 300 mg/kg BW) (Figure 2).

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The effect of THA treatment on plasma levels of total bilirubin and blood urea nitrogen (BUN) are shown in Figure 3a and b, respectively. Mice which were intragastrically given sublethal doses of THA (37-300 mg/kg BW) for 30 consecutive days showed dose-related increases in both total bilirubin and BUN as compared to those in controls. However, the increase was slight and they were all in the normal ranges (0-1.5 mg % for total bilirubin and 10-20 mg % for BUN) (Appendix XXIII). Both parameters were significantly different from those in the control group only in the highest dose group.

After treatment with various doses of THA, the liver triglyceride content (Figure 4) appeared to be progressively increased with the doses. The statistically significant differences among the THA-treated groups and the control were observed at a dose of 75 mg/kg BW onward. The increased triglyceride concentration by the highest dose of THA was almost twice of that in control. The liver GSH content was significantly increased and the highest content was observed when treated with THA at a dose of 37.5 mg/kg BW. Higher dose of THA progressively had less effects and THA at 300 mg/kg BW had no significant effect on hepatic glutathione content (Figure 5).

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Table 2. Effect of 2,4,6-trihydroxyacetophenone treatment on body weight, liver weight and plasma ALT, AST, BUN, Bilirubin and liver triglyceride and glutathione contents in adult male mice.

Parameters	Control	2,4,6-Trihydroxyacetophenone (mg/kgBW)				
		0	37.5	75	150	300
Body weight (g)	31.3±1.22	32.4±1.42	32.2±1.18	31.6±1.22	32.0±1.31	35.9±0.97
Liver weight (g/ 100 g BW)	4.8±0.04	4.9±0.04	4.9±0.04	4.8±0.02	4.9±0.08	5.2±0.06
ALT (IU/ml)	42.5±1.35	49.7±1.88+	54.0±1.27	59.0±0.77	74.7±3.3*	79.8±1.65*
AST (IU/ml)	74.2±2.44	87.8±2.43+	91.4±1.79	98.1±1.08	120.3±4.24*	134.71±1.27*
BUN (mg %)	7.5±0.17	8.3±0.13	9.6±0.07	10.6±0.16	11.2±0.16*	12.0±0.17*
Total bilirubin (mg %)	0.9±0.02	0.9±0.02	1.2±0.01	1.2±0.01	1.4±0.01*	1.6±0.01*
Liver triglyceride (mg/ g liver)	10.3±0.03	10.9±0.20	12.9±0.12	15.3±0.21*	15.8±0.35*	18.8±0.21*
Liver glutathione (µmol/ g liver)	0.2±0.01	0.2±0.01	0.5±0.02*	0.4±0.02*	0.3±0.03*	0.2±0.02

2,4,6-Trihydroxyacetophneone was intragastrically administered once a day for 30 consecutive days.

Values are means ± SEM from 8-10 animals.

+P < 0.05 significantly different from the normal control.

*P < 0.05 significantly different from the solvent control.

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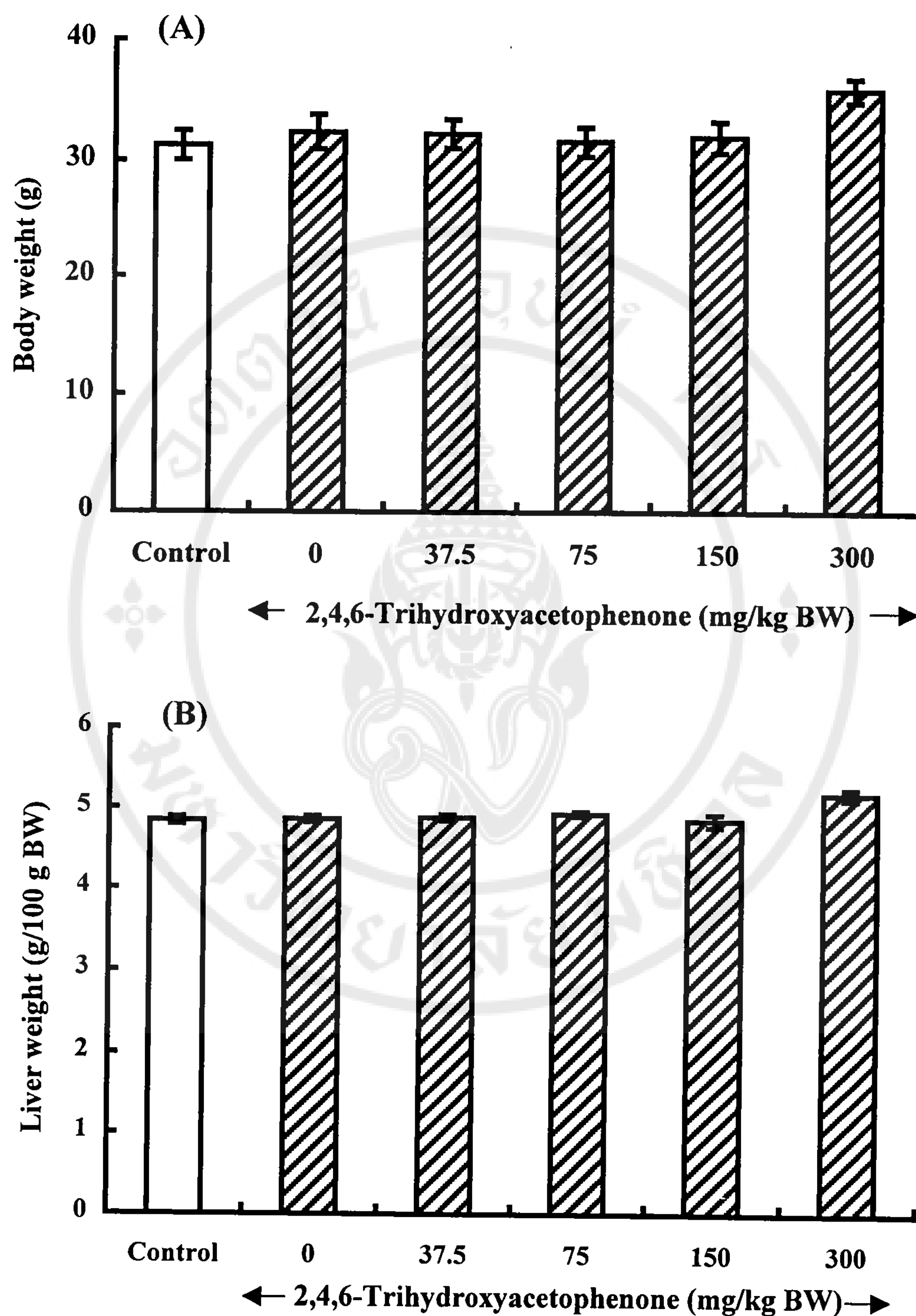

Figure 1. Effect of 2,4,6-trihydroxyacetophenone (THA) treatment on body weight (A) and liver weight (B) in adult male mice. THA was intragastrically given to the animals once a day for 30 consecutive days. Values are means $\pm$ SEM from 8-10 animals.

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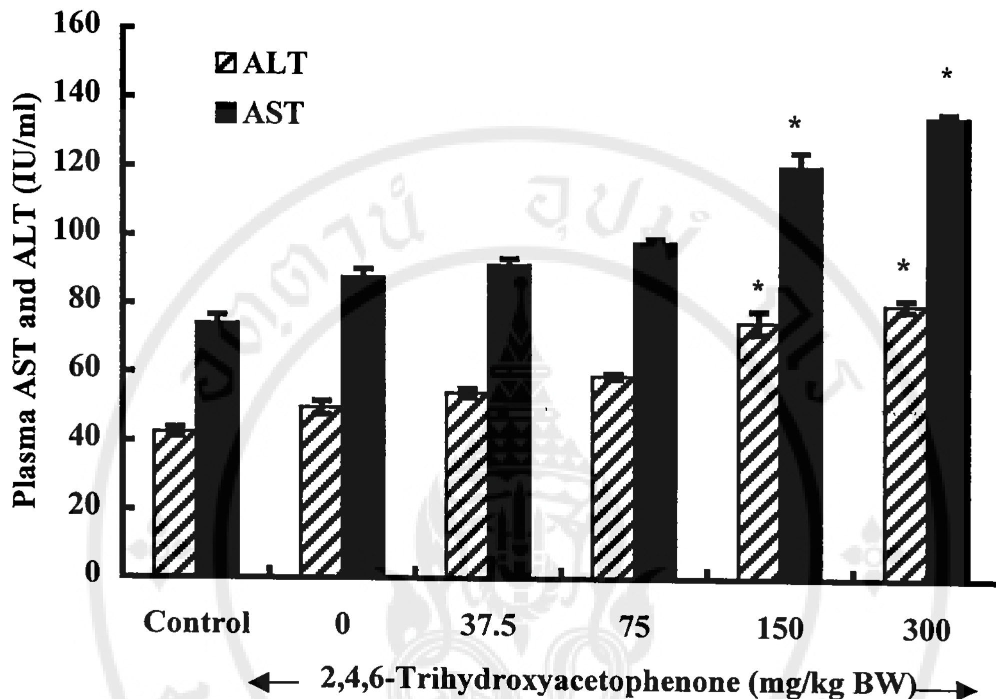

Figure 2. Effect of 2,4,6-trihydroxyacetophenone (THA) treatment on plasma activities of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) in adult male mice. THA was intragastrically given to the animals once a day for 30 consecutive days. Values are means ± SEM from 8-10 animals.

*$p < 0.05$ significantly different from the control group.

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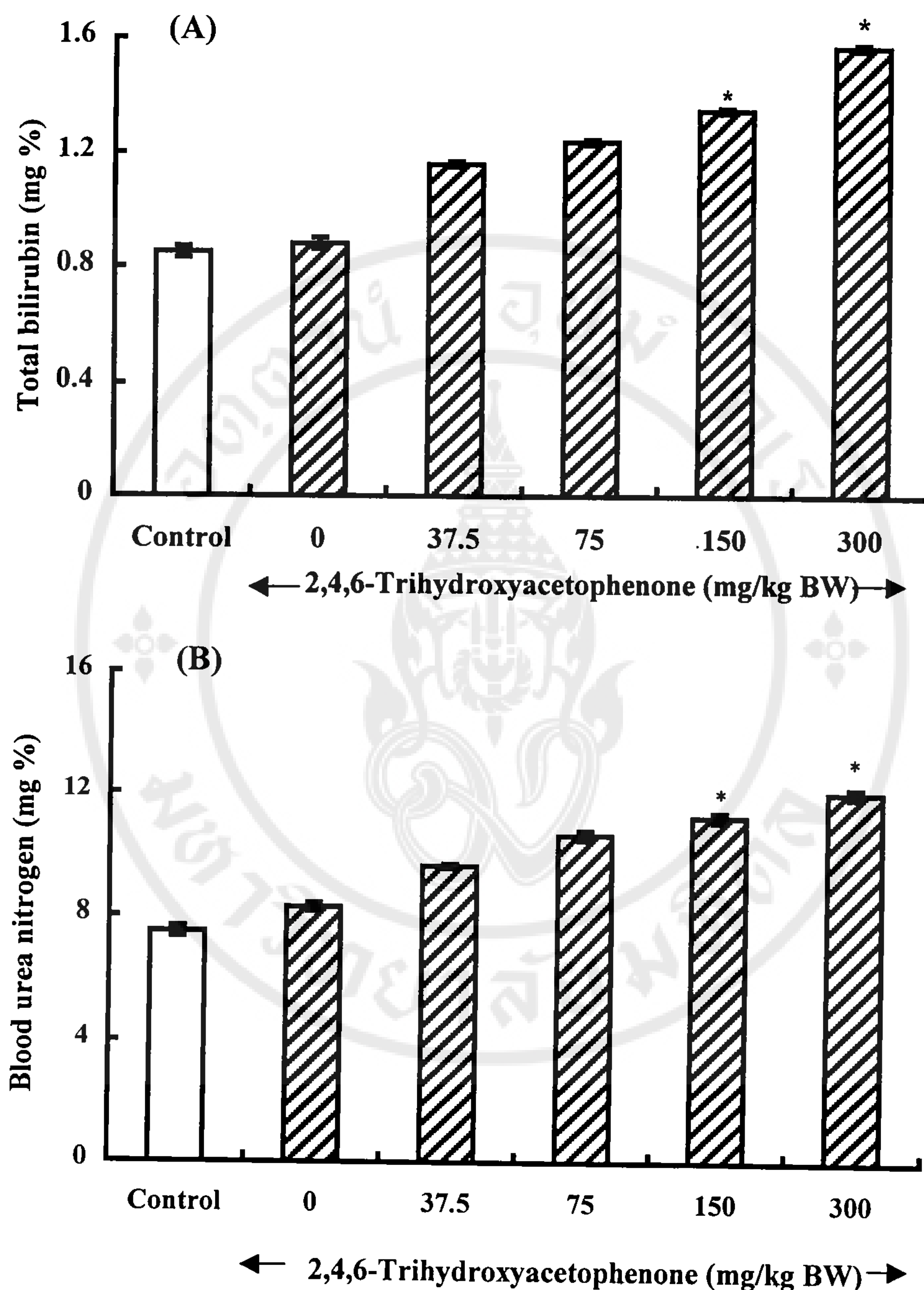

Figure 3. Effect of 2,4,6-trihydroxyacetophenone (THA) treatment on plasma level of total bilirubin (A) and blood urea nitrogen (B) in adult male mice. THA was intragastrically given to the animals once a day for 30 consecutive days. Values are mean ± SEM from 8-10 animals.

*$p < 0.05$ significantly different from the control group.

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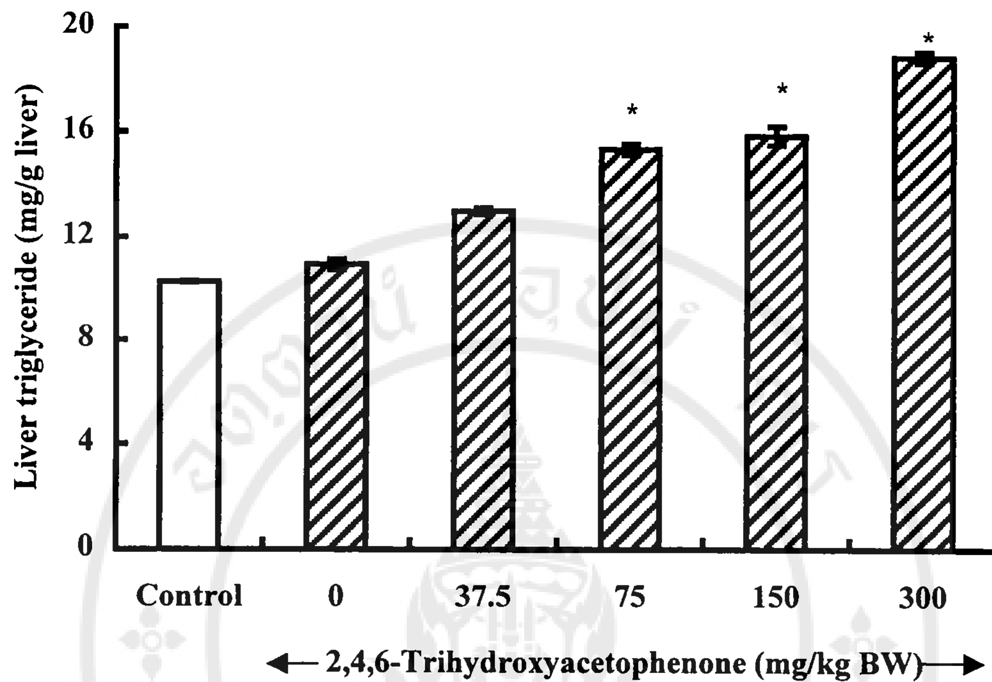

Figure 4. Effect of 2,4,6-trihydroxyacetophenone (THA) treatment on liver triglyceride in adult male mice. THA was intragastrically given to the animals once a day for 30 consecutive days. Values are mean ± SEM from 8-10 animals.

*$p < 0.05$ significantly different from the control group.

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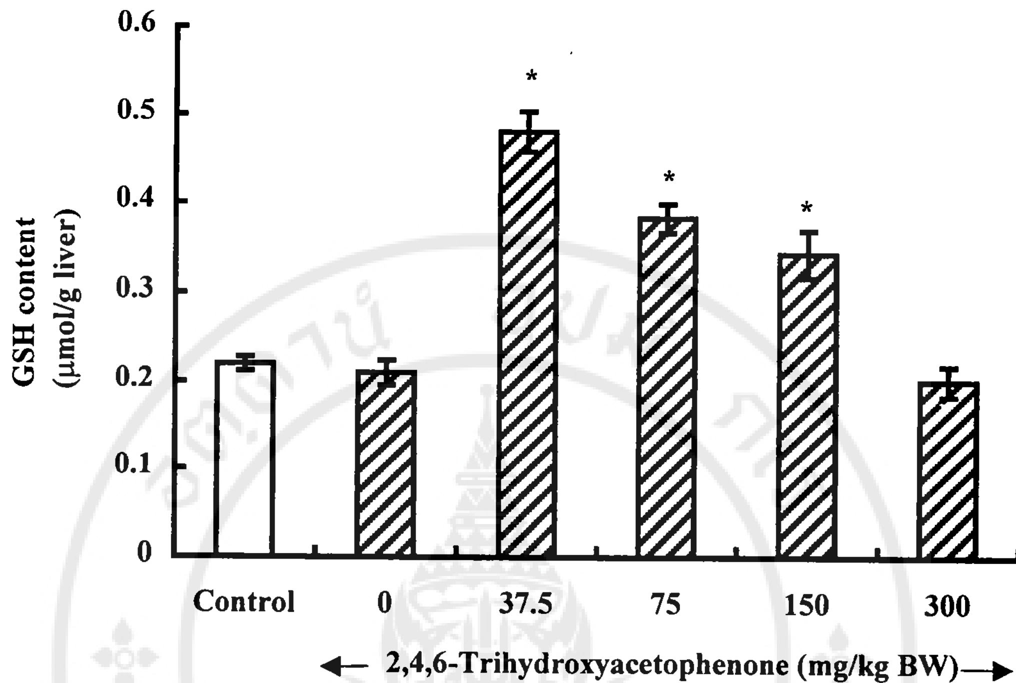

Figure 5. Effect of 2,4,6-trihydroxyacetophenone (THA) treatment on liver glutathione content in adult male mice. THA was intragastrically given to the animals once a day for 30 consecutive days. Values are mean ± SEM from 8-10 animals.

*$p < 0.05$ significantly different from the control group.

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Histopathological Examinations

Histopathology of various tissues obtained after intragastric administration of THA at sublethal doses ranging from 37-300 mg/kg BW for 30 consecutive days to mice were examined and compared to those of the solvent. THA at low dose (37.5 or 75 mg/kg BW) did not induce any changes in histomorphology of liver, kidney, pancreas, and cells in mucosal layer of gastrointestinal tract (gastric gland in the fundus part of stomach and small intestine). However, at higher doses of THA treatment, liver showed prominent histologic changes. The changes were also observed in kidney, pancreas, and gastric gland in the fundus part of stomach. In normal mouse liver, parenchyma cells mice oriented in hepatic cords which were composed of a single row of cells. They clearly radiated from central vein (Figure 6a). The hepatic cords are separated from vascular sinusoid by endothelial cells. As blood flow through the sinusoids from portal to central venous regions, distribution of hepatocyte along sinusoids are differences in receiving supply of nutrients, metabolic function and sensitivity to toxin.

THA treatment at low dose (37.5 and 75 mg/kg BW) slightly affected hepatocytes in periportal area. The affected area were pale in color and cells were slightly swollen (Figure 6b and c). THA at high dose (150 mg/kg BW) caused a marked changes of hepatocytes. The main affected area is at periportal which contained a number of cytoplasmic vacuoles which were not known whether they were phagocytic vacuoles or degenerated nuclei (Figure 6d). In severe case, the affected area expanded from the periportal area into the central vein area. As the hepatocytes in the periportal region were the first area which received blood supply

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from portal vein, they were exposed to the highest concentration of absorbed nutrients as well as toxins from the gastrointestinal lumen. Based on evidence of lesion in periportal region, it was suggested that the blood-borne toxin or the absorbed THA from the gastrointestinal lumen was attributed to the observed effect on hepatocytes, not its metabolite after being biotransformed by hepatocytes.

Figure 7 illustrates the histology of kidneys in control and after treatment with THA. The cortical parenchyma of control kidney consisted of renal corpuscles, proximal convoluted tubules, and distal convoluted tubules. The renal corpuscle consisted of a glomerulus surrounded by Bowman's capsule and it was sectioned through the vascular pole then the afferent arteriole was seen in adjacent to the distal convoluted tubule. The simple columnar epithelium of the proximal convoluted tubule possessed a thick brush border, which almost filled the lumen (Figure 7a). Figure 7b demonstrates histopathologic changes in mouse kidney treated with THA at a dose of 150 mg/kg BW. Hydropic degeneration of epithelial cells in cortex including those in proximal convoluted tubules, Bowman's capsule, and glomerulus were observed. In addition, cloudy swelling of nucleus were also apparent. In Figure 8, both exocrine and endocrine glands of pancreas in THA treated mouse are partially affected. Cells with pyknotic nuclei were observed in the Islet's gland whereas cells in the exocrine glands region showed clumping of glands with some atrophy. It was also the case of parietal cells in the gastric glands showing disorganization of unhealthy cells in the glands (Figure 9). The epithetial cells in the intestinal glands at ileum were slightly affected, as shown in Figure 10. Globlet cells seems to be more active whereas some were slightly swollen.

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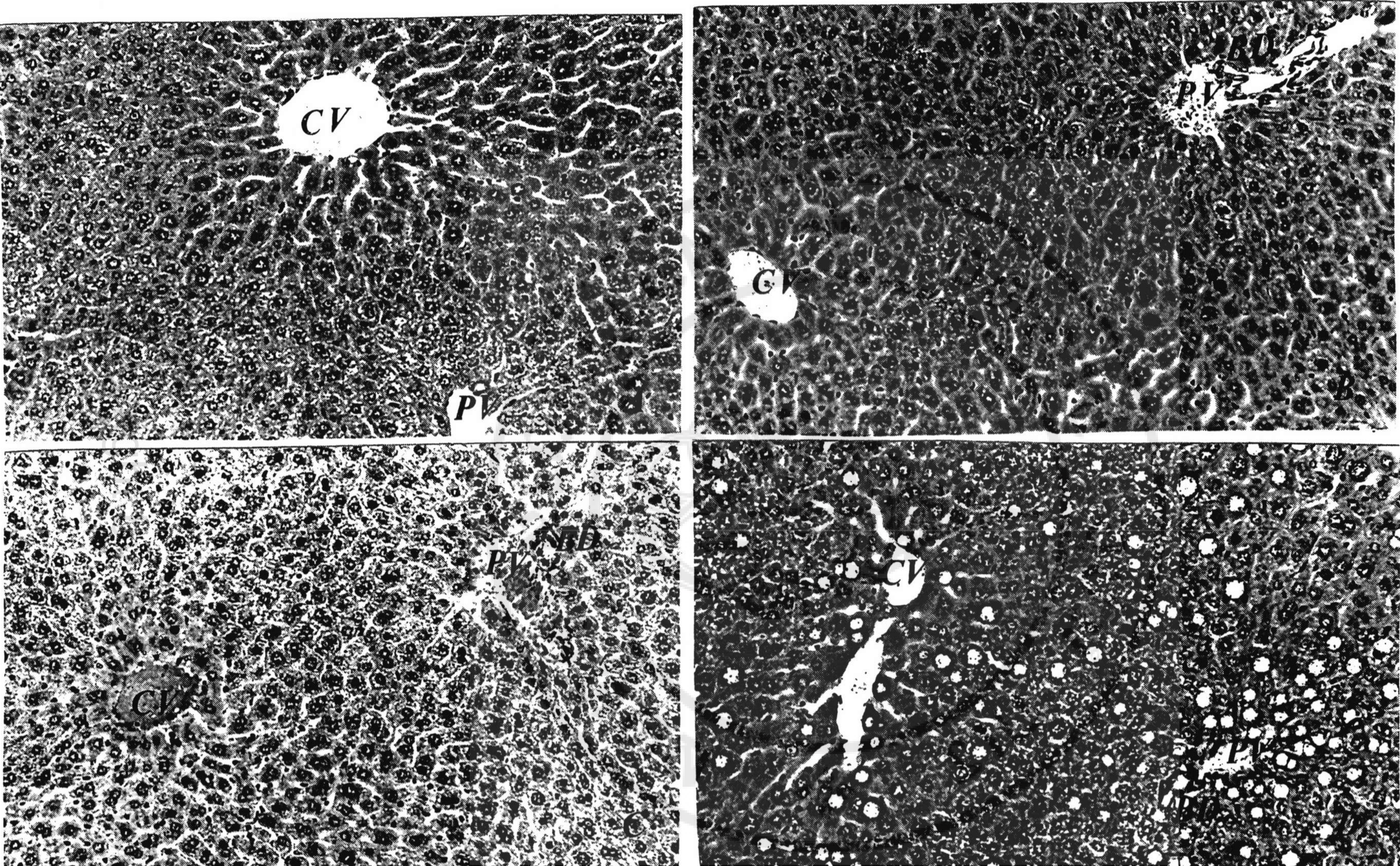

Figure 6. Light micrographs of liver sections from control mouse (a), mouse treated with 2,4,6-trihydroxyacetophenone (THA) at doses of 37.5 mg/kg BW (b), 75 mg/kg BW (c), and 150 mg/kg BW (d), respectively. THA was intragastrically given once a day for 30 consecutive days. Note a normal appearance of liver with low dose of THA-treatment. High dose of THA-treatment caused a marked of hepatocytes with vacuoles. The affected area is at periportal area (PV) and extended deeply into central vein (CV) (arrowhead), (H & E, 200x).

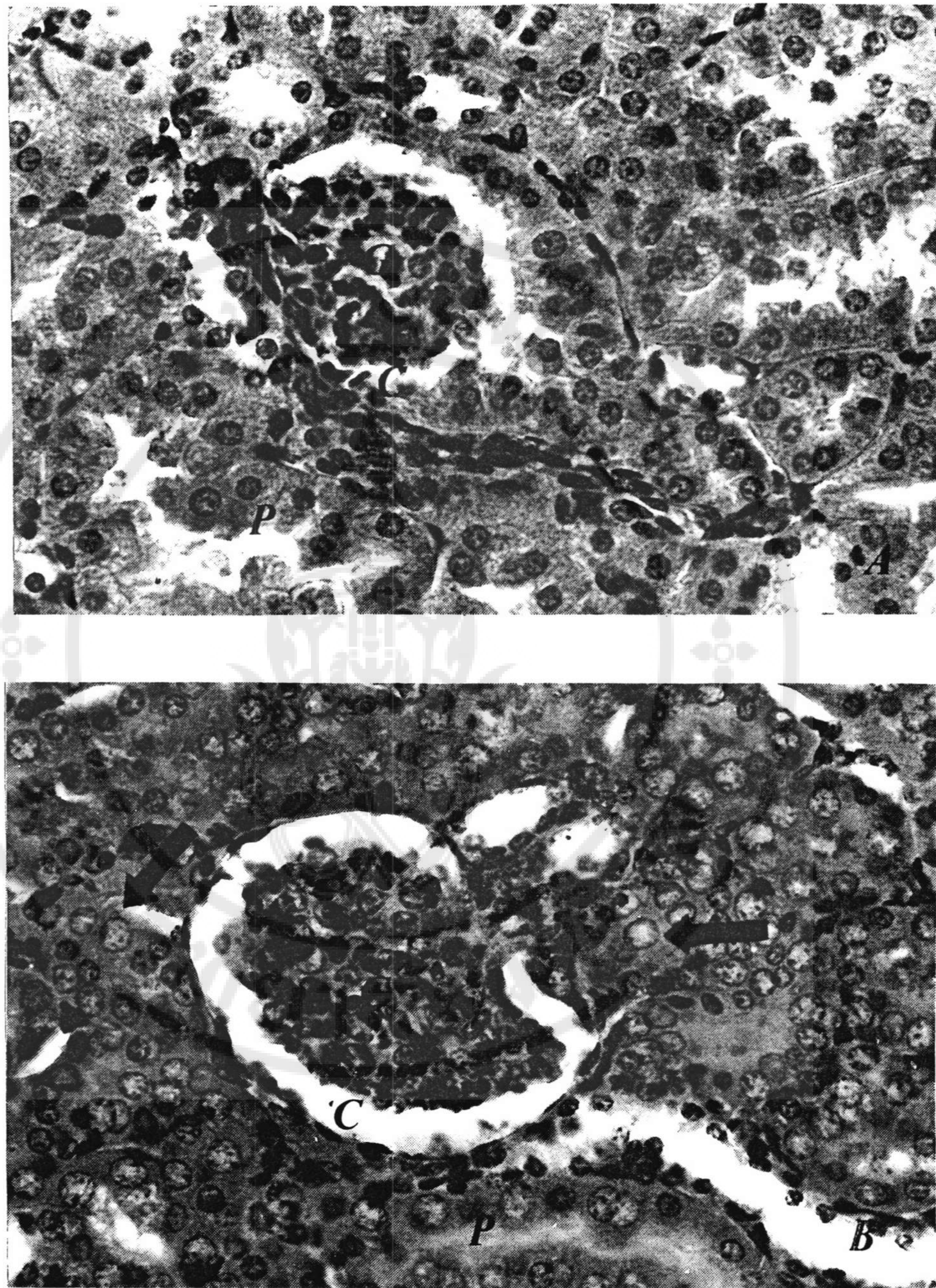

Figure 7. Light micrographs of renal cortex from control mice (a), and mice treated with 2,4,6-trihydroxyacetophenone (THA) at a dose of 150 mg/kg BW (b). THA was intragastrically given once a day for 30 consecutive days. Note the hydropic degeneration of epithelial cell in cortex including proximal convoluted tubules (P), Bowman's capsule (C), and glomerulus (G) (arrowhead), (H&E, 400x).

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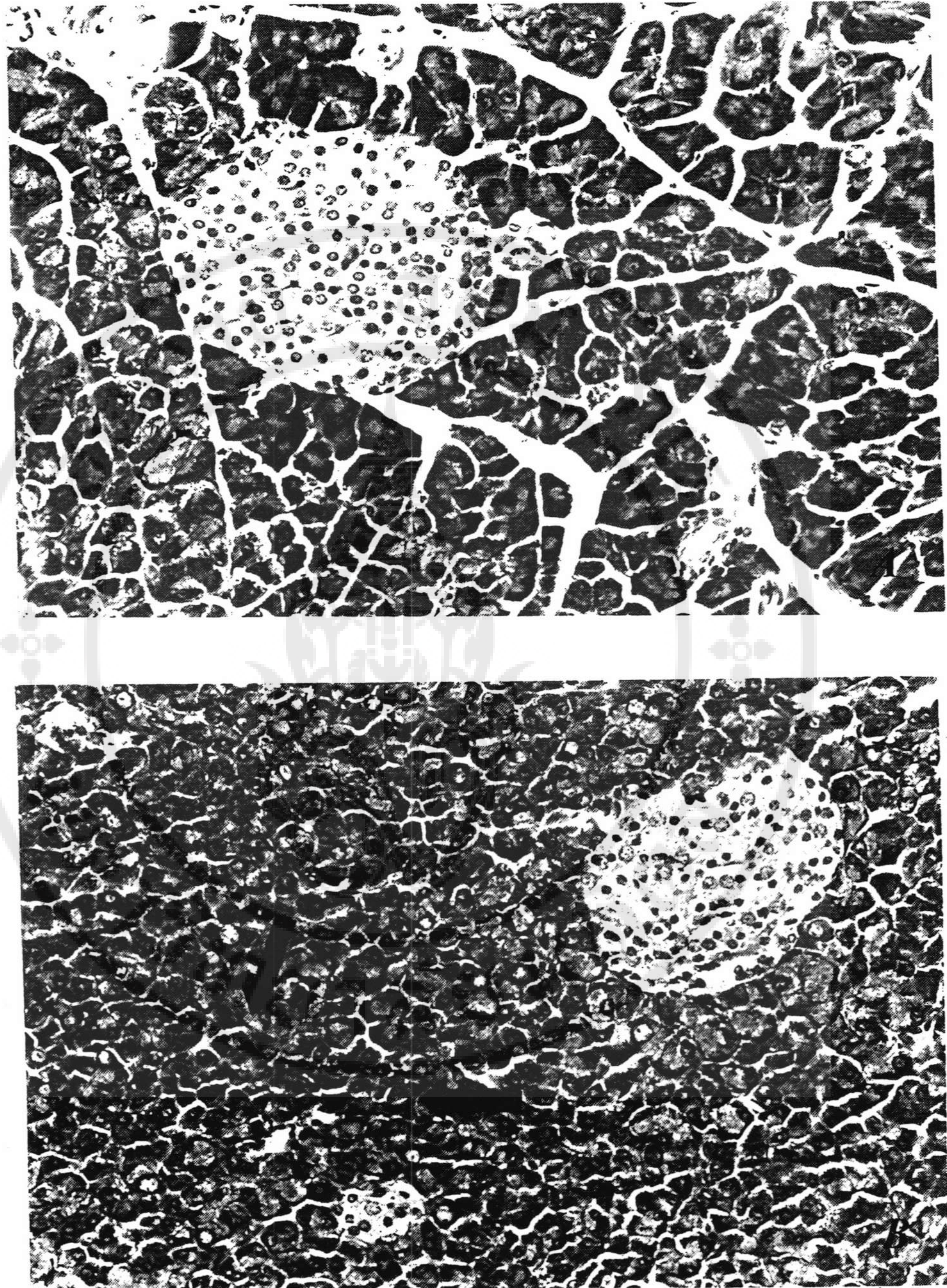

Figure 8. Light micrographs of pancreas from control mice (a), and mice treated with 2,4,6-trihydroxyacetophenone (THA) at a dose of 150 mg/kg BW (b). THA was intragastrically given once a day for 30 consecutive days. Note cells with pyknotic nuclei were observed in Islet's gland whereas cells in exocrine glands showed some acinar atrophy (arrowhead) (H&E, 200x).

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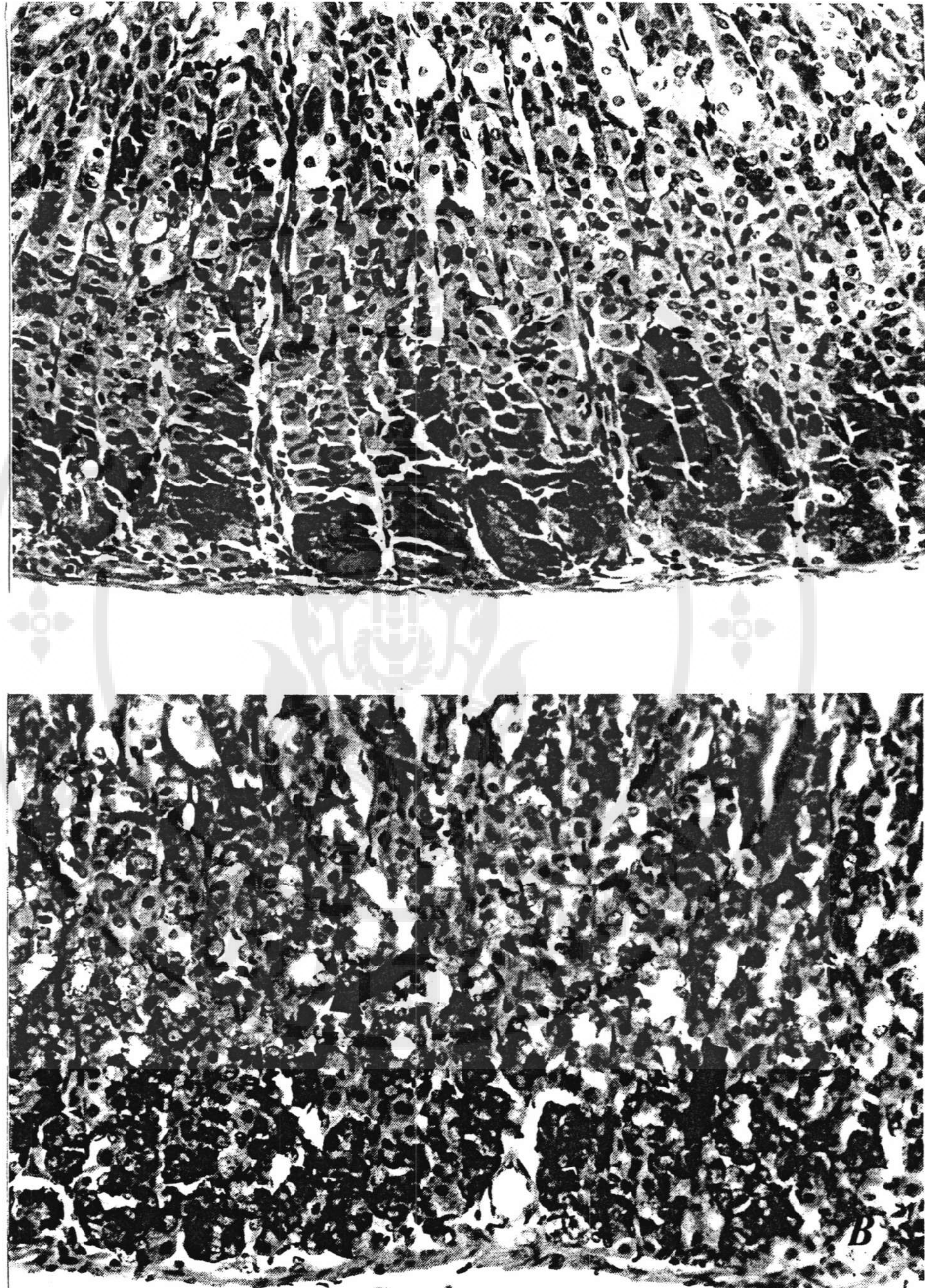

Figure 9. Light micrographs of gastric glands in the fundus part stomach in control mice (a), and mice treated with 2,4,6-trihydroxyacetophenone (THA) at a dose of 150 mg/kg BW (b). THA was intragastrically given once a day for 30 consecutive days. Note a disorganization and atrophy of cells lining in the lower part gastric glands in THA-treated group (arrowhead) (H&E, 200x).

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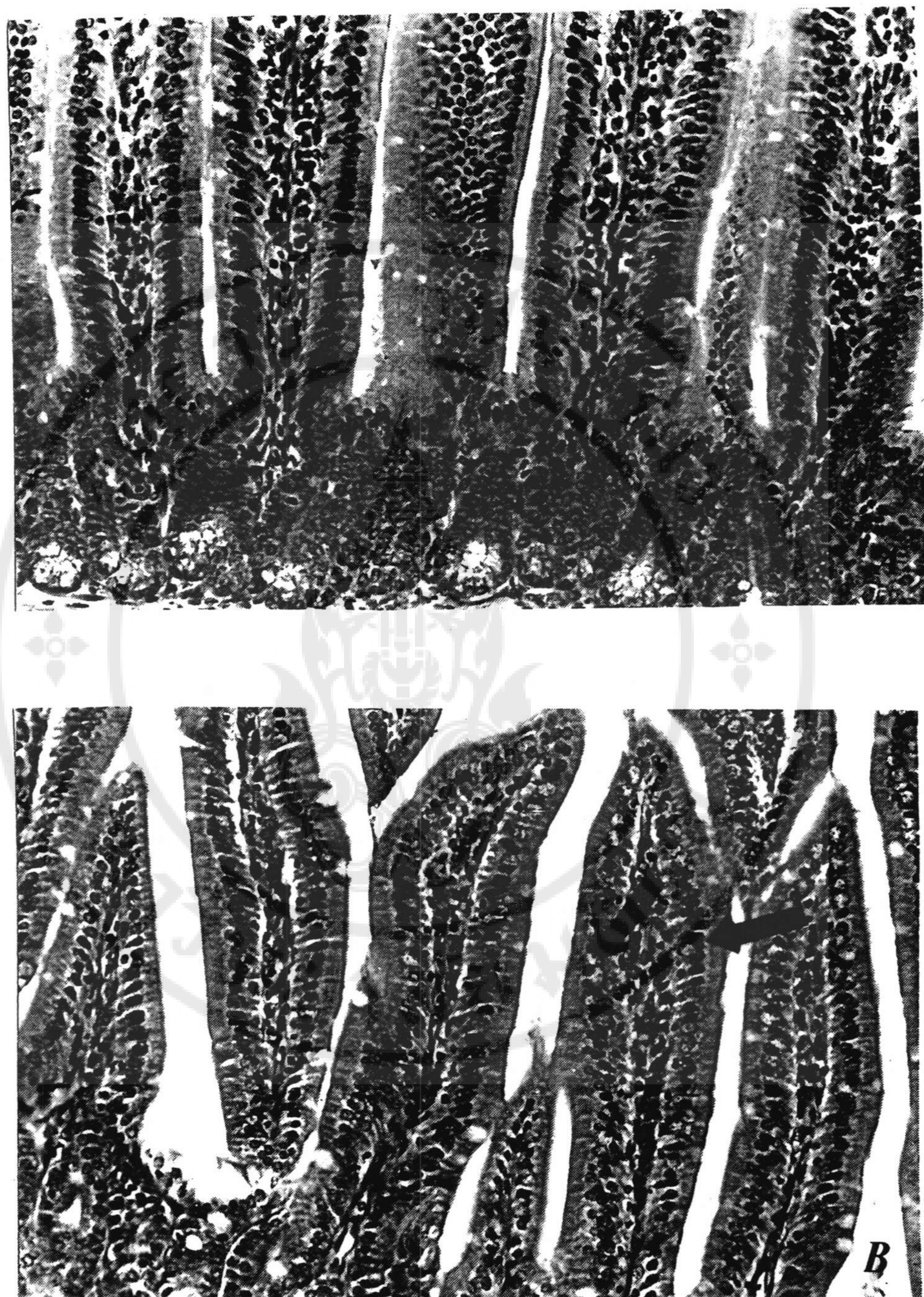

Figure 10. Light micrographs of the small intestine from control mice (a), and mice treated with 2,4,6-trihydroxyacetophenone (THA) at a dose of 150 mg/kg BW (b). THA was intragastrically given once a day for 30 consecutive days. Note active globlet cells with slightly swollen of nuclei of small intestinal mucosal cells in THA-treated group (arrowhead) (H&E, 200x).

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Experiment III: Effect of 2,4,6-Trihydroxy-Acetophenone Treatment on Hepatic Excretory Function in Normal Rats and in Ethinylestradiol-Induced Cholestatic Rats.

As 2,4,6-trihydroxyacetophenone (THA) induced an immediate stimulatory action on bile secretion, it is of interest to investigate whether THA treatment had beneficial or deteriorates effect to liver function. Therefore, this experiment is designed to evaluate the bile secretory capability and hepatic excretory function after an acute single and multiple THA administration in normal and cholestatic conditions. The hepatic excretory function was evaluated by using sulfobromophthalein (BSP) dye clearance.

I. Bile flow rate

Table 3 shows the effect of THA treatment on bile flow rate. In control animals, basal bile flow rate (BFR) was approximately 62.7$\pm$0.2 μl/kg. min (ranging from 62.3$\pm$0.4 to 69.6$\pm$0.3 μl/kg. min). It was steadily maintained at this level throughout 2 h of experimental period. An intraduodenal administration of THA immediately increased BFR, which peaked at 15 min after administration. BFR at peak was significantly increased to the level of 120.1$\pm$2.6 μl/kg. min (192% of control before THA administration) and 136.4$\pm$0.2 μl/kg. min (218% of control) by THA at doses of 50 and 100 mg/kg BW, respectively (Figure 11). After peak, BFR rapidly fell down in 15 min and then gradually declined to their base-line levels. Unlike the effect of a single acute THA administration, multiple administrations of THA for 5 days at a dose of 50 or 100 mg/kg BW did not significantly change basal secretion of bile or BFR, although they were slightly higher than that in control without THA

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treatment. As shown in Figure 12, basal BFRs in 5-days THA-treated animals at doses of 50 and 100 mg/kg BW were slightly elevated to the levels of 65.4±0.5 µl/kg. min and 67.6±0.5 2l/kg. min, respectively, as compared to 62.7±0.2 µl/kg. min in control. As THA had an immediate stimulatory effect on bile secretion, the marked changes essentially appeared within the period of 15 min. The slight change of BFR by multiple administrations, was probably due to the effect was not determined right after the THA administration. It represented the basal bile secretion.

In Figure 13, rats which were induced cholestasis by given ethinylestradiol, (EE 5 mg/kg BW), subcutaneously for 5 days showed a significant suppression of BFR to 36.6±0.2 µl/kg. min (58% of control without treatment) or 42% decrease as compared to that of control (62.7±0.2 µl/kg. min), indicating a cholestatic condition. The partial increase in the basal BFR was observed in animals those were concurrently given THA at doses of either 50 or 100 mg/kg BW, for 5 days of experimental period. The BFRs were significantly increased by both doses of THA as compared to those in the corresponding EE-treated rats. The improvement of BFR in EE-treated rats showed a dose-dependent to THA, indicating a beneficial effect of the concurrent THA treatment, although the obtained BFR at the employed doses were still lower than that of basal BFR in control (Figure 14). However, the acute effects of these same doses (THA at dose of 50 or 100 mg/kg BW) which immediately stimulated BFR was able to increase BFR in the EE-induced cholestatic rats, at peaked or at 15 min after THA administration to the basal level of control (Figure 15). Moreover, the peak of BFR stimulated by high dose of THA was even higher than that of basal control level. It was noted that after peak, BFR did not abruptly return to

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base-line levels, it was slowly declined. Both basal and peak levels of BFR in various experimental animals are summarized in Figure 16.

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Table 3. Effect of 2,4,6-trihydroxyacetophenone (THA) treatment on bile flow rate.

Treatment	Time (min)						
	0	15	30	45	60	90	120
Control	63.5±0.12	62.5±0.07	62.5±0.08	62.7±0.26	62.8±0.24	62.3±0.38	62.5±0.39
THA 50 mg/kg BW (single dose)	65.0±0.33	120.1±2.59*	80.5±0.12*	72.8±3.25	72.5±0.12	70.4±0.08	68.5±0.13
THA 100 mg/kg BW (single dose)	69.4±0.08	136.4±0.15*	103.8±0.15	85.2±0.24	80.6±0.06	77.8±0.65	74.3±0.07
EE 5 mg/kg BW (multiple dose)	37.8±0.31*	36.7±0.20*	36.4±0.06*	36.4±0.15*	36.4±0.09*	36.4±0.11*	36.1±0.20*
EE + THA 50 mg/kg BW (single dose)	37.4±0.39	63.5±1.22+	60.3±0.69	58.2±1.10	55.5±1.30	51.9±1.00	47.8±1.01
EE + THA 100 mg/kg BW (single dose)	37.5±0.56	72.6±0.16+	71.1±0.42	68.1±0.28	64.8±0.36	60.9±0.45	58.9±0.68
THA 50 mg/kg BW (multiple dose)	64.9±0.20	65.5±0.13	65.4±0.17	64.9±1.17	65.8±0.79	65.8±0.89	65.5±0.24
THA 100 mg/kg BW (multiple dose)	67.4±0.14	67.0±1.39	67.9±0.56	67.9±0.47	67.9±0.51	67.6±0.10	67.4±0.12
EE + THA 50 mg/kg BW(multiple dose)	42.9±0.47	42.2±1.13	42.9±0.44	42.7±0.52	42.8±0.24	42.7±0.14	43.8±1.75
EE + THA 100 mg/kg BW (multiple dose)	52.8±0.94	52.9±0.32	53.4±0.23	53.2±0.22	53.7±0.90	53.2±0.93	52.2±0.27

In single dose treatment, an acute stimulatory effect of THA-treatment on bile secretion was determined whereas in the multiple dose treatment, THA was given for 5 days and changes of bile flow rate were observed.

Values are means ± SEM from 8-10 animals.

*P < 0.05 significantly different from solvent control.

+P < 0.05 significantly different from the corresponding EE-treated rat.

EE = 17α ethinylestradiol

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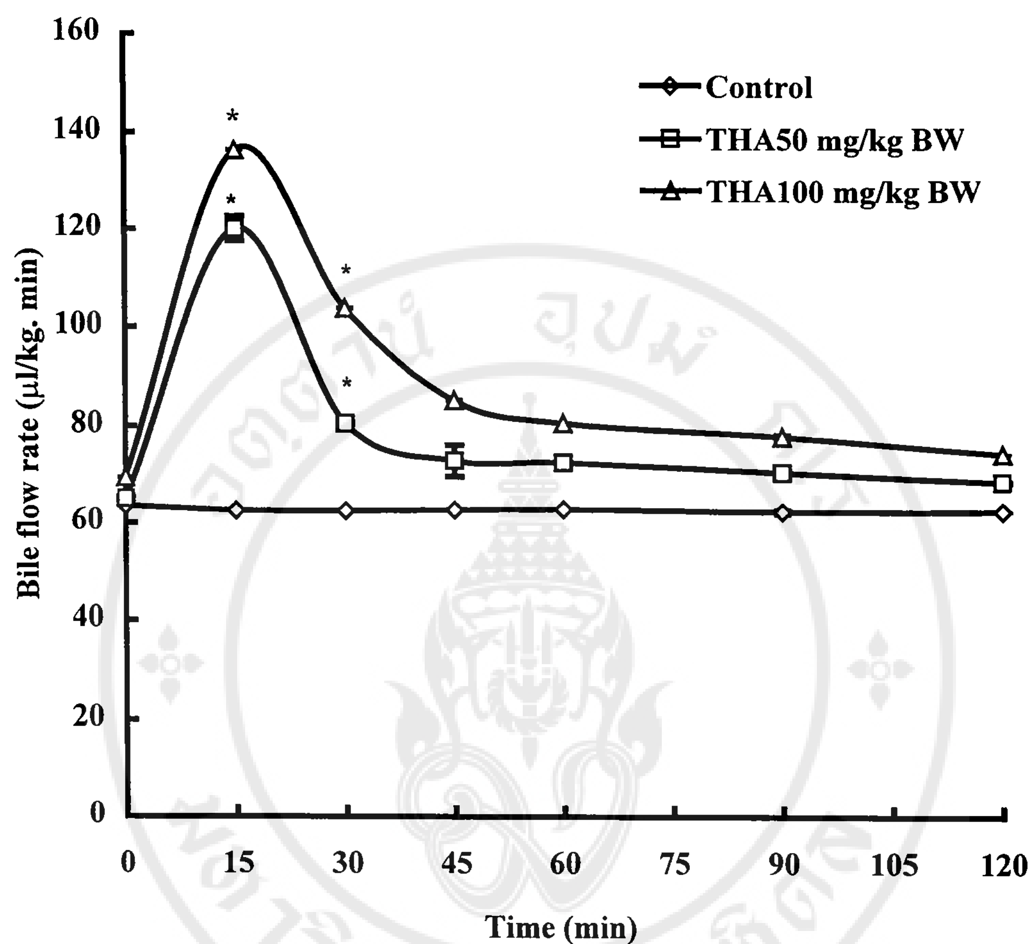

Figure 11. Acute effect of 2,4,6-trihydroxyacetophenone (THA) administration on bile flow rate (μl/kg. min) in adult male rats. THA at a dose of 50 or 100 mg/kg BW was intraduodenal administered. Values are means ± SEM from 8-10 animals.

*$p < 0.05$ significant difference from solvent control.

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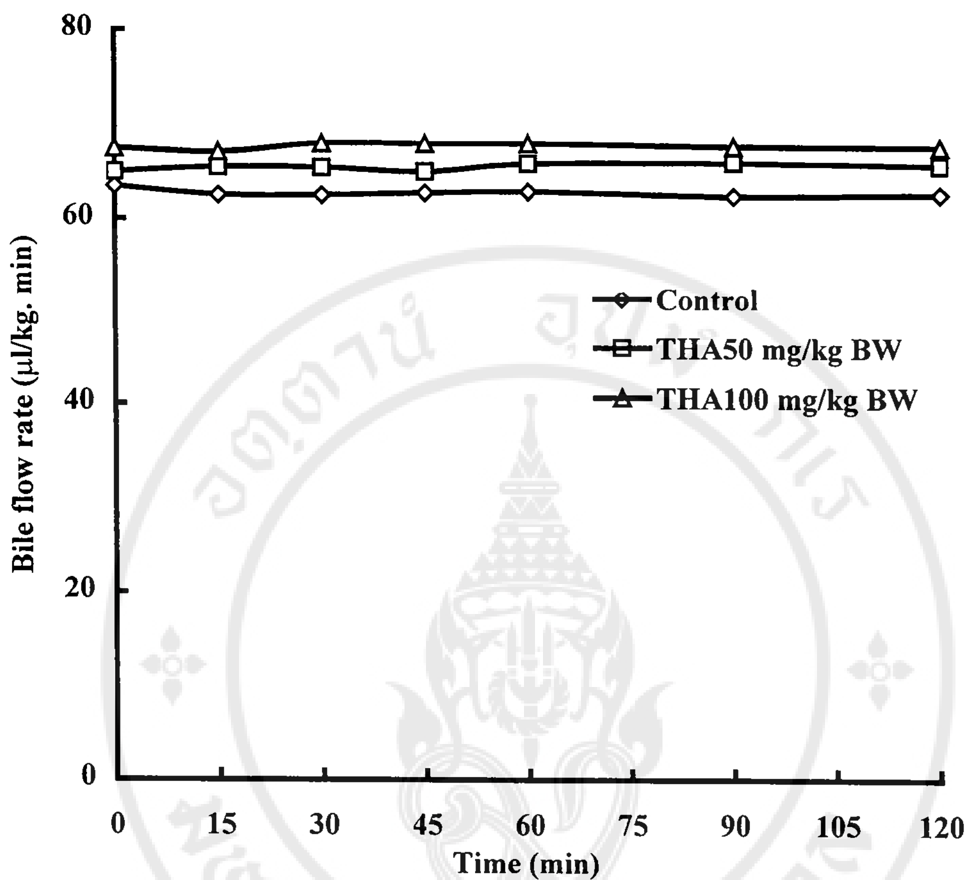

Figure 12. Basal bile flow rate (µl/kg. min) in control and 2,4,6-trihydroxyacetophenone (THA) treated rats. THA at a dose of 50 or 100 mg/kg BW were intragastrically administered for 5 days. Values are means ± SEM from 8-10 animals.

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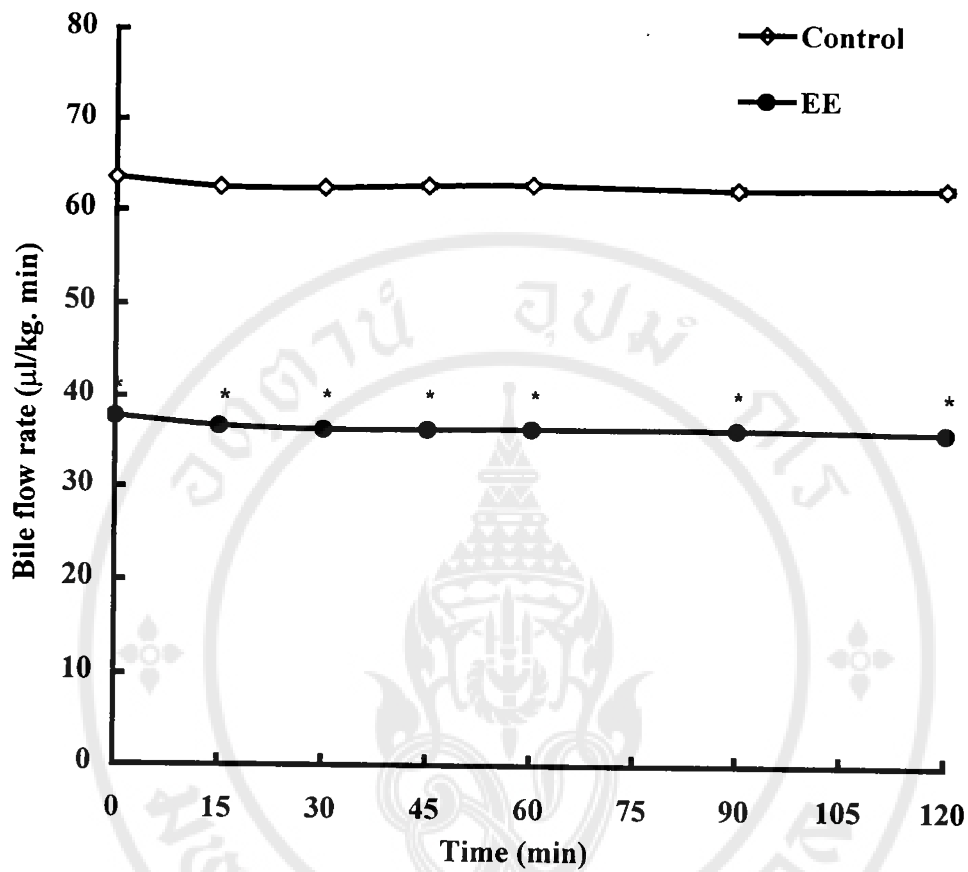

Figure 13. Bile flow rate (µl/kg. min) in control and ethinylestradiol (EE)-induced cholestatic rats (EE 5 mg/kg BW, subcutaneously, for 5 days). Values are mean ± SEM from 8-10 animals.

*$p < 0.05$ significant difference from solvent control.

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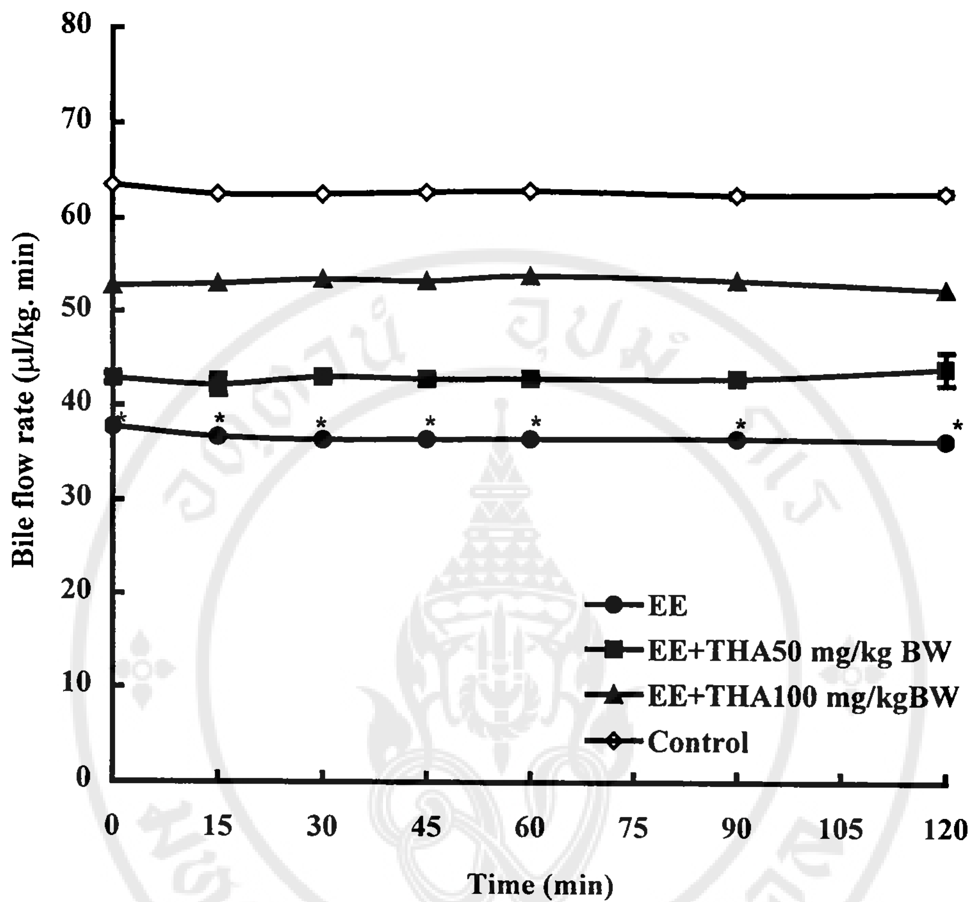

Figure 14. Effect of 2,4,6-trihydroxyacetophenone (THA) treatment on bile flow rate (µl/kg. min) in ethinylestradiol (EE)-induced cholestasis (EE 5 mg/kg BW, subcutaneously, for 5 days) with and without concurrent treatment with multiple doses of THA (50 or 100 mg/kg BW, intragastrically). Values are means ± SEM from 8-10 animals.

*$p < 0.05$ significant different from solvent control.

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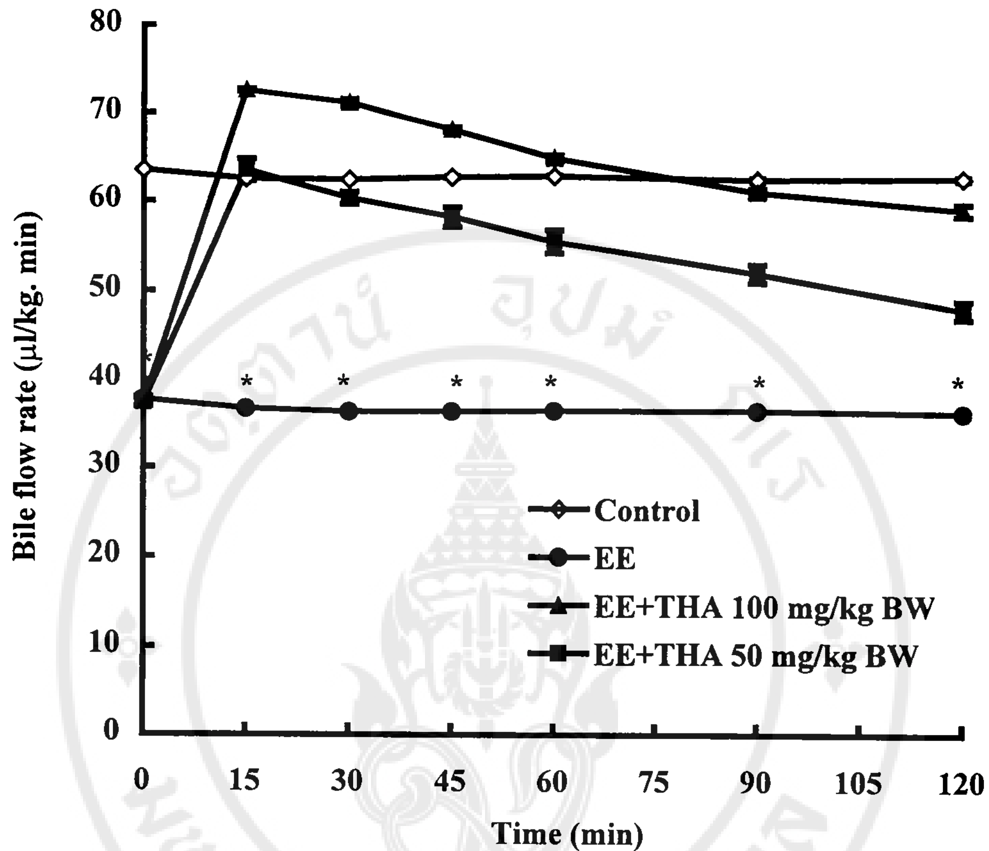

Figure 15. Effect of 2,4,6-trihydroxyacetophenone (THA) treatment on bile flow rate (µl/kg. min) in ethinylestradiol (EE)-induced cholestasis (EE 5 mg/kg BW, subcutaneously, for 5 days) with and without acute single THA administration (50 or 100 mg /kg BW, intraduodenal). Values are means ± SEM from 8-10 animals.

*$p < 0.05$ significant different from solvent control.

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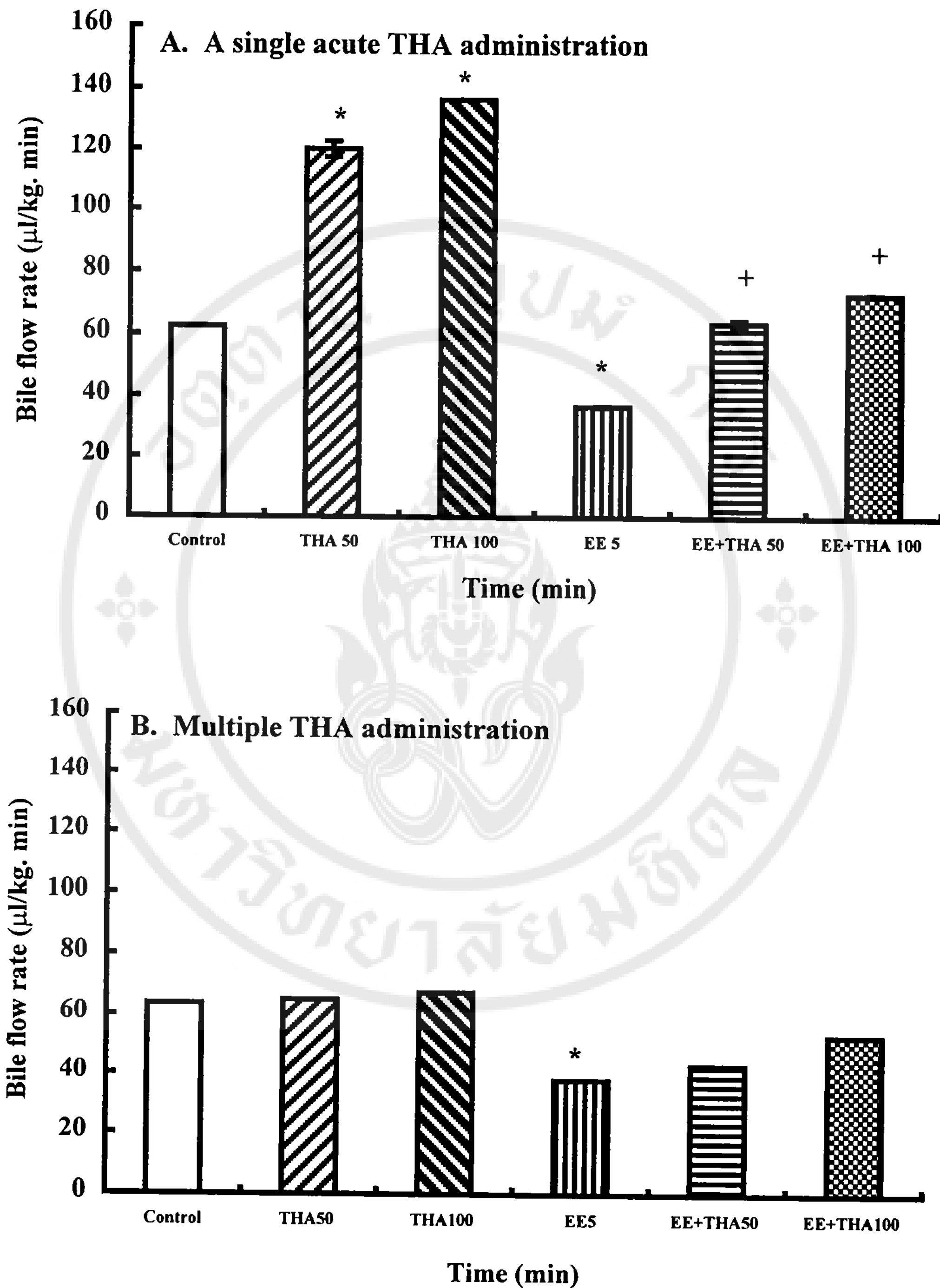

Figure 16. Effect of a single acute and multiple administration of 2,4,6-trihydroxyacetophenone (THA) on bile secretion. (A) BFR obtained at peak 15 min after a single intraduodenal (i.d.) THA administration and (B) basal BFR in multiple intragastric (i.g.) THA administration in both normal and EE-induced cholestatic rats.
$^{*}p < 0.05$ significantly different from normal control.
$^{+}p < 0.05$ significantly different from its corresponding EE-control.

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II. Plasma BSP concentration and biliary appearance rate of BSP

Table 4 and Figure 17 demonstrate profile changes of plasma BSP concentration with time after intravenous injection at a dose of 6.25 mg/kg BW (108). The initial plasma concentration of BSP which were obtained within first minute after BSP injection were comparable in various experimental groups including control rats, rats treated with a single intraduodenal of THA (50 or 100 mg/kg BW) with and without ethinylestradiol (EE)-induced cholestasis. In control rats, plasma BSP concentration rapidly decreased in first 15 min, it decreased from 1.38±0.03 mg % (100% of control) to 0.70±0.03 mg % (51% of control). At 45 min after injection, plasma BSP concentration was 0.18±0.05 mg % (13% of control). THA, which was known to stimulate bile secretion, enhanced clearance of BSP from plasma. At 15 min after injection, plasma BSP concentration decreased to 0.52±0.03 mg % (36% of control) and 0.42±0.03 mg % (29% of control) by THA administration at doses of 50 and 100 mg/kg BW, respectively. At 45 min after injection, BSP, which was almost cleared from plasma, the percent retention was 7% and 5% in THA treated at doses of 50 and 100 mg/kg BW, respectively. These two values were much lower than that in control.

In contrast, EE which is known to induce cholestasis significantly decreased clearance of BSP from plasma. At 15 min and 45 min injection, plasma BSP was decreased to 1.12±0.03 mg % (82% of control) and 0.59±0.03 mg % (43% of control), respectively. However, THA administration apparently increased BSP clearance in EE-cholestatic rats. Administration THA at doses of 50 and 100 mg/kg BW decreased plasma BSP concentration to 0.66±0.03 mg % (49% of control) and

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0.56±0.04 mg % (42% of control), respectively at 15 min and to 0.15±0.04 mg % (11% of control) and 0.12±0.03 mg % (9 % of control), respectively at 45 min. It was evident that acute THA treatment improved BSP clearance in EE-cholestasis to the level, which was comparable to that of control.

Table 6 and Figure 18 demonstrate appearance rates of BSP in bile with time or the biliary excretion rates of BSP after intravenous injection at a dose of 6.25 mg/kg BW in control rats, rats treated with a single dose of THA in ethinylestradiol (EE)-induced cholestatic rats with and without concurrent treatment with THA. In control rats, the biliary excretion rate of BSP or the appearance rate was 0.74±0.03 and 1.17±0.05 mg/min/kg BW at 15 min and 45 min after administration, respectively. In rats treated with THA at a dose of 50 mg/kg BW, the appearance rate of BSP in bile was significantly increased to 1.01±0.04 and 1.36±0.03 mg/min/kg BW at 15 and 45 min, respectively. THA at a higher dose of 100 mg/kg BW, further increased the appearance rate of BSP in bile to 1.14±0.03 and 1.38±0.03 mg/min/kg BW at 15 and 45 min, respectively. In EE-induced cholestasis rats, the appearance rate in bile was very low which was 0.45±0.04 and 0.76±0.04 mg/min/kg BW at 15 and 45 min, respectively. The appearance rate of BSP in bile after concurrent treatment with THA at a dose of 50 mg/kg BW was increased to 0.66±0.02 and 1.02±0.04 mg/min/kg BW at 15 and 45 min, respectively. THA at a higher dose of 100 mg/kg BW, further increased appearance rate of BSP in bile to 0.96±0.04 and 1.21±0.05 mg/min/kg BW at 15 and 45 min, respectively.

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Table 5 and Figure 19 demonstrate profile changes of plasma BSP concentration with time after intravenous injection at a dose of 6.25 mg/kg BW (108).

The initial plasma concentrations of BSP which were obtained within first minute after BSP injection were comparable in various experimental groups including control rats, rats-treated with THA (50 or 100 mg/kg BW) with and without ethinylestradiol (EE)-induced cholestasis. In control rats, plasma BSP concentration rapidly declined in first 15 min, it decreased from 1.38±0.03 mg % (100% of control) at the beginning to 0.70±0.03 mg % (51% of control). After this first 15 min, the decreasing concentration was slow down and at 45 min after injection, plasma BSP concentration was approximately 0.18±0.04 mg % (13% of control). It was anticipated that THA, which was known to stimulate bile secretion, would enhance plasma clearance of BSP. Indeed, at 15 min after THA administration at a dose of 50 or 100 mg/kg BW, plasma BSP concentrations were decreased to 0.64±0.05 mg % (44% of control) and 0.40±0.04 mg % (27% of control), respectively, indicating a clearance rate was enhanced in a dose-dependent by THA. At 45 min after injection, BSP, which was almost cleared from plasma, was approximately 9% and 7% left in THA-treated at doses of 50 and 100 mg/kg BW, respectively.

In contrast, EE, which is known to induce cholestasis significantly, decreased plasma clearance of BSP. At 15 min and 45 min after injection, plasma BSP concentration was decreased to 1.1±0.03 mg % (83% of control) and 0.59±0.03 mg % (43% of control), respectively (Table 5). However, THA administration apparently increased BSP clearance in EE-cholestatic rats. Administration THA at a dose of 50 or 100 mg/kg BW enhanced plasma clearance of BSP by decreasing plasma BSP concentration to 1.03±0.03 mg % (77 % of control) and 0.90±0.02 mg % (67 % of

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control), respectively, at 15 min and to 0.48±0.03 mg % (35% of control) and 0.32±0.04 mg % (23 % of control), respectively, at 45 min.

BSP is the compound, which is disposed via biliary excretion. Disappearance of BSP from plasma would accompany with its appearance in bile. Table 7 and Figure 20 demonstrate an appearance rates of BSP in bile with time or the biliary excretion rate of BSP after intravenous injection at a dose of 6.25 mg/kg BW. In control rats, the biliary appearance rates of BSP or cumulative concentrations in bile were 0.75±0.03 and 1.19±0.04 mg/min/kg BW at 15 min and 45 min after administration, respectively. THA was the compound known to enhance bile secretion and BSP in plasma is eliminated via bile. Thus, it was anticipated that THA would increase BSP excretion in bile. Indeed, THA treatment at a dose of 50 mg/kg BW increased the appearance rate of BSP in bile to 1.00±0.02 and 1.31±0.03 mg/min/kg BW at 15 and 45 min after THA administration, respectively, while administration of THA at a dose of 100 mg/kg BW increased the appearance rate of BSP in bile to 1.11±0.03 and 1.37±0.04 mg/min/kg BW at 15 and 45 min, respectively. In EE-induced cholestatic rats, the biliary appearance rate of BSP was approximately 0.40±0.03 and 0.73±0.04 mg/min/kg BW at 15 and 45 min, respectively. The enhancing effect of THA on BFR and biliary excretion of BSP were observed in concurrent EE treatment with THA at dose of 50 or 100 mg /kg BW. The appearance rate of BSP in bile by concurrent treatment EE with THA at a dose of 50 mg/kg BW was 0.48±0.02 and 0.86±0.03 mg/min/kg BW at 15 and 45 min, respectively. After concurrent administration with THA at a dose of 100 mg/kg BW,

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the higher appearance rate of BSP in bile was observed, it was 0.60±0.03 mg/min/kg BW and 1.02±0.04 mg/min/kg BW at 15 and 45 min, respectively.

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Table 4. Plasma BSP concentration (mg %) after an acute single THA treatment.

Treatment	Plasma BSP concentration (mg %)							
	0 (min)	% retention	15 (min)	% retention	30 (min)	% retention	45 (min)	% retention
Control	1.38±0.03	100	0.70±0.03	51	0.36±0.03	26	0.18±0.05	13
THA 50 mg/kg BW	1.46±0.05	100	0.52±0.03	36	0.22±0.04	16	0.10±0.04	7
THA 100 mg/kg BW	1.45±0.04	100	0.42±0.03	29	0.15±0.03	11	0.07±0.03	5
EE 5 mg/kg BW (5 days)	1.36±0.03	100	1.12±0.03	82	0.82±0.03	59	0.59±0.03	43
EE+ THA 50 mg/kg BW	1.34±0.05	100	0.66±0.03	49	0.29±0.03	21	0.15±0.04	11
EE + THA 100 mg/kg BW	1.33±0.03	100	0.56±0.04	42	0.26±0.03	19	0.12±0.03	9

Values are means ± SEM from 8-10 animals.

Table 5. Plasma BSP concentration (mg %) after multiple THA treatment for 5 days.

Treatment	Plasma BSP concentration (mg %)							
	0 (min)	% retention	15 (min)	% retention	30 (min)	% retention	45 (min)	% retention
Control	1.38±0.03	100	0.70±0.03	51	0.36±0.03	26	0.18±0.05	13
THA 50 mg/kg BW	1.44±0.02	100	0.64±0.05	44	0.25±0.03	18	0.13±0.04	9
THA 100 mg/kg BW	1.47±0.04	100	0.40±0.04	27	0.15±0.03	11	0.09±0.03	7
EE 5 mg/kg BW (5 days)	1.32±0.02	100	1.10±0.03	83	0.82±0.03	59	0.59±0.03	43
EE + THA 50 mg/kg BW	1.34±0.03	100	1.03±0.03	77	0.70±0.03	51	0.48±0.03	35
EE + THA 100 mg/kg BW	1.34±0.02	100	0.90±0.02	67	0.58±0.03	42	0.32±0.04	23

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Table 6. Biliary excretion rate (mg/min/kg BW) of BSP after an acute single THA treatment.

Treatment	Biliary excretion rate of BSP (mg/min/kg BW)			
	0(min)	15(min)	30(min)	45(min)
Control	0	0.74±0.03	1.12±0.03	1.17±0.04
THA 50 mg/kg BW	0	1.01±0.04	1.29±0.04	1.36±0.03
THA 100 mg/kg BW	0	1.14±0.03	1.34±0.04	1.38±0.03
EE 5 mg/kg BW (5 days)	0	0.45±0.04	0.68±0.04	0.76±0.04
EE + THA 50 mg/kg BW	0	0.82±0.02	1.15±0.04	1.19±0.04
EE + THA 100 mg/kg BW	0	0.96±0.04	1.19±0.04	1.21±0.05

Values are means ± SEM from 8-10 animals.

Table 7. Biliary excretion rate (mg/min/kg BW) of BSP after multiple THA treatment for 5 days.

Treatment	Biliary excretion rate of BSP (mg/min/kg BW)			
	0(min)	15(min)	30(min)	45(min)
Control	0	0.75±0.03	1.13±0.03	1.19±0.04
THA 50 mg/kg BW	0	1.00±0.02	1.25±0.04	1.31±0.03
THA 100 mg/kg BW	0	1.10±0.03	1.35±0.04	1.37±0.04
EE 5 mg/kg BW (5 days)	0	0.40±0.03	0.68±0.04	0.73±0.04
EE + THA 50 mg/kg BW	0	0.48±0.02	0.80±0.03	0.86±0.03
EE + THA 100 mg/kg BW	0	0.60±0.03	0.92±0.03	1.02±0.04

Values are means ± SEM from 8-10 animals.

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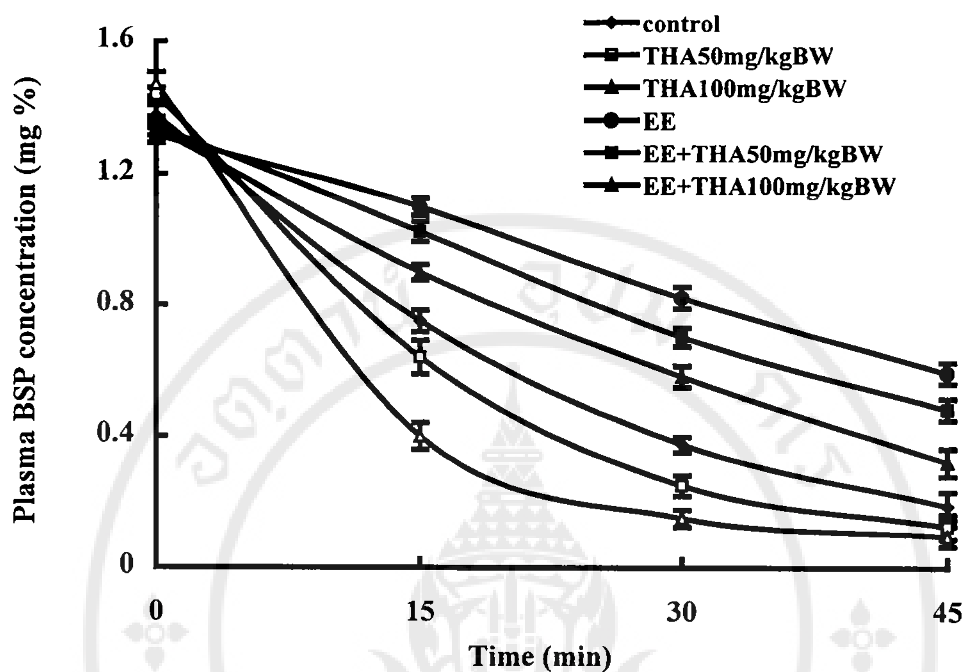

Figure 17. Effect of an intraduodenal administration of 2,4,6-trihydroxyacetophenone (THA) (50 or 100 mg/kg BW) on plasma concentration of BSP in adult male rat, and rats with and without concurrent treatment with ethinylestradiol (EE) in EE-induced cholestasis. The cholestatic rats were induced by subcutaneous injection with EE 5 mg/kg BW for 5 days. Values are means $\pm$ SEM from 8-10 animals.

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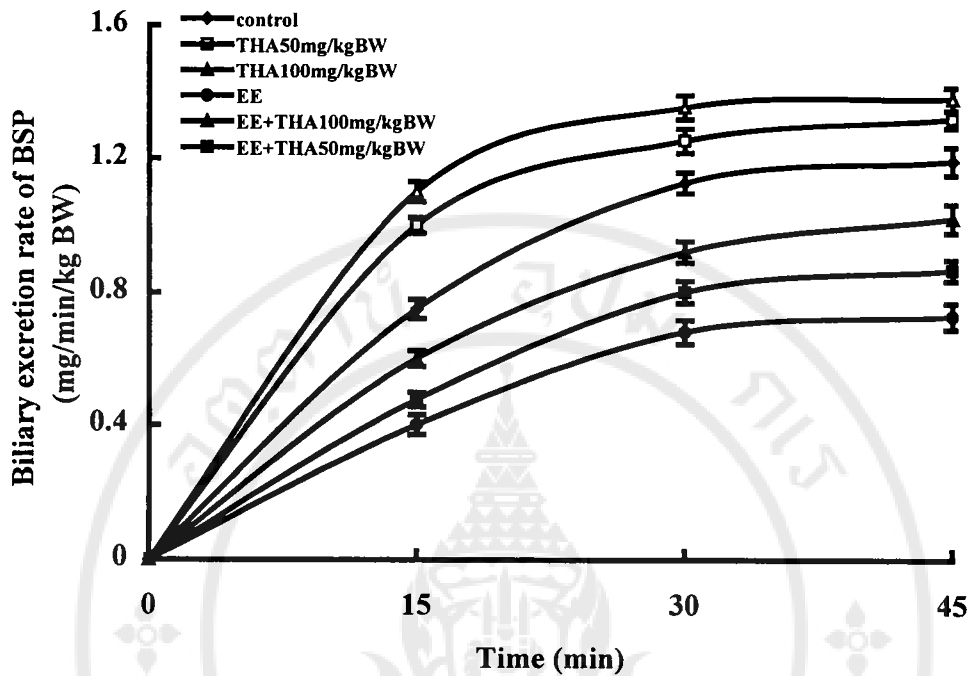

Figure 18. Effect of 2,4,6-trihydroxyacetophenone (THA) treatment (50 or 100 mg/kg BW, intraduodenal (i.d.), single dose) on the biliary excretion of BSP in adult male rat, and rats with and without concurrent treatment with ethinylestradiol (EE) in EE-induced cholestasis. The cholestatic rats were induced by subcutaneous injection with EE 5 mg/kg BW for 5 days. Values are means ± SEM from 8-10 animals.

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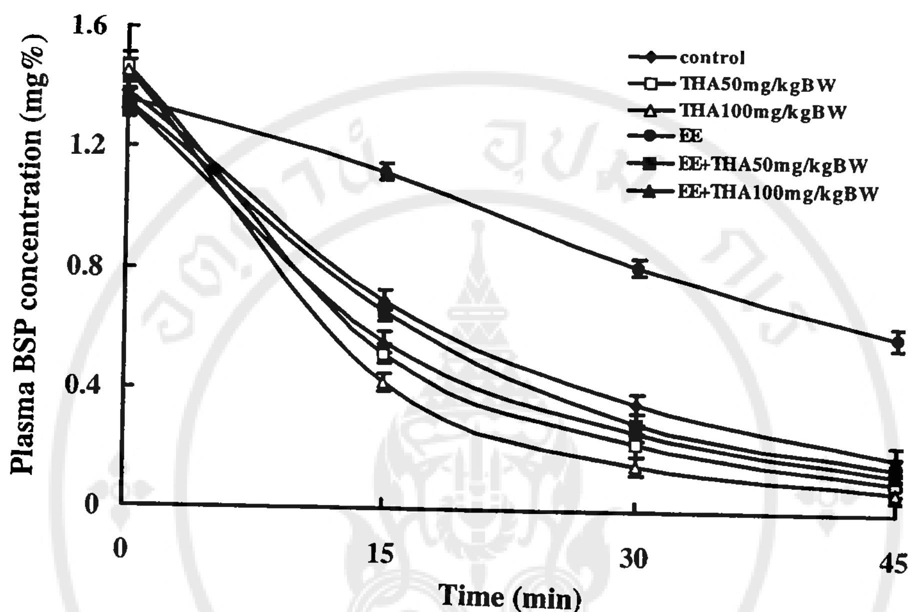

Figure 19. Effect of 2,4,6-trihydroxyacetophenone (THA) treatment (50 or 100 mg/kg BW, i.g., for 5 days) on plasma concentration of BSP in adult male rat, and rats with and without concurrent treatment with ethinylestradiol (EE) in EE-induced cholestasis. The cholestatic rats were induced by subcutaneous injection with EE 5 mg/kg BW for 5 days. Values are means ± SEM from 8-10 animals.

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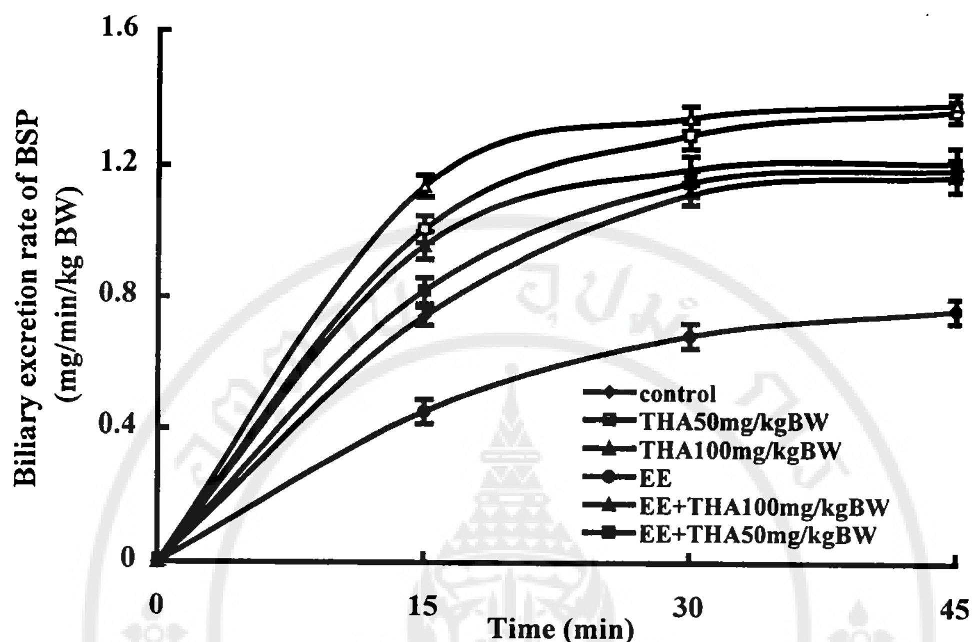

Figure 20. Effect of 2,4,6-trihydroxyacetophenone (THA) treatment (50 or 100 mg/kg BW, intragastrically, for 5 days) on the biliary excretion of BSP in adult male rat, and rats with and without concurrent treatment with ethinylestradiol (EE) in EE-induced cholestasis. The cholestatic rats were induced by subcutaneous injection with EE 5 mg/kg BW for 5 days. Values are means ± SEM from 8-10 animals.

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III. Effect of 2,4,6-trihydroxyacetophenone (THA) on plasma alkaline phosphatase (AP) activity

Effects of THA on plasma levels of alkaline phosphatase in both acute and 5 days treatment are shown in Table 8 and Figure 21a. In control rats, the basal enzyme activity was approximately 34.0±0.5 IU. A single administration of THA at either 50 or 100 mg/kg BW did not significantly alter the enzyme activity as compared to that of control. In contrast, the enzyme activity in rats-treated with EE was significantly increased to 172.8±4.3 IU. However, a single administration of THA at dose of 50 or 100 mg/kg BW to these EE-induced cholestasis decreased the enzyme activities which were obtained at 1 h after THA administration to control level (172.8±4.3 IU to 36.3±0.2 IU and 172.8±4.3 to 36.2±0.4 IU, respectively).

Table 8 and Figure 21b show the effect of multiple THA treatment (50 and 100 mg/kg BW, intragastrically for 5 days) on plasma alkaline phosphatase activities in rats with and without ethinylestradiol (EE)-treatment in EE-induced cholestasis. THA treatment at either dose of 50 or 100 mg/kg BW did not significantly affect the plasma alkaline phosphatase activity, they were 36.3±1.2 IU, and 36.7±0.9 IU, respectively. In EE-induced cholestasis, the plasma activity, which was significantly elevated to 172.8±4.3 IU, was not reversed by THA treatment. The concurrent treatment with THA at either dose of 50 or 100 mg/kg BW slightly decreased to 142.8±1.7 IU and 115.8±0.2 IU, respectively, but they were statistically significant difference from the corresponding EE-treatment ($P < 0.05$). From the results, it indicated that THA partially corrected the rise of plasma alkaline phosphatase activity in EE-treated rats.

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IV. Effect of 2,4,6-trihydroxyacetophenone (THA) on total plasma bilirubin

Table 9 and Figure 22a demonstrate effect of THA-treatment on total plasma bilirubin in rats with and without ethinylestradiol (EE)-treatment in EE-induced cholestasis. In control rats, total plasma bilirubin was approximately 0.94±0.02 mg %. THA-treatment at a single dose of either 50 or 100 mg/kg BW, intraduodenal did not significantly affect the total plasma bilirubin as compared to that of control. In contrast, in rats treated with EE, the total plasma bilirubin was significantly increased to 1.73±0.04 mg %. A single administration of THA at doses of 50 or 100 mg/kg BW, or a concurrently treatment with EE, significantly affect the total plasma bilirubin as compared to that of EE-treatment. They were slightly reduced to 1.63±0.02 mg % and 1.58±0.03 mg %, by THA at a dose of 50 and 100 mg/kg BW, respectively.

Table 9 and Figure 22b demonstrate the effect of multiple THA-treatment (50 and 100 mg/kg BW, intragastrically for 5 days) with and without ethinylestradiol (EE)-treatment in EE-induced cholestasis. In control rats, treatment with THA at doses of 50 or 100 mg/kg BW slightly increased total plasma bilirubin, but no statistically significant differences. The values were increased from 0.94±0.02 mg % in control to 1.04±0.02 mg % and 1.12±0.02 mg %, respectively. In EE-induced cholestasis rats, the total plasma bilirubin which was significantly increased (1.73±0.04 mg %) was slightly decrease by concurrent treatment with THA either dose of 50 or 100 mg/kg BW. The total plasma bilirubin was decreased to 1.66±0.02

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mg % and 1.59±0.02 mg %, respectively, which were significantly higher than that in control.

Therefore, a single dose of THA administration could effectively decreased the increased of plasma alkaline phosphatase in EE-cholestatic rats but not for bilirubin. The multiple treatment of THA could not be able to correct the increased of plasma bilirubin in EE-cholestatic rats.

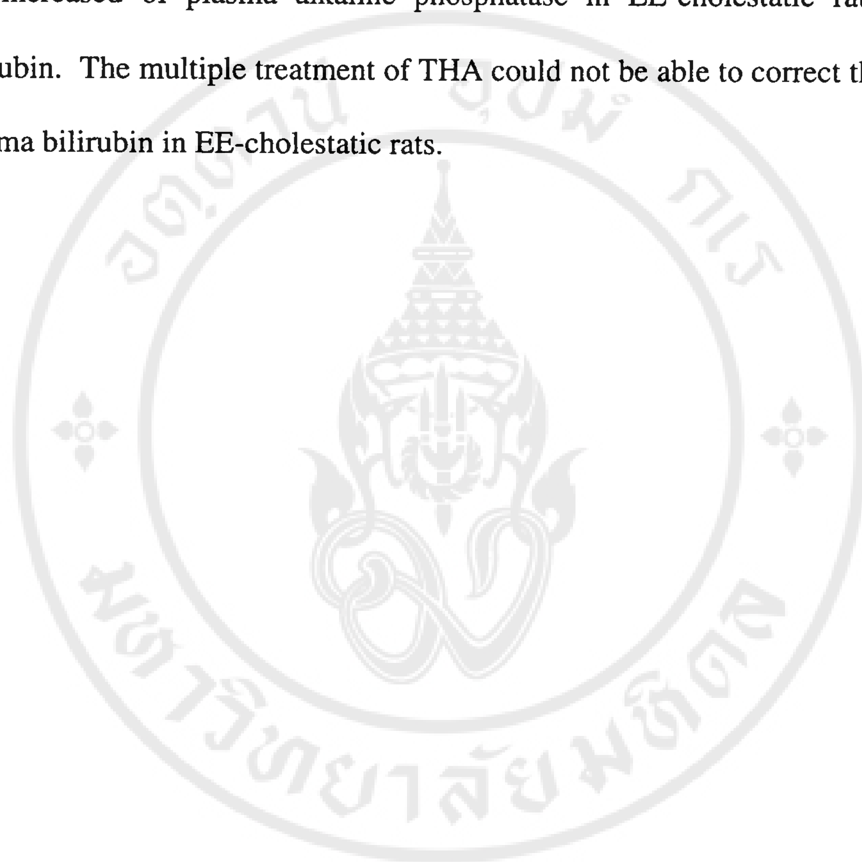

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Table 8. Effect of 2,4,6-trihydroxyacetophenone (THA) on plasma activity of alkaline phosphatase.

	Alkaline Phosphatase (IU/ml)	
	Acute treatment	**5-Days treatment**
Control	34.04±0.53	34.04±0.53
THA 50 mg/kg BW	34.84±0.79	36.34±1.18
THA 100 mg/kg BW	36.34±0.98	36.70±0.91
EE 5 mg/kg BW	172.80±4.32*	172.80±4.32*
EE+ THA 50 mg/kg BW	36.26±0.23⁺	142.78±1.71
EE + THA 100 mg/kg BW	36.22±0.37⁺	115.82±0.21

Values are means ± SEM from 8-10 animals.

*P < 0.05 significant difference from control.

⁺P < 0.05 significant difference from EE-treatment.

Table 9. Effect of 2,4,6-trihydroxyacetophenone (THA) on total plasma bilirubin concentration.

	Total bilirubin (mg %)	
	Acute treatment	**5-Days treatment**
Control	0.94±0.02	0.94±0.02
THA 50 mg/kg BW	0.95±0.02	1.04±0.02
THA 100 mg/kg BW	0.99±0.04	1.12±0.02
EE 5 mg/kg BW	1.73±0.04*	1.73±0.04*
EE + THA 50 mg/kg BW	1.63±0.02	1.66±0.02
EE + THA 100 mg/kg BW	1.58±0.03	1.59±0.02

Values are means ± SEM from 8-10 animals.

*P < 0.05 significant difference from control.

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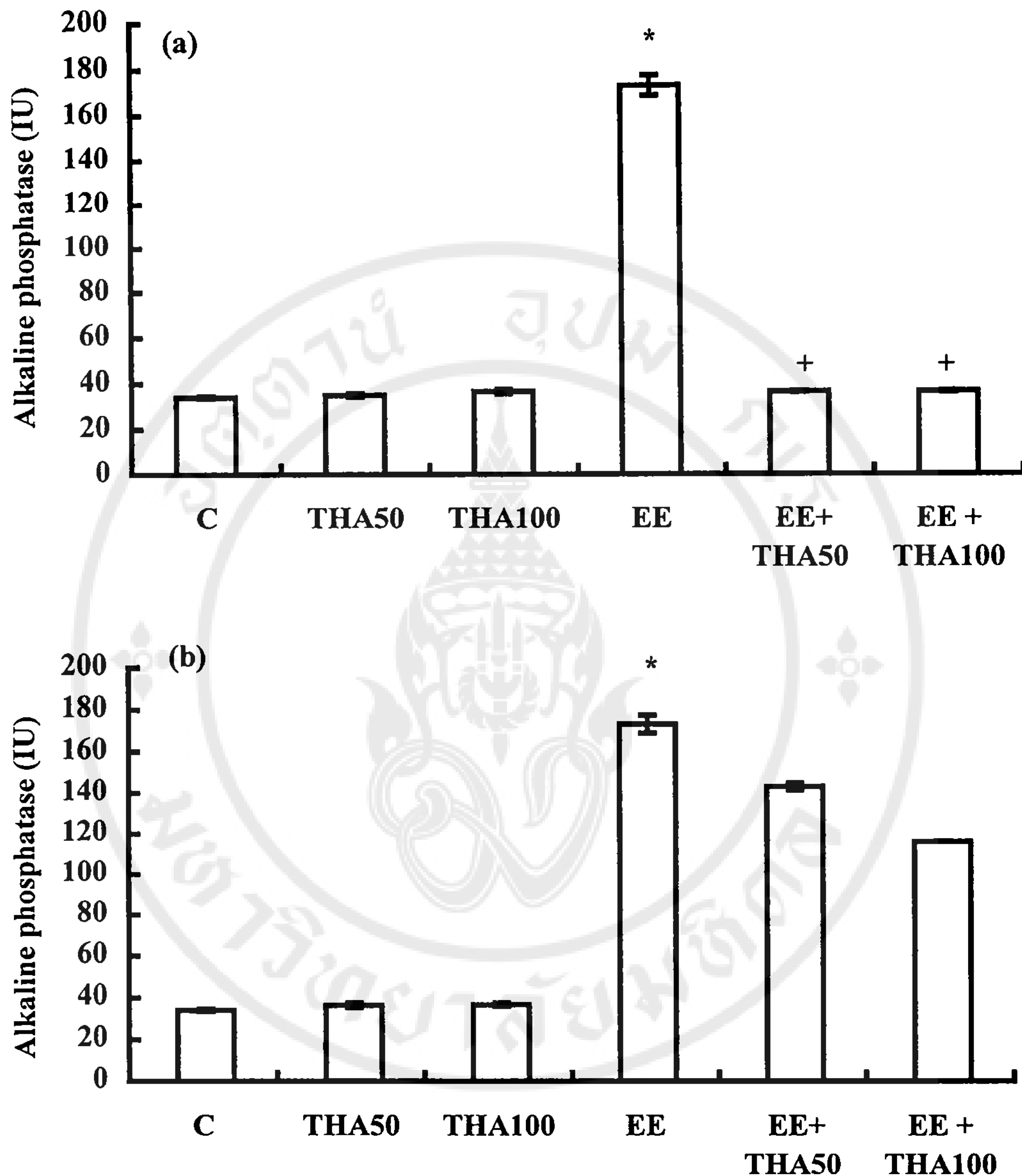

Figure 21. Effect of 2,4,6-trihydroxyacetophenone (THA) treatment on plasma activity of alkaline phosphatase in (a) an acute treatment and (b) multiple treatment for 5 days. THA at a dose of 50 or 100 mg/kg BW was administered to rats with and without concurrent treatment with ethinylestradiol (EE) in EE-induced cholestasis. The cholestatic rats were induced by subcutaneous injection with EE mg/kg BW for 5 days. Values are means $\pm$ SEM from 5 animals.
$*p < 0.05$ significantly different from normal control.
$^{+}p < 0.05$ significantly different from corresponding EE cholestasis control.

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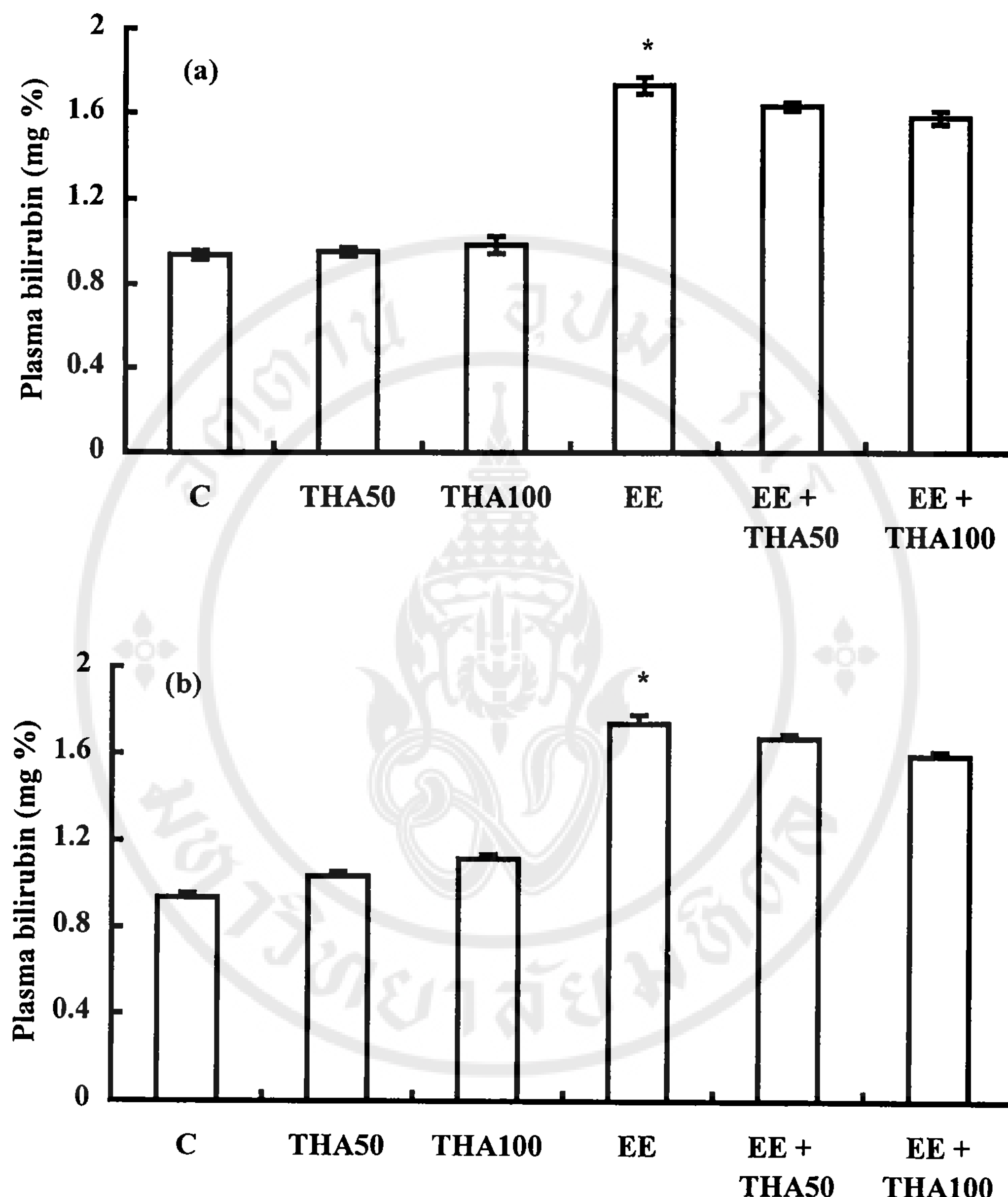

Figure 22. Effect of 2,4,6-trihydroxyacetophenone (THA) treatment on total plasma bilirubin in (a) an acute treatment and (b) multiple treatment for 5 days. THA at a dose of 50 or 100 mg/kg BW was intraduodenally and intragastrically administration to rats with and without ethinylestradiol (EE) in EE-induced cholestasis. The cholestatic rats were induced by subcutaneous injection with EE mg/kg BW for 5 days. Values are means + SEM from 5 animals.
*$p < 0.05$ significantly different from normal control.

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CHAPTER VI

DISCUSSION

Phloracetophenone (2,4,6-trihydroxyacetophenone, THA), the aglycone part of phloracetophenone glucoside, a naturally occurring compound has previously been reported to stimulate bile secretion by enhancing bile acid excretion which in turn, decreased plasma cholesterol. The compound with such actions has the potential for development as a therapeutic agent for treatment cholestasis and lowering plasma cholesterol in hypercholesterolemic patients. However, there has been no information available regarding toxicity of THA. Therefore, this study aims to investigate acute and subacute toxicity of THA and its effect on hepatic excretory function.

Acute Toxicity of THA

This study investigated acute toxicity of THA in various animals species including mice, rats and hamsters of both sexes using two routes of administration, either intraperitoneally or intragastrically. The result indicated that acute toxicity of THA essentially depended upon species and sexes of animals and also the route of administration. However, no age differences in response to toxic effect of THA was observed. According to toxicity rating chart (109), the result of the present study indicated that THA was practically classified as a non-toxic compound to rats when it was given by an oral route. The LD_{50} value in adult male and female rats were more than 6 g/kg BW. The data on acute toxicity of THA in this study was consistent with

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an earlier report in our laboratory (107), in which the acute toxicity of THA was evaluated by giving the compound either i.p. or i.g. to Swiss albino male mice. LD_{50} values by i.p. administration was 365-370 mg/kg BW and was 3.2 g/kg BW for i.g. route (107). Likewise, THA administration induced an increase in locomotor activity and convulsion in adult male mice. In addition, in the present study the susceptibility of the animals to the THA was found to vary with species of animals. Among three animals species tested; hamsters and mice appeared to be more sensitive to THA than rats. LD_{50} value via intraperitoneal (i.p.) route in adult male hamsters and mice were 338 and 365 mg/kg BW, respectively whereas the rats showed the least sensitive to THA. LD_{50} value in adult rats was much higher than those in mice and hamsters. LD_{50} value for i.g. was more than 6 g/kg BW. The species differences in response to toxic chemicals might be due to the variations in detoxification mechanisms or the efficiency of the detoxification system in each animal species (110). Remarkable qualitative and quantitative differences in enzymatic activities between species have been reported in a number of studies (111). For example; among mammalian species, guinea pig has higher activity of UDPG-glucoronosyl transferase (112). In contrast, cats are deficient in glucuronyl transferase activity, but have high sulfotransferase activity (113). A deficiency in one pathway can shift to other pathway since similar functional groups are conjugated by these two enzyme system. In addition, other factors such as absorption, distribution, excretion, and metabolism by bacteria in the gastrointestinal tract also played parts of toxicity in several animals species. For example, Stevioside, a sweetening agent is not toxic to rats but very toxic to hamsters

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as the bacteria in the intestinal lumen of hamsters could convert stevioside into steviol which was toxic and probably responsible for the observed toxic effects (114). In addition, cholesterol containing diet which effectively induced hypercholesterolaemia in hamsters, but not in rats which has a tight regulation of plasma cholesterol. Rats had higher basal activity of hepatic cholesterol 7-α hydroxylase activity to convert cholesterol into bile acid and excrete out into feces (115). The variation in the detoxification mechanisms among animals species reflected their genetic components which might account for our finding on the species differences in response to THA. The susceptibility of the animals to the THA were also found to vary with sexes of animals. In all of our animals studied, males appeared to be more sensitive than females. The LD_{50} values in males were consistently less than those in females in all cases (Table 1). Sex-related differences have frequently been observed in responses to toxic chemicals and appeared to relate to hormone. It also can be substrate dependent. For example, hexobarbital sleeping time is markedly prolonged in the female rats as compound to male rats (116). Plasma half-life of the compound was considerably longer in the female and the rate of biotransformation was significantly lower over that measured in males (117). It may involve both Phase I and Phase II enzyme systems which are be susceptible to levels of androgens and estrogens (118). In contrast, the sulfate conjugation of steroid hormones has been reported to have sex differences. Female rats have five-fold higher activity for cortisol metabolism than male rats. This sex difference in cortisol metabolism is apparently due to a suppression of sulfotransferase by male hormone whereas ovarian hormone may has

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stimulating activity (119, 120). Therefore, the sex differences in response to THA might relate to hormone-related metabolic pathway of THA.

Administration of THA by different routes also influenced toxicity in hamsters and mice. LD_{50} values by i.g. were approximately 8 to 10 times higher than that by i.p. THA was most toxic when given i.p., but the amount to induce toxicity was increased when it was administered by i.g.. The reason for high toxicity of THA by i.p. route might be due to a rapid absorption of THA by this route. Absorption from the peritoneum is, in general, more rapid than oral absorption (30). By oral route, the compound may be subjected to the first-pass metabolism by liver. Thus, it is difficulties in achieving significant plasma levels as slow oral absorption may be overcome without significant qualitative differences. In addition, the higher concentration of THA preparation tended to form colloid in aqueous media and as a consequence, less absorption might occur. However, the preparation of THA used for i.p. injection was miscible with the aqueous solution. General signs and symptoms were found to be similar among these animal species after being treated with THA. The similarity in neurological responses included hyperactivity, drowsiness, and convulsion, which were observed in all animals species treated with THA by different routes. These finding indicated that the effects were probably produced by the similar substances, while solvent used had no detectable effect. The exactly toxic effect of THA to animals was not known. However, the present result demonstrated that after i.p. administration of the toxic dose of THA, the animals became excited and finally

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develop convulsion within 30-60 min. These results indicated that THA might act on the nervous system. The cause of death was identified to be due to respiratory paralysis.

Subacute Toxicity of THA

Eventhough the data on the acute toxicity following the LD_{50} value indicated that THA was the practically a non-toxic compound. However, the acutely non-toxic compound may be toxic after prolonged exposure, even at low doses, due to accumulation and disruption of physiological and biochemical homeostasis. Biochemical effects of toxicants can occur in any part of an organism, which constitute the basis of an organism's response to toxic substances. Therefore, biochemical interactions are very important in the establishment of target organ toxicity. In this study the biochemical adverse effects which were resulted from repeated exposure of mice to THA at different doses for 30 consecutive days were examined.

Effect of THA-treatment on body weight and liver weight

Body weight and liver weight are frequently employed as indications of adverse effects after exposure to the toxic chemicals. Reduction of body weight gain is a simple and sensitive index of toxicity in general. In addition, the relative organ weights (to body weight) are a valuable tool for identifying possible target organs (121). The body weight data may associate with feed consumption data and may indicate changes in appetite as well as changes in the efficiency of feed utilization by

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the body. In the present study, THA-treatment to mice via i.g. route for 30 consecutive days did not induce any statistically significant difference in both body weight and liver weight from those in controls (Table 2 and Figure 1). In addition, no mortality of animal was observed throughout the period of treatment. From these finding it suggested that THA might not interfere in the process of food absorption and general metabolism of the body which allowed all experimental animals gaining their body weights.

Effect of THA-treatment on plasma activities of AST and ALT

Elevated levels of serum enzymes, which have a specific tissue origin, are of the great value in identifying conditions of the target organs insulted by toxic chemicals. For example, an increase in serum ALT out of normal range level may indicate hepatotoxicity. While an increase in serum AST activitie may suggest a general tissue damage. Because AST is present in a wide variety of tissues, including heart, skeletal muscle, kidney, and brain in addition to liver, while ALT appears to be localized primarily in the liver (122). In the present study, THA treatment for 30 consecutive days might alter the structural integrity of the hepatocellular membrane. It was evident by the increases in plasma ALT and AST levels after treatment. For the toxic action of THA, THA itself might act directly at liver cells. This was supported by the histopathological changes of liver that high dose of THA caused zonal degeneration at periportal area. The affected area was associated with vacuolization of hepatocyte and degenerated nuclei. In addition, the hepatocyte alteration was also associated with plasma membrane leakage, which can be detected biochemically by

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determination of liver cytosol-derived enzymes (123). Changes of hepatocyte and plasma hepatic enzyme in the present study were consistent with the features of the hepatic degeneration. The main affected area of liver was at the periportal region or metabolic zone 1 and sometimes extended deeply into central vein area (Figure 6). With high dose of THA (150 mg/kg BW), the periportal hepatocytes which were extensively damaged contained a number of vacuolization, probably in cytoplasm. It was not known whether they were phagocytic vacuoles or degenerated nuclei. In the present study, THA specifically damaged cells in zone 1 which suggested that it might have been THA it self, not its metabolites, to exert the toxic effects. Although the mechanism by which THA caused hepatocyte alteration was not clear, two different reasons have generally been proposed to explain the periportal cell damages. Firstly, the periportal area is the first area of the hepatic lobular to be exposed to a toxin being delivered via the blood stream. These periportal hepatocytes may receive the largest dose of the toxin whereas the cells further down the sinusoid may be partly protected by removal of the toxicant in the periportal area. Secondly, it may be due to metabolic zonation since higher oxygen tension in the portal than in the central lobular area of the liver. Both reasons might be the case of the present study to account for the degeneration of hepatocytes by THA since the effects were observed at high dose and showed zonal degeneration. From diversity of chemical structures and the biochemical basis for hepatoxicity, the metabolically generated toxins can be classified as free radicals or as containing strong electrophilic centers. CCl_4 is the best studied for the proposed free radical types of toxins (124). For electrophilic toxins, their molecules contain centers that are capable of accepting an incoming pair of

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electrons. In the present study, it was not clear whether THA molecule contained strong electrophilic centers, which would undergo accepting electron pairs causing the zonal changes of hepatocytes. However, the characteristic of the zonal alterations with high dose of THA could also be due to the high concentration of THA reaching hepatocytes, which might not be related to the degree of oxygenation of blood.

Effect of THA-treatment on total bilirubin

Bilirubin is the bile pigment in which hemoglobin from damaged or aged erythrocytes is metabolized through a series of biochemical reactions. Bilirubin is transported to the liver via the bloodstream or via a saturable and probably active transport process. The bilirubin is conjugated with glucuronide in the liver before being excreted into the bile and it is used to differentiate variety of jaundice such as intrahepatic jaundice, extrahepatic jaundice etc. The intrahepatic jaundice usually occurs because of hepatic conjugation failures, for example, neonatal physiological jaundice, transport disorders hepatocellular damage by viral hepatitis, toxic hepatitis, and cirrhosis, and intrahepatic obstruction. Extrahepatic or posthepatic jaundice usually occurs because of bile duct obstructions by stones, neoplasm. Serum bilirubin has been reported to increase with administration of high amount of chemical compounds including acetaminophen, acetylphenylhydrazine, aminopyrine, arsenic, cadmium (125), and complex chemical wastes (125) which injures liver. In this study total plasma bilirubin was slightly increased after subacute exposure of THA. Although, total plasma bilirubin at the high dose group was increased almost double, these values were within the normal range (Figure 3a). It is known that the hepatocytes have very high constitutive enzyme activities of many phases that add a

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polar group to a molecule and render them to be more readily excreted than the parent compound. Likewise, bilirubin after getting into the hepatocytes was conjugated by glucuronide and excreted into bile (125). The level of plasma bilirubin was dependent on the ability of liver to conjugate and eliminate it into bile. The slightly increased plasma bilirubin suggested that the conjugation activity of liver was slightly affected. Although the histopathological changes of liver in animals treated with high dose of THA demonstrated that THA caused a marked changes of liver cells when compared to control, it slightly interfered with bilirubin metabolism.

Effect of THA-treatment on blood urea nitrogen (BUN)

Blood urea nitrogen is derived from normal metabolism of protein and is excreted in the urine. Elevated BUN usually indicates glomerular damage. However, poor nutrition and hepatotoxicity can also affect its level, which are common effects of many toxicants. From the present result, histopathological examination of kidney demonstrated that after treatment of THA at high dose (150 mg/kg BW), a mild hydropic degeneration of epithelial cells in proximal convoluted tubules, Bowman's capsule and glomerulus area (Figure 7) were appeared. The value of BUN level was slightly increased by the high dose of THA treatment (Figure 3b) and the changes was still within the normal range. However, the histopathologic change in kidneys has been reported that do not always correlate with BUN. It has also been reported that the serum urea nitrogen levels may not change significantly until 50% of renal function is impaired (125) and BUN is much less sensitive than urea clearance in determining renal function. This might be the case of our study.

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Effect of THA-treatment on liver triglyceride (TG)

A number of agents that induce liver injury also cause the accumulation of abnormal amounts of fat, predominantly triglyceride, in the parenchyma cells. In general, triglyceride accumulation can be thought of as a result from an imbalance between the rate of synthesis and the rate of release of triglyceride by the parenchyma cells into the system circulation (126). However, in this study after subacute exposure of THA to mice the liver triglyceride was increased about twice of that in control by the highest dose of THA (Figure 4). However, the increased triglyceride concentration was still in the normal range. This was consistent with liver histopathology showing no fatty liver, which implicated the status of triglyceride accumulation in the liver cells. From this finding, it is suggested that THA had little effect on the increase of the liver triglyceride.

Effect of THA-treatment on liver glutathione content (GSH)

In general, GSH is a tripeptide, γ-glutamylcysteinylglycine, which is essential for the survival of all aerobic cells and is implicated in many cellular functions. It is the most prevalent cellular thiol and most abundant low-molecular weight peptide present in the cells. GSH participates in many critical cellular functions, acts as a reducing agent and antioxidant, serves as a reservoir for cysteine, participates in detoxification reactions for xenobiotics, including defense against toxins and free radicals. In addition, this peptide plays a role on metabolism of numerous cellular compounds, protection against tissue damage and also involve in modulation of cell cycle (127). Administration of xenobiotics those might be metabolized to free radicals, initiate lipoperoxidation, and toxic to cells. GSH and other cytoprotective

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substances would deactivate those toxic compounds. With this form of liver injury the levels of GSH often fall. Nutritional depletion and liver injury limit hepatic GSH synthesis (128). However, this pattern was not found after administration of THA to mice for 30 days. In present study, the liver GSH level was increased by a low dose of THA at 37 mg/kg BW whereas it was reduced with increasing the dose. The highest dose of THA reduced GSH to the level of control. Thus, the interfering effect of THA in the present study did not correlate with changes of GSH content in liver. This finding suggested that THA might not be a toxic compound.

In conclusion, through THA might be considered as a non-toxic compound, subacute exposure of THA for a long period was able to cause the dose-related impairment of liver function. From the changes of hepatocytes in zone 1, it was suggested that the toxic effect might be attributed by THA itself, not its metabolite. THA only at the high dose induced the statistically significant toxic effect. From these finding suggested that subacute toxic effect of THA was seen with high dose and prolonged treatement.

Consideration on the toxicity of phloracetophenone (2,4,6-trihydroxyacetophenone, THA), its chemical structure related to acetophenone that consisted of a benzene ring nucleus with functional ketone group, and three OH groups at 2,4,6-position on benzene ring. At present, the mechanism by which THA induced toxicity to animals was not known. It might relate to the chemical structure and properties of THA molecule. After THA administration, this compound would rapidly be absorbed and biotransformed by liver and producing metabolites which might be responsible for toxicity (129). In general, the biotransformation is the

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mechanism for converting poorly excretable lipophilic xenobiotics to readily excretable water-soluble compound (130), which they divided into two distinct phases. Phase I reactions result in an addition of specific functional groups that are required for subsequent metabolism by Phase II enzymes. Phase II reactions are biosynthesis. These Phase I and II reactions are often coordinated, with the product of one reaction becoming the substrate of the other. Combinations of Phase I and Phase II reactions have a concerted effect on the detoxification and/or excretion of foreign compounds. Many compounds can be metabolized by Phase II reactions without first undergoing a Phase I reaction. This might be the case of THA. The metabolic pathway of THA might go pass Phase I into Phase II reactions, because this compound had already contained OH groups which might be an appropriate functional group amenable for conjugation; glucuronidation or sulfation. The products might be readily to be excreted in urine or bile. This contention was supported by its rapid action in increasing BFR after administration of THA and the appearance of the biliary glucuronide conjugate of its analog in a similar study (131). However, this result demonstrated that only after high dose of THA, the changes of hepatocytes at periportal area or in zone 1 of liver plate was appeared. Metabolic zonation is considered as a functional consequence of the gradients of components both in blood and in hepatocytes. Blood entering the liver consists of oxygen-depleted blood from the portal vein (60 to 70 percent of hepatic blood flow) and oxygenated blood from the hepatic artery (30 to 40 percent). Oxygen concentrations in zone 1 are approximately 9 to 13 percent O_2 as compared with only 4 to 5 percent O_2 in zone 3 (132). Hepatocytes in zone 3 will be exposed to substantially lower concentrations of

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oxygen than hepatocytes in zone 1. In addition, zone 1 stores more iron than zone 3, therefore, this zone has more capability of oxidation. It was possible to induce oxidation of OH group in THA such as epoxidation to generate epoxides, which were relatively reactive compounds. They are capable of binding covalently to proteins and nucleic acids, causing alteration of liver cells or toxic response. Alternatively, with high dose of THA, Phase II reaction for conjugation might be saturated with high amount of THA and caused high accumulation for inducing toxic effect.

Effect of THA Treatment on Hepatic Excretory Function in Normal Rats and in Ethinylestradiol-Induced Cholestatic Rats

2,4,6-trihydroxyacetophenone (THA) is a acetophenone which contains three hydroxy groups and it was the most effective choleretic agent among tested analogues with varying number and position of hydroxyl groups (7). It remarkably stimulated the bile secretion by enhancing bile acid excretion which subsequently led to decreased plasma cholesterol (7). In order to ensure the beneficial effect of this compound and for further development as a therapeutic agent as choleretic and hypolipidemic drugs, it is necessary to obtain more information of the compound particularly in pathologic conditions, in which the compound is intended to be used for treatment. Therefore, the effect of THA treatment on hepatobiliary excretory function was evaluated in normal rats and rats-induced bile stasis condition.

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Effect of THA on bile flow

Effect of THA on bile flow rate in rats has previously been studied and demonstrated that a single intraduodenal administration of THA induced a dose-dependent increase in bile flow rate. THA at a dose of 100 mg/kg BW gave a maximal increase of bile flow rate which was about 200% of control (107). Likewise, in the present study, THA at doses of 50 and 100 mg/kg BW also demonstrated a dose-related increase in bile flow rate which peaked at 15 min after administration (Figure 11). These results confirmed the previous study and suggested that THA is indeed a choleretic agent, its onset was rapid but duration of action was also short. The stimulatory effect was acutely appeared and would be gone in a few hours. Prolonged treatment by the multiple administration of THA for 5 days did not give the character of acute response, did not show any peak of action as found in the acute treatment. THA just slightly increased the bile flow rate over the basal secretion in control animals. Recently, it is evident that, compounds which posses stimulatory action on bile secretion such as ursodeoxycholic acid (UDCA) (133), epomediol (134) etc. can improve biochemical and histological parameters in a number of cholestasis disorders and primary biliary cirrhosis (133, 134). In this study, the effect of THA in cholestatic rats, which were induced defect in bile secretion by given 17α-ethinylestradiol, EE, was also evaluated. Subcutaneous administration of EE (5 mg/kg BW) for 5 days caused a marked reduction of bile flow rate or cholestatic condition (Table 3 and Figure 13). Induction of intrahepatic cholestasis in man and animals by EE has been documented. The mechanism of the cholestatic effect of EE has been reported to increase the cholesterol content and decrease the lipid fluidity and Na^{+}-K^{+}-ATPase

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activity of plasma membranes (135). Furthermore, EE has been shown to reduce bile flow as well as a maximal capacity to excrete organic anions, such as BSP, bilirubin, and bile salts. A recent study on $E_2$17G-mediated cholestasis demonstrated that it does not directly inhibit BSP-mediated bile acid transport. The canalicular transporter Mdr 1a/1b are not essential for $E_2$17G cholestasis. However, the data indicated that the process of Mrp2-mediated transport of high concentrations of $E_2$17G is essential for its induction of cholestasis (136). It is not clear whether EE induced cholestasis is mediated by a similar mean to that of $E_2$17G. In addition to selective interfere with excretion into the cannaliculi, EE may also interfere with hepatic uptake from the blood of substances to be excreted and defects in metabolic transformation within the hepatocyte. Meanwhile, morphological changes in hepatic structure of cholestasis are reported to be usually little or no inflammation (137). However, in the present study after concurrent treatment with EE, THA either an acute single dose or 5 days treatments was able to reverse the impairment of bile flow rate being suppressed by EE (Table 3, Figures 14 and 15), particularly high dose of THA.

Effect of THA on plasma BSP concentration and biliary appearance rate of BSP

The effect of THA on hepatobiliary excretory function were explored by using sulfobromophthalein (BSP). BSP is a phthalein dye that is removed from blood predominantly by the liver and excreted into the bile (138). Removal is impaired in the presence of hepatocellular damage, and BSP retention in blood has been proved to be a sensitive index of the hepatic dysfunction (139). In this study, liver excretory

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function was determined from the percent retention of plasma BSP or plasma BSP concentration. After intravenous administration of BSP, a disappearance of BSP from plasma would correspond with its appearance in bile. THA, which increased BFR, enhanced disappearance of BSP from plasma and appearance of BSP in bile. In EE-induced cholestatic rats, although a marked delay in disappearance of BSP from plasma or high retention of BSP were observed (Table 4 and 6), this defect was effectively reversed by an acute THA treatment. The removal of BSP or liver transport function of BSP from the blood into the bile depends on several individual factors including extracellular binding to serum albumin (140-1), transport across the liver sinusoidal membrane (142), intracellular binding to ligandin (143), intracellular conjugation with glutathione (144-5), and release of the dye via the bile canalicular membrane mainly in its conjugated form (146-7). However, after concurrent treatment of EE with THA at either an acute single dose or 5-days of multiple treatment, it markedly reduced the percent retention of BSP in plasma with the increasing appearance rate of BSP in bile. The high dose-treatment gave more effect than the low dose-treatment (Tables 4-7, Figures 17-20). Both acute and multiple THA treatments gave a similar BSP clearance effect, it was probably due to the effective BSP clearing system in rats or the amount of the injected BSP was rather low. The slight increase in basal bile flow in the multiple THA treatment could induce a comparable clearing rate to those are challenged by an acute single THA administration. The difference of BSP clearance was seen only in cholestatic condition.

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Effect of THA on plasma alkaline phosphatase activity

Alkaline phosphatase enzyme is present in many tissues. It is not an organ specific enzyme. In the liver, it is closely associated with lipid membranes in the canalicular zone, so that any interference with plasma membrane fluidity would lead to an increase in plasma alkaline phosphatase (148). In our EE-induced cholestatic rats, plasma alkaline phosphatase activity was significantly increased when compared to that in control (Table 8 and Figure 21). The mechanisms of the increases of alkaline phosphatase in cholestatic condition have frequently been reported. The cholestasis resulted in a failure to clear or excrete the hepatic alkaline phosphatase, thereby producing an elevation in the circulating level of the enzyme (148). In the present study, it was interesting that an acute single THA administration at either 50 or 100 mg/kg BW to EE-rats completely abolished the increased plasma alkaline phosphatase activity to the control level. The exactly mechanism of THA on the returning of plasma alkaline phosphatase level in EE cholestatic rats was not clear. It was possible to be due to the rapid choleretic effect of THA. THA was known to effectively stimulated bile flow rate and the increased excretion of bile acid might help in reducing the detergent effect of the accumulated bile acid in the lipid membranes which was reported to be the cause of cholestasis. However, this effect was not seen in the concurrent treatment of EE with 5-days of THA administration. It was possible that the complete clearing of the increased plasma alkaline phosphatase occurred during high BFR in acute THA administration. In the prolonged treatment it was observed 24 h later after THA administration. It showed only slight decrease.

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Effect of THA on total plasma bilirubin

Bile formation is one of the most sophisticated functions of the liver, it is also one of the most readily disrupted. Such a disruption becomes clinically evident as yellow discoloration of the skin and sclera owing to retention of pigmented bilirubin, and as cholestasis. In cholestasis, the retention was not only bilirubin but also included other solutes eliminated in bile (148). The elevated of total plasma bilirubin which was observed in our EE-induced cholestatic rat could be referred to bile secretory failure (Table 9 and Figure 22) as bile constitutes the primary pathway for elimination of bilirubin. However, after concurrent EE-treatment with either an acute single and 5 days of multiple THA were not fully reversed the total plasma bilirubin to control level, probably the transportation for bilirubin was failed. The increased BFR did not associate with bilirubin excretion process.

In conclusion, THA at biologically active choleretic dose had low toxicity, it might be safe for further development as a therapeutic agent for a short period of treatment in preventing cholestasis. The beneficial for long term treatment of ethynylestradiol-induced cholestasis still remains to be proven.

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CHAPTER VI

CONCLUSION

Study of toxicity of phloracetophenone (2,4,6-trihydroxyacetophenone, THA) which is the aglycone part of phloracetophenone glucoside, a naturally occurring compound from *Curcuma comosa* family Zingiberaceae was concluded.

1. Toxicity of THA varied according to species and sexes of animals, and route of administration. Susceptibility to THA toxicity in males were higher than in females via i.p. route. However, no age difference was observed in response to toxic of THA.

2. In acute toxicity, THA was classified as a practically non-toxic compound to rats when given via i.g. route. LD_{50} values in both male and female rats were more than 6 g/kg BW. Therefore, rats were least sensitive to THA among three species were used in this study. Male hamsters and male mice were more sensitive than male rats.

3. Toxic signs were the neurological responses including hyperactivity, drowsiness and convulsion were similar in i.p. and i.g. routes of THA administration

4. The cause of animal death in acute toxicity test after administration of the lethal dose of THA via i.p. route was due to respiratory failure.

5. In subacute toxicity, THA only at high dose (150 mg/kg BW) induced a marked changes of hepatocytes at periportal area including vacuolization of hepatocyte and nuclear degeneration. In addition, biochemical parameters which are

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indices of liver function were slightly increased and significantly different from control values at only high dose.

6. THA stimulated bile secretion after an acute single i.d. administration. The maximal effect of THA was observed at peak 15 min after administration.

7. The acute administration of THA increased bile flow and enhanced hepatic clearance of BSP with decreasing plasma alkaline phosphatase in EE-cholestatic rats to normal level, however not affect on bilirubin.

8. For prolonged treatment of THA for 5 days treatment increased basal bile flow rate and partially improved BSP clearance in EE-cholestatic rats.

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REFERENCES

1. Berkowitz BA and Katzung BG. Basic and clinical evaluation of new drugs. In: Katzung BG, ed. Basic and Clinical Pharmacology. 5th ed. New York: Prentice Hall, 1992. p. 60-8.
2. Manuel FB, Douglas K, and Norman RF. Plant-derived natural products in drug discovery and development. In: Douglas K, Manuel F, and Norman RF, eds. Human Medicinal Agents from Plants. 2nd ed. Washington, DC: American Chemical Society, 1993. p. 1-11.
3. Piyachaturawat P, Ercharuporn S, and Suksamrarn A. Uterotrophic effect of *Curcuma comosa* in rats. Int J Pharmacog 1995; 33(4): 334-8.
4. Piyachaturawat P, Gansar R, and Suksamrarn A. Choleretic effect of *Curcuma comosa* rhizome extract in rats. Int J Pharmacog 1996; 34 (3): 174-8.
5. Piyachaturawat P, Teeratagolpisal N, Toskulkkao C, and Suksamrarn A. Hypolipidemic effect of *Curcuma comosa* in mice. Artery 1997; 22: 233-41.
6. Suksamrarn A, Eiamong S, Piyachaturawat P, and Byrne LT. A phloracetophenone glucoside with choleretic activity from *Curcuma comosa*. Phytochemistry 1997; 45(1): 103-5.
7. Piyachaturawat P, Chai-ngam N, Chuncharunee A, Komaratat P, and Suksamrarn A. Choleretic activity of phloracetophenone in rats: structure-function studies using acetophenone analogues. Eur J Pharmacol 2000; 387: 221-7.

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8. Hikino H, Kiso Y, Kato N, et al. Antihepatotoxic action of gingerols and diarly heptanoids. J Ethnopharmacol 1985; 14: 31-9.

9. Ozaki Y. Antiinflamatory effect of *Curcuma xanthorrhiza* (Roxb.) and its active principle. Chem Pharm Bull 1990; 38: 1045-48.

10. Woo WS, Lee FB, Shin KH, and Chi HJ. A review of research on plants for fertility regulation in korea. Korean J Pharmacog 1981; 21: 153-170.

11. U.S. Environmental Protection Agency. Health and Environmental Effects Document for Acetophenone. ECAO-CIN-G001. Environmental Criteria and Assessment Office, Office of Health and Environmental Assessment, Office of Research and Development, Cincinnati, OH. 1987.

12. The Merck Index. An Encyclopedia of Chemical, Drugs, and Biologicals. 11th ed. S. Budavari. Merck and Co. Inc., Rahway, NJ. 1989.

13. U.S. Department of Health and Human Service. Hazardous Substances Data Bank (HSDB, online database). National Toxicology Information Program, National Library of Medicine, Bethesda, MD. 1993.

14. M. Sittig. Handbook of Toxic and Hazardous Chemicals and Carcinogens. 2nd ed. Noyes Publications, Park Ridge, NJ. 1985.

15. U.S. Department of Health and Human Service. Registry of Toxic Effects of Chemical Substances (RTECS, online database). National Toxicology Information Program, National Library of Medicine, Bethesda, MD. 1993.

16. U.S. Environmental Protection Agency. Integrated Risk Information System (IRIS) on Acetophenone Environmental Criteria and Assessment Office,

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Office of Health and Environmental Assessment, Office of Research and Development, Cincinnati, OH. 1993.

17. Calixto JB, Mignel OG, Yunes RA, and Rac GA. Action of 2-hydroxy-4,6-dimethoxyacetophenone isolated from *Sebastiania schottiana.* Planta Medica 1990; 56: 31-5.
18. Jurenka RA, Neal WJ, Howard RW, Oliver JE, and Blomquist GJ. In vitro inhibition of prostaglandin H synthase by compound from the exocrine secretions of lace bugs. Biochem Physiol 1989; 93(2): 253-5.
19. Swat P, Merwek JV, Swat AC, Todres PC, and Hofmeyr JHS. Inhibition of cytochrome P-45011β by some naturally occurring acetophenones and plant extracts from the shrub *Salsola turculatiformis.* Planta Medica 1993; 59: 134-43.
20. Lin YL, and Lin TC. Two acetophenone glucosides, cynanonesides A and B, from *Cynanchum taiwanianum* and revision of the structure for cynandion A. J Nat Prod 1997; 60: 368-70.
21. Manahan SE. Biochemical action and transformation of toxicants. In: Stanley EM, ed. Toxicological Chemistry. 3rd ed. Michigan: Lewis Publishers, 1990. p. 69-84.
22. Geoffrey KG and Beresford HS. Inhibition of aminopyrine and ethylmorphine-*N*-demethylases by 2,6-dihydroxyacetophenone. Biochem Pharmacol 1979; 28: 1421-5.

Copyright by Mahidol University

23. Malmfors T and Teiling A. LD_{50}: Its value for the pharmaceutical industry in safety evaluation of drugs. Acta Pharmacol Toxicol 1983; 52(suppl 2): 229.

24. Ping KC and Hayes AW. Principles and methods for acute toxicity and eye irritancy. In Hayes AW, 2nd ed. Principles and Methods of Toxicology. New York: Raven Press, 1989: 169-86.

25. Donald JE. Acute toxicity studies. In: Donald JE, ed. The Basic of Toxicity Testing. New York: CRC Press, 1992: 35-50

26. Mcnamara BP. Concepts in health evaluation of commercial and industrial chemicals. In: Mchlnan MA, Shapiro RE, and Blumenthal H, eds. New Concepts in Safety Evaluation. Washington, DC: Hemishere, 1976: 61-140.

27. Donald JE. Subchronic and chronic studies. In: Donald JE, ed. The Basic of Toxicity Testing. New York: CRC Press, 1992: 61-81.

28. Miller HI, and Young FE. The drug approval process at the food and drug administration: new biotechnology as a paradigm of a science-based activist approach. Arch Intern Med 1989; 149: 655.

29. James AP and Russcell CC. Hepatobiliary System. In: James AP, ed. Handbook of Toxicologic Pathology. New York. Academic Press, 1991: 279-314.

30. Frank CL. Absorption, distribution and excretion of toxicants. In: Frank CL, ed. Basic Toxicology fundamentals, targets organs, and risk assessment. 3rd ed. Washington, DC: Taylor & Franicis, 1996: p. 13-25.

31. Braner RW, Krebs JS, and Pessotti RL. Bromosulfophthalein as a tool for study of liver physiology. Fed Proc 1950; 9: 259.

32. Klaassen CD and Watkins JB. Mechanisms of bile formation, hepatic uptake, and biliary excretion. Pharmacol Rev 1984; 36: 1-67.

Copyright by Mahidol University

33. Boyger JL, Graff J, and Meier PJ. Hepatic transport systems regulating pH_i, cell volume, and bile secretion. Annu Rev Physiol 1992; 54: 415-38.

34. Rowntree LG, Hurwitz SH, and Bloomfield AL. An experimental and clinical study of the value of phonoltetrachlorphthalein as a test of hepatic function. Bull Johns Hopkins Hosp 1913; 24: 327.

35. Rosenthal SM, and White EC. Clinical application of bromsulphthalein test for hepatic function. JAMA 1925; 84: 1112.

36. Nishida T, Hardenbrook C, Gatmaitau Z, and Aris IM. ATP-dependent organic anion transport system in normal and TR rat liver canalicular membranes. Am J Physiol 1992; 262: G629-G635.

37. Tiribelli G, Lunazzi GC, and Sottocasa GL. Biochemical and molecular aspects of the hepatic uptake of organic anions. Biochim Biophys Acta 1990; 1031: 261-75.

38. Yamazaki M, Suzuki H, Sugiyama Y, Iga T, and Hanano M. Uptake of organic anions by isolated rat hepatocytes a classification interm of ATP-dependency. J Hepatol 1992; 14: 41-7.

39. Garbiel LP and Michiel C. Detection and Evaluation of Chemically Induced Liver Injury. In: Hayes AW, ed. Principles and Methods of Toxicology, 3rd ed. New York: Raven Press, 1994: 847-51.

40. Deutsch E. A fractional bromsulphthalein test to determine liver damage in the non-jaundice patients. New Engl J Med 1941; 225: 171.

Copyright by Mahidol University

41. Goodman RD and Kingsley GR. Sulfobromophthalein clearance test. JAMA 1953; 153: 462.

42. Klassen CD and Doull J. Toxic responses of the liver. In: Doull J, Klassen CD, and Amdur MO, eds. 3rd ed. Casarett and Doull's Toxicology: The Basic Science of Poisons. New York: Raven Press, 1986. p. 412.

43. Neville B and Affred B. Basic function of the liver. In: Gary G, Douglos RL, and Frank GN, eds. Diseases of the liver and biliary tract. New York: Mosby-Yearbook; 1992: 34-7.

44. Zimmerman HJ. Indirecct hepatotoxins-cholestatic. In: Zimmerman HJ, ed. Hepatotoxicity 2nd ed. New York: Preutice-Hally, 1978. p. 259.

45. Arias IM, Fleischner G, Listowsky I, et al. On the structure and function of ligandin and Z protein. In: Taylor W, ed. The Hepatobiliary System: Fundamental and Pathological Mechanisms. New York: Plenum Press, 1975: 81-107.

46. Scharschmidt BF, Waggoner JG, and Berk PD. Hepatic organic anion uptake in the rats. J Clin Invest 1975; 56: 1280.

47. Wheeler HO. Secretion of bile. In: Schiff L, ed. Diseases of the liver 4th ed. Philadelphia: Lippincott, 1975: 87-110.

48. Taylor W. The hepatobiliary system: retrospect and prospect. In: Taylor W, ed. The Hepatobiliary System: Fundamental and Pathological Mechanisms. New York: Plenum Press, 1975: 1-8.

49. Prandi D, Erlinger S, Glasinovic JC, and Dumont M. Canalicular bile production in man. Eur J Clin Invest 1975: 1-8.

Copyright by Mahidol University

50. Forker EL. Canalicular anion transport pathogenitic mechanisms and a steady state distributed model for measuring kinetics. In: Goresky CA and Fisher MM, eds. Jaundice. New York: Plenum Press, 1975; 229-43.

51. Slater TF and Eakins MN. Biochemical studies on bile secretion. In: Taylor W, ed. The Hepatobiliary System: Fundamental and Pathological Mechanisms. New York: Plenum Press, 1975; 61-80.

52. Phillips MJ, Oda M, Mak E, and Steiner JW. Fine structure of the biliary tree. In: Taylor W, ed. The Hepatobiliary System: Fundamental and Pathological Mechanisms. New York: Plenum Press, 1976. p. 245-65.

53. Popper H, Schaffner F, and Denk H. Molecular pathology of cholestasis. In: Taylor W, ed. The Hepatobiliary system: Fundamental and Pathological Mechanisms. New York: Plenum Press, 1975. p. 605-29.

54. Boyer JL and Bloomer JR. Canalicular bile secretion in man studies utilizing the biliary clearance of ^{14}C mannitol. J Clin Invest 1974; 54: 773.

55. Schaffner F, Popper H, and Perez V. Changes in bile canaliculi produced by norethadralone: Electron microscopic study of human and rat liver. J Lab Clin Med 1960; 56: 623.

56. Palmer R. Toxic effects of lithocholate on the liver and biliary tree. In: Taylor W, ed. The Hepatobiliary system: Fundamental and Pathological Mechanisms. New York: Plenum Press, 1975. p. 221-43.

57. Schaffner F and Raisfeld IH. Drugs and the liver: A review of metabolism and adverse reactions. Adv Int Med 1969; 15: 211.

Copyright by Mahidol University

58. Bullock G, Eakins MN, Sawyer BC and Slater TF. Studies on bile secretion with the aid of the isolated perfused liver. I. Inhibitory action of sporidesmin and icterogenin. Proc Roy Soc London Biol 1974; 186: 333.

59. Erlanger S, Glasinovic JC, Poupon R, and Dumont M. Hepatic transport of bile acids. In: Taylor W, ed. The Hepatobiliary system: Fundamental and Pathological Mechanisms. New York: Plenum Press, 1975. p. 433-52.

60. Gumucio JJ and Validivieso VD. Studies on the mechanism of the ethinylestradiol impairment of bile flow and bile salt excretion in the rat. Gastroenterology 1971; 61: 339.

61. Zimmerman HJ. Clinical and laboratory manifestations of hepatoxicity. Ann NY Acad Sci 1971; 61: 339.

62. Dreyfuss F, Klein H, and Winnik HZ. Preventing chlorpromazine jaundice. Lancet 1956; 2: 1211.

63. Danhof IE. Factors in the etiology of chlorpromazine jaundice: An experimental study. Texas Rep Biol Med 1958; 16: 443.

64. Plaa GL. Hyperbilirubinemia and cholestasis, a different from of liver injury produced in animals. Essays Toxicol 1970; 2: 137.

65. Plaa GL and Priestly BG. Intrahepatic cholestasis induced by drugs and chemicals Pharmacol Rev 1976; 28: 207-73.

66. Kottra LL and Kapas A. Estrogen pharmacology. III. Effect of estradiol on plasma disappearances rate of sulfobromophthalein in man. Arch Intern Med 1966; 117: 333-75

Copyright by Mahidol University

67. Mueller MN and Kappas A. Estrogen pharmacology. I. The influence of estradiol and estriol on hepatic disposed of sulfobromophthalein (BSP) in man. J Clin Invest 1964; 43: 1905-14.

68. Larsson-Cohn U and Stenram U. Jaundice during treatment with oral contraceptive agents. J Am Med Ass 1965; 193: 422-6.

69. Ockner RK and Davidons CS. Hepatic effects of oral contraceptives. New Engl J Med 1967; 276: 331-4.

70. Gallagher TF, Mueller MN, Kappas A. Estrogen pharmacology. IV. Studies of the structural basis for estrogen-induced impairment of liver function. Medicine 1966; 45: 417-9.

71. Kreek MJ, Peterson RE, Sleisenger MH, et al. Effects of ethinylestradiol induced cholestasis on bile flow and biliary excretion of estradiol and estradiol glucuronide by the rat. Proc Soc Exp Biol Med 1969; 131: 646-50.

72. Harkavy M, Jaritt NB. Effect of ethinylestradiol on hepatic excretory function of the rat. In: Salhanick DM, et al. eds. Metabolic effects of gonadol hormones and contraceptive steriods. New York Plenum, 1969. p. 11-8.

73. Forker EL. The effect of estrogen on bile formation in the rat. J Clin Invest 1969; 48: 654-63.

74. Gunucio J, Valdivieso V. Studies on the mechanisms of ethinylestradiol impairment of bile flow and bile salt excretion in the rat. Gastroenterology 1971; 61: 339-44.

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75. Kern F, Eriksson H, Curstedt T, et al. Effect of ethinylestradiol on biliary excretion of bile acids, phosphatidylcholines and cholesterol in the bile fistula rat. J Lipid Res 1977; 18: 623-34.

76. Reichen J, Panmgartner G. Relationship between bile flow and Na^+ K^+ adenosine triphosphatase in liver plasma membranes enriched in the bile canaliculi. J Clin Invest 1977; 60: 429-34.

77. Davis R, Kern F. Effects of ethinylestradiol an phenobarbital on bile acid synthesis and biliary bile acid and cholesterol excretion. Gastroenterology 1976; 70: 1130-5.

78. Simon FR, Arias IM. Alteration of bile canalicular enzymes in cholestasis. A possible cause of bile secretory failure. J Clin Invest 1973; 52: 765-75.

79. Jaeschke H, Trummer E, and Krell H. Increase in biliary permeability subsequent to intrahepatic cholestasis by estradiol valerate in rats. Gastroenterology 1987; 93: 533-8.

80. Scharschmidt BF, Stephens JE. Transport of sodium, chloride, and taurocholate by cultured rat hepatocytes. Proc Natl Acd Sci USA 1981; 78: 986-90.

81. Ruifrok PG, Meijer DKF. Sodium coupled uptake of taurocholate by rat liver plasma membrane vesicles. Liver 1982; 2: 28-34.

82. Inoue M, Kinne R. Tran T, et al. Taurocholate transport by rat liver sinusoidal membrane vesicles, evidence of sodium cotransport. Hepatology 1982; 2: 572-9.

Copyright by Mahidol University

83. Duffy M, Blitzer BL, Boyer JL. Direct determination of the driving forces for taurocholate uptake into rat liver plasma membrane vesicles. J Clin Invest 1983; 72: 1470-81.

84. Meier PJ, Meier-Abt AS, Barrett C, et al. Mechanisms of taurocholate transport in canalicular and basolateral rat liver plasma membrane vesicles evidence for an electronic canalicular organic anion carrier. J Biol Chem 1984; 259: 614-22.

85. Inoue M, Kinne R, Tran T, et al. Taurocholate transport by rat liver canalicular membrane vesicles. J Clin Invest 1984; 73: 659-63.

86. Davis RA, Kern F Jr, Showalter r. Alterations of hepatic Na^+ K^+-ATPase and bile flow by estrogen: effects on liver surface, membrane lipid structure and function. Proc Natl Acad Sci USA 1978; 75: 4130-4.

87. Heikel TAJ, Lathe GH. The effect of 17-ethinyl substituted steroids on adenosine triphosphatases of rat liver plasma membrane. Biochem J 1970; 118: 187-9.

88. Reichen J, Paumgartner G. Uptake of bile acids by perfused rat liver. Am J Physiol 1976; 231: 734-42.

89. Heikel TAJ. Lathe GH. The effect of oral contraceptive steroids on bile secretion and bilirubin Tm in rats. Br J Pharmacol 1970; 38: 593-601.

90. Gumuciol J, Accatino L, Mahcio AM, et al. Effect of phenobarbital on the ethinylestradiol induced cholestasis in the rat. Gastroenterology 1973; 65: 651-7.

Copyright by Mahidol University

91. Simon FR, Gonzalez M, Sutherland E, et al. Reversal of ethinylestradiol-induced bile secretory failure with Triton WR-1339. J Clin Invest 1980; 65: 851-9.

92. Hay RV, Pottenger LA, Reingold AL, et al. Degradation of I^{125}-labelled serum low density lipoprotein in normal and estrogen-treated male rats. Biochem Biophys Res Comm 1971; 44: 1471-7.

93. Kovanen PT, Brown MS, Goldstein JL. Increased binding of LDL to liver membranes from rats treated with 17-ethinylestradiol. J Biol Chem 1979; 254: 11367-73.

94. Chao YS, Windler EE, Chen GC, et al. Hepatic catabolism of rat and human lipoproteins in rats treated with 17α-ethinylestradiol. J Biol Chem 1979; 254: 11360-6.

95. Keeffe EB, Scharschmidt BF, Blankenship NM, et al. Studies of relationships among bile flow, liver plasma membrane Na^+ K^+-ATPase and membrane microviscosity in the rat. J Clin Invest 1979; 64: 1590-8.

96. Boelsterli UA, Rakhit G, Balazs T. Modulation by *S*-adenosylmethionine of hepatic Na^+ K^+-ATPase, membrane fluidity and bile flow in rats with ethinylestradiol-induced cholestasis. Hepatology 1983; 3: 12-7.

97. Eisenfeld AJ, Alen R, Weinberger M, et al. Estrogen receptor in the mammalian liver. Science 1976; 191: 862-5.

98. Porter LE, Elm MS, Van Thiel DH, et al. Characterization and quantitation of human hepatic estrogen receptor. Gastroenterology 1983; 84: 804-12.

99. Menard J, Corrol P, Foliot A, et al. Effects of estrogen on renin substrate and uterrine weights in rats. Endocrinology 1973; 93: 747-51.

Copyright by Mahidol University

100. Corval PL, Chromback A, Rodbard D, et al. Physical properties and binding capacity of testosterone-estradiol-binding globulinin human plasma

determined by polyacrylamide gel electrophoresis. J Biol Chem 1971; 246: 3435-43.

101. Glinoer D, MacGuire RA, Gershengorn MC, et al. Effects of estrogen on thyroxine-binding globulin metabolism in rhesus monkeys. Endocrinology 1977; 100: 9-17.

102. Song CS, Rifkind AB, Gillette PN, et al. The effect of estrogens, progestines and pregnancy on hepatic function. Am J Obstet Gynecol 1969; 105: 813-47.

103. Meier PJ, Sztul ES, Reuben A, et al. Structural and functional polarity of canalicular and basolateral plasma membrane vesicles isolated in high yield from rat liver. J Cell Biol 1984; 98: 991-1000.

104. Meier PJ, Ruetz ST, Hugentobler O, et al. Identification and isolation of the putative canalicular bile acid carrier from rat liver (abstr). Hepatology 1985; 5: 958.

105. Wieland T, Nassal M, Kramer W, et al. Identity of hepatic membrane transport systems for bile salts, pholloidin, and antamanide by photoaffinity labelling. Proc Natl Acad Sci USA 1984; 81: 5232-6 .

106. Litchfield JT and Wilcoxon F. A simplified method of evaluating dose effect experiments. J Pharmacol Exp Ther 1949; 69: 99-113.

107. Piyachaturawat P, Suwan-ampai P, Komaratat P, Chuncharunee A, and Suksamrarn A. Effect of phloracetophenone on bile flow and biliary lipids in rats. Hepatology 1998; 12: 198-206.

Copyright by Mahidol University

108. Kaplowitz N, Eberle D, and Yamada T. Biochemical tests for liver disease. In: Zakim D and Boyer L, eds. Hepatology a Textbook of Liver Disease. New York: Raven Press, 1982. p. 583-587.

109. Klassen CD and Doull J. Evaluation of safety: toxicology evaluation. In: Doull J, Klassen CD, and Amdur MO, eds. 3rd ed. Casarett and Doull's Toxicology: The Basic Science of Poisons. New York: Raven Press, 1986. p. 13.

110. William RT. Inter-species variation in the metabolism of xenobiotics. Biochem Soc Trans 1974; 2: 359-377.

111. Calwell J. The current status of attempts to predict species differences in drug metabolism. Drug Metab Rev 1981; 12: 221-237.

112. Donald J and Hayes JR. Metabolism: A Determinant of Toxicity. In: Hayes AW, ed. 2nd ed. Principles and Methods of Toxicology. New York: Raven Press, 1989. p. 45.

113. Donald J and Hayes JR. Metabolism: A Determinant of Toxicity. In: Hayes AW, ed. 2nd ed. Principles and Methods of Toxicology. New York: Raven Press, 1989. p. 44-46.

114. Nakayama K, Kasahara D, and Yamamoto F. Absorption, distribution, metabolism and excretion of stevioside in rats. J Food Hyg Japan 1986; 27: 1-8.

115. Srivoraphan P. Study of the effect of phloracetophenone on lipid metabolism in hypercholesterolaemic hamsters. (M.S. Thesis in Physiology). Bangkok: Faculty of Graduate Studies, Mahidol University, 1998.

Copyright by Mahidol University

116. Kato R and Gillette JR. Sex differences in the effects of abnormal physiological states on the metabolism of drugs by rat liver microsomes. J Exp Ther 1965; 150: 285-291.

117. Kato R. Sex-related differences in drug metabolism. Drug Metab Rev 1974; 3: 1-32.

118. Kato R. Characteristics and differences in the hepatic mixed function oxidase of different species. Pharmacol Ther 1979; 6: 41-98.

119. Singer SS and Bruns L. Enzymatic sulfation of steroids-VII. Hepatic cortisol sulfation and glucocortocoid sulfotransferases in old and young male rats. Exp Gerontol 1978; 13: 425-429.

120. Singer SS and Sylvester S. Enzymatic sulfation of steroids: II. The control of the hepatic cortisol sulfotransferase activity and of the individual hepatic steroid sulfotransferases of rats by gonads and gonadal hormones. Endocrinology 1976; 99: 1346-1352.

121. Gad SC. An approach to the design and analysis of screening studies in toxicology. J Am Coll Toxicol 1988; 8: 127-138.

122. Suber RL. Clinical pathology methods for toxicology studies. In: Hayes AW, ed. 3rd ed. Principles and Methods of Toxicology. New York: Raven Press, 1994. p. 760-761.

123. Suber RL. Clinical pathology methods for toxicology studies. In: Hayes AW, ed. 3rd ed. Principles and Methods of Toxicology. New York: Raven Press, 1994. p. 762.

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124. Cheeseman KH, Albano EF, Tomas A, and Slater TF. Biochemical studies on the metabolic activation of halogenated alkanes. Environ Health Persp 1985; 64: 85-101.

125. Suber RL. Clinical pathology methods for toxicology studies. In: Hayes AW, ed. 3rd ed. Principles and Methods of Toxicology. New York: Raven Press, 1994. p. 752-4, 761-2.

126. Plaa GL and Charbonneau M. Detection and evaluation of chemically induced liver injury. In: Hayes AW, ed. 3nd ed. New York: Raven Press, 1994. p. 852-853.

127. Deneke SM and Fanburg BL. Regulation of cellular glutathione. Pharmacol Rev 1989; 36: L163-L173.

128. Meister A. Glutathione deficiency produced by inhibition of its synthesis, and its reversal, application in research and therapy. Pharmacol Ther 1991; 51:155-194.

129. Donald JE. Biotransformation. In: Donald JE, ed. The Basis of Toxicity Testing. New York: Raven Press, 1992. p. 24.

130. Donald JE. Biotransformation. In: Donald JE, ed. The Basis of Toxicity Testing. New York: Raven Press, 1992. p. 26.

131. Tanphichai K. Stimulatory effect and mechanisms of acetophenones on bile secretion in rats. (M.S. Thesis in Physiology). Bangkok: Faculty of Graduate Studies, Mahidol University, 2000.

Copyright by Mahidol University

132. Klassen CD and Doull J. Toxic responses of the liver. In: Doull J, Klassen CD, and Amdur MO, eds. 3rd ed. Casarett and Doull's Toxicology: The Basic Science of Poisons. New York: Raven Press, 1986. p. 405.

133. Kinbara S, Ishizaki K, and Sakakura H. Improvement of estradiol-17-β-D-glucuronide-induced cholestasis by sodium tauroursodeoxycholate therapy in rats. Scand J Gastroenterol 1997; 32: 947-952.

134. Zanninelli GA and Tavanti ME. Effect of the terpenic compound epomediol on bilary secretion and bile composition in the rat. J Hepatology 1992; 14: 64-70.

135. Arunee C, Sherrie D, and Mary V. Characterization of estradiol binding sites in basolateral and canalicular liver plasma membranes. Biochem Pharmacol 1990; 6: 1219-1225.

136. Huang L, Smit JW, Meijer DK and Vore M. Mrp_2 is essential for estradiol-17β (β-D-glucuronide)-induced cholestasis in rats. Hepatology 2000, 32: 66-72.

137. Klassen CD and Doull J. Toxic responses of the liver. In: Doull J, Klassen CD, and Amdur MO, eds. 3rd ed. Casarett and Doull's Toxicology: The Basic Science of Poisons. New York: Raven Press, 1986. p. 410.

138. Klassen CD and John BW. Mechanism of bile formation, hepatic uptake, and biliary excretion. Pharmacol Rev 1984; 36: 28-29.

139. Klassen CD and John BW. Mechanism of bile formation, hepatic uptake, and biliary excretion. Pharmacol Rev 1984; 36: 27.

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140. Keeffe EB, Scharschmidt BF, Blankenship NM, et al. Studies of relationships among bile flow, liver plasma membrane NA^+ K^+-ATPase, and membrane microviscosity in the rats. J Clin Invest 1979; 64: 1590.

141. Rosenthal SM and White EC. Studies in hepatic function: The pharmacological behavior of certain phthalein compounds in the estimation of hepatic function. J Pharmacol Exp Ther 1924; 24: 265.

142. Davis RA, Kern FJ, Showalter R, et al. Alterations of hepatic NA^+ K^+-ATPase and bile flow by estrogen: effects of line surface membrane lipid structure and function. Proc Natl Acd Sci 1987; 75: 4130.

143. Rosenthal SM and White EC. Clinical application of the bromsulfophalein test for hepatic function. J Am Med Ass 1925; 84: 1112.

144. Schwarz LR, Summer KH, and Schwenk M. Transport and metabolism of bromosulfophthalein by isolated rat liver cells. Eur J Biochem 1979; 94: 617-622.

145. Schwenk M, Schwarz L, and Pfaff E. Uptake of bromosulfophthalein by isolated liver cells. Eur J Biochem 1976; 64: 189-197.

146. Combes B. The importance of conjugation with glutathione for sulfobromophthalein sodium (BSP) transfer from blood to bile. J Clin Invest 1965; 44: 1214-1224.

147. Combes B and Stakelum GS. Maturation of the sulfobromophthalein sodium-glutathione conjugating system in rat liver. J Clin Invest 1962; 41: 750.

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148. Raymond JM, Niesine J, and Mannfred AH. Hepatotoxicology: mechanisms of liver toxicity and methodological aspects. In: Raymond JM, ed. Toxicology: Principles and Applications. New York: CRC Press, 1996. p. 703-21.

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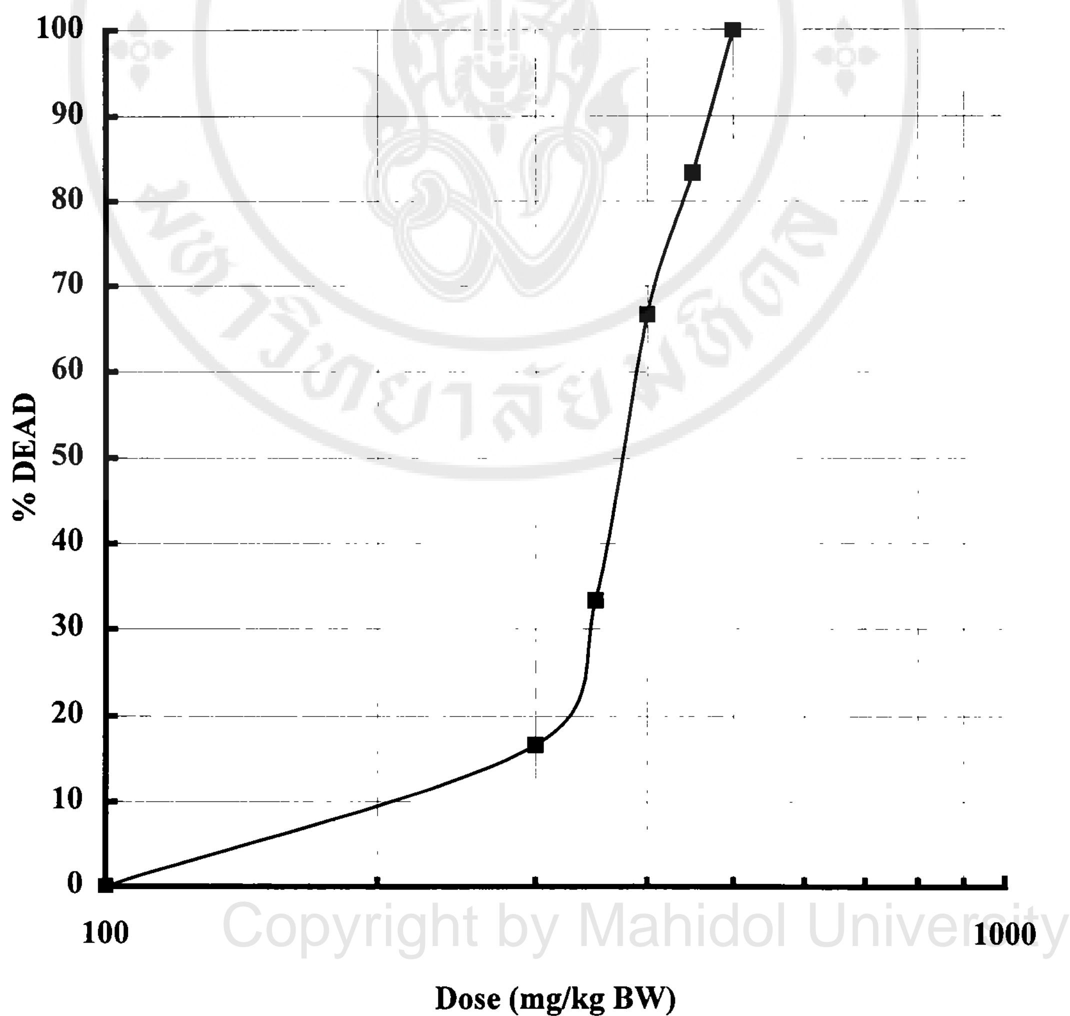
APPENDIX I

LD_{50} of 2,4,6-trihydroxyacetophenone (THA) in male weanling Swiss albino mice (i.p.)

Dose (mg/kg BW)	% Dead
500	100
450	83.33
400	66.67
350	33.33
300	16.67
100	0



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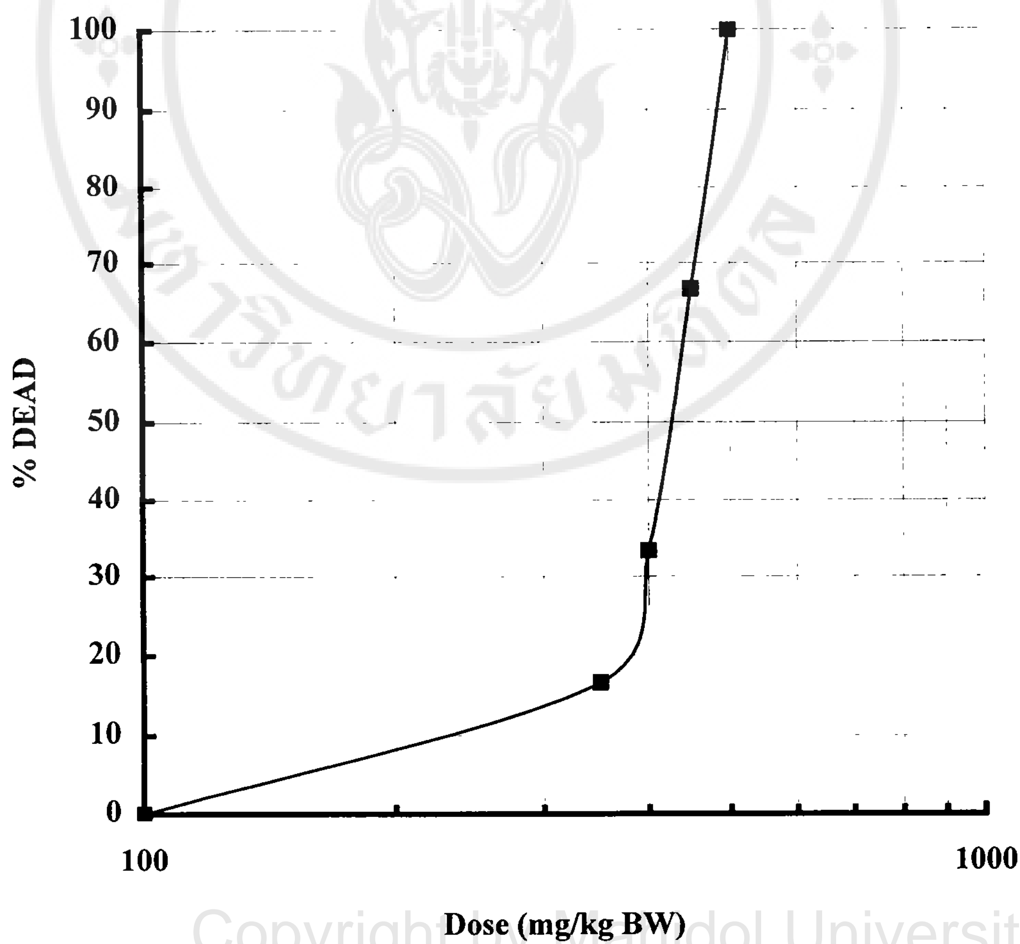
LD_{50} = 370 mg/kg BW

APPENDIX II

LD_{50} of 2,4,6-trihydroxyacetophenone in female weanling Swiss albino mice (i.p.)

Dose (mg/kg BW)	% Dead
500	100
450	66.67
400	33.33
350	16.67
100	0



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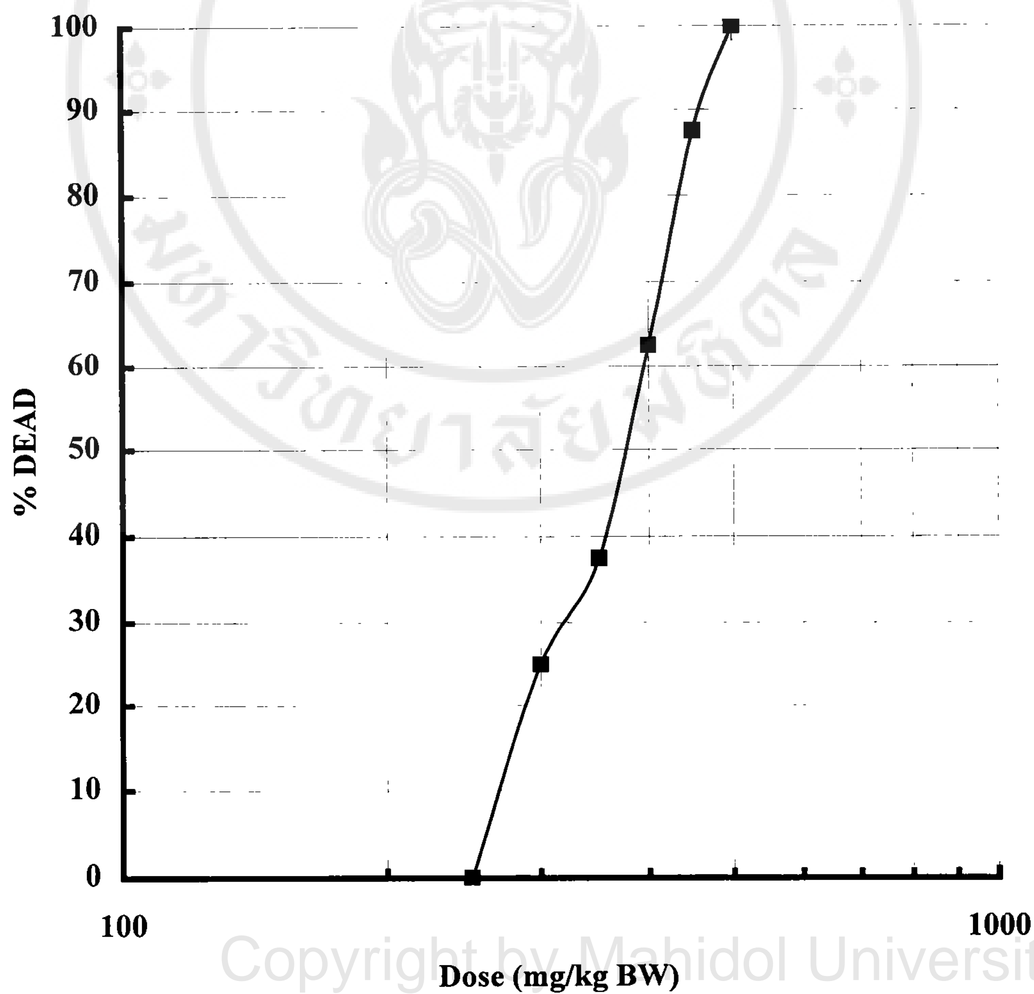
LD_{50} = 430 mg/kg BW

APPENDIX III

LD_{50} of 2,4,6-trihydroxyacetophenone in male Swiss albino mice (i.p.)

Dose (mg/kg BW)	% Dead
500	100
450	87.5
400	62.5
350	37.5
300	25
250	0



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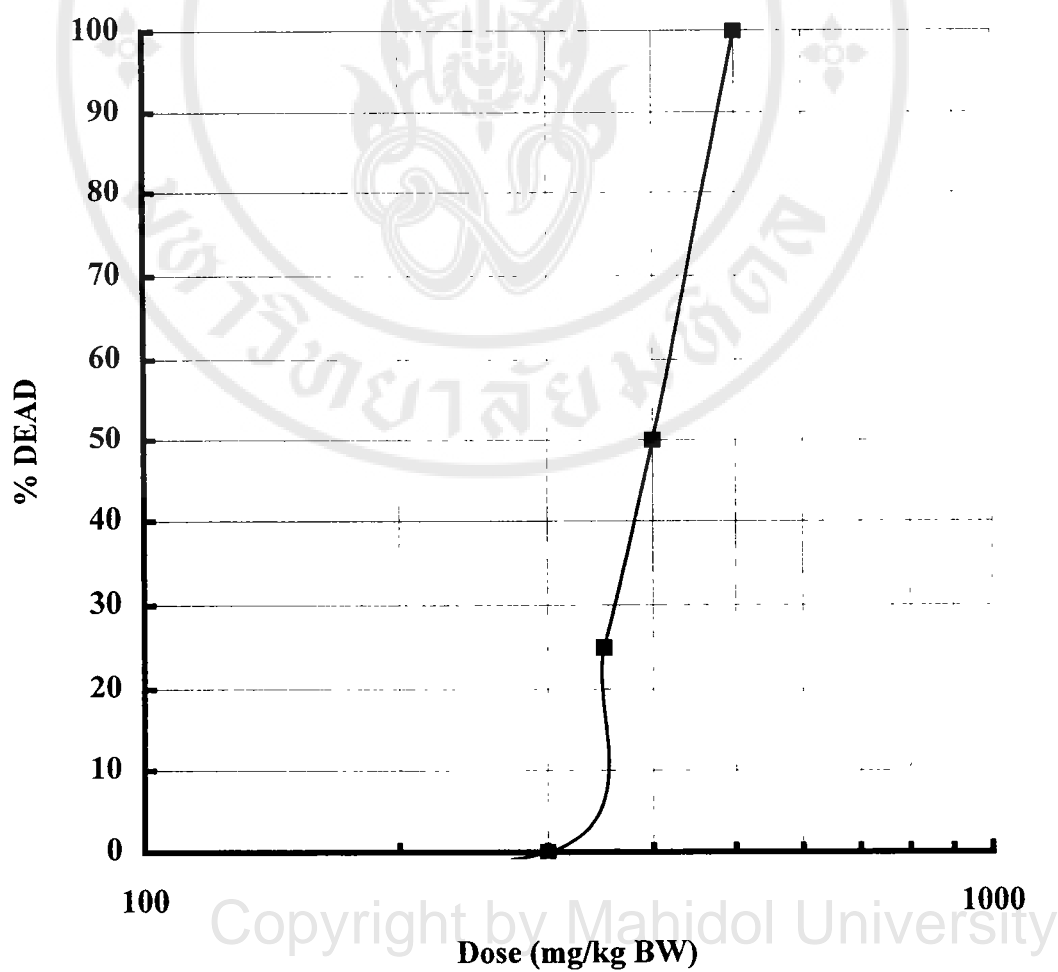
LD_{50} = 365 mg/kgBW

APPENDIX IV

LD_{50} of 2,4,6-trihydroxyacetophenone in female Swiss albino mice (i.p.)

Dose (mg/kg BW)	% Dead
500	100
400	50
350	25
300	0
50	0



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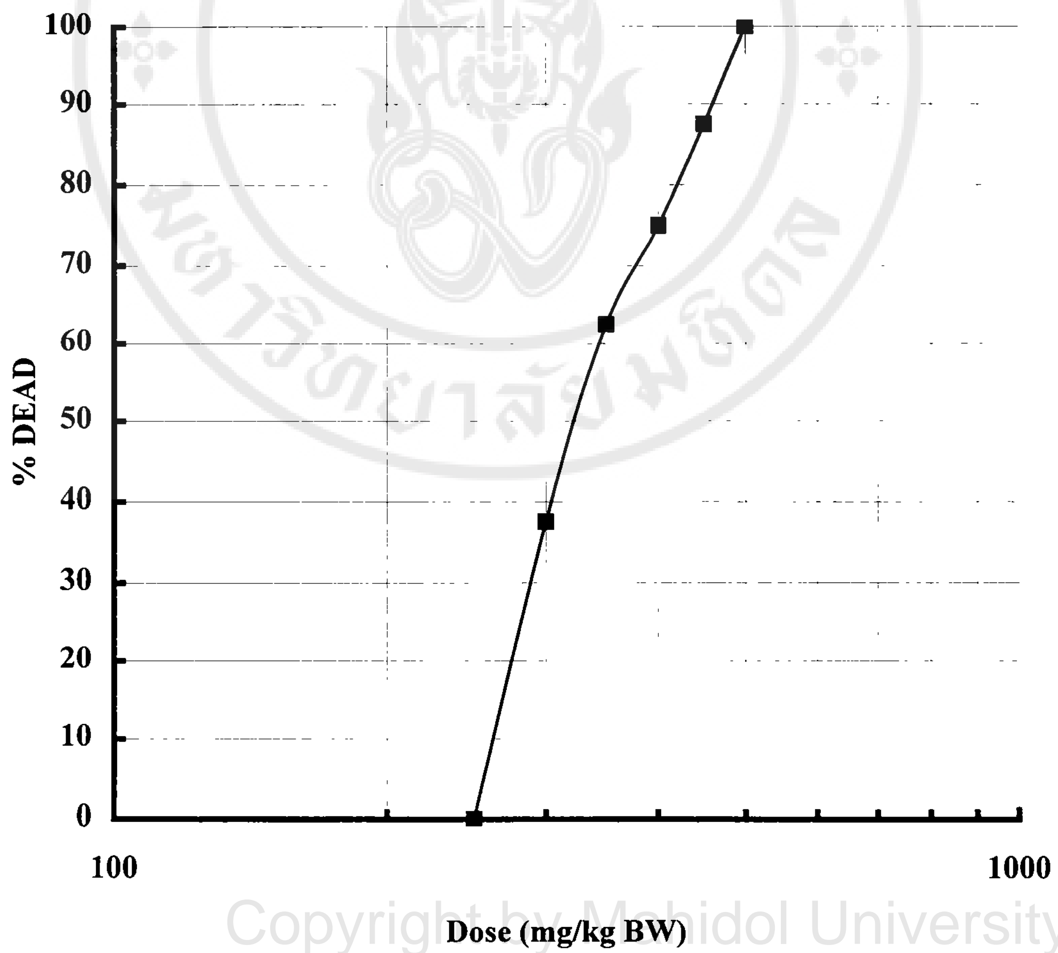
LD_{50} = 400 mg/kg BW

APPENDIX V

LD_{50} of 2,4,6-trihydroxyacetophenone in male Syrian golden hamster (i.p.)

Dose (mg/kg BW)	%Dead
500	100
450	87.5
400	75
350	62.5
300	37.5
250	0



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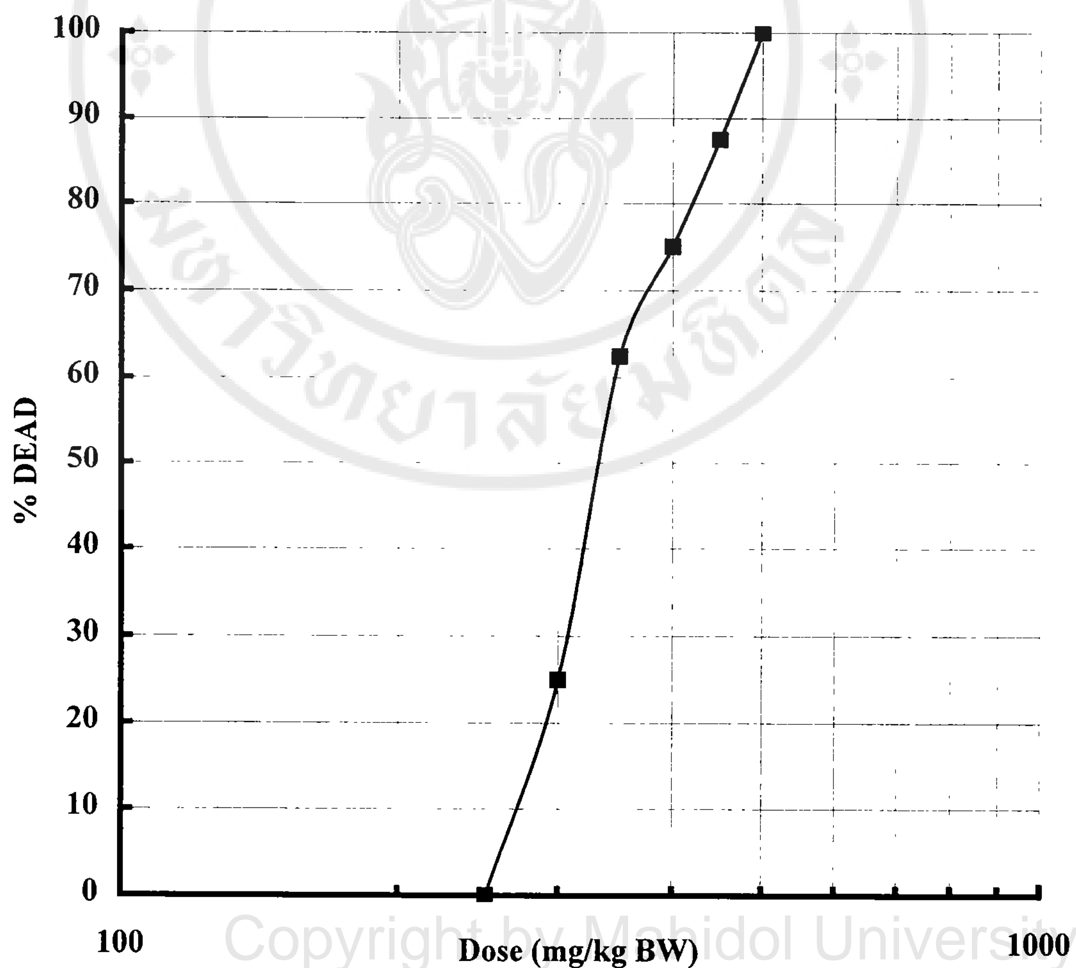
LD_{50} = 338 mg/kg BW

APPENDIX VI

LD_{50} of 2,4,6-trihydroxyacetophenone in female Syrian golden hamster (i.p.)

Dose (mg/kg BW)	% Dead
500	100
450	87.5
400	75
350	62.5
300	25
250	0



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LD_{50} = 371 mg/kg BW

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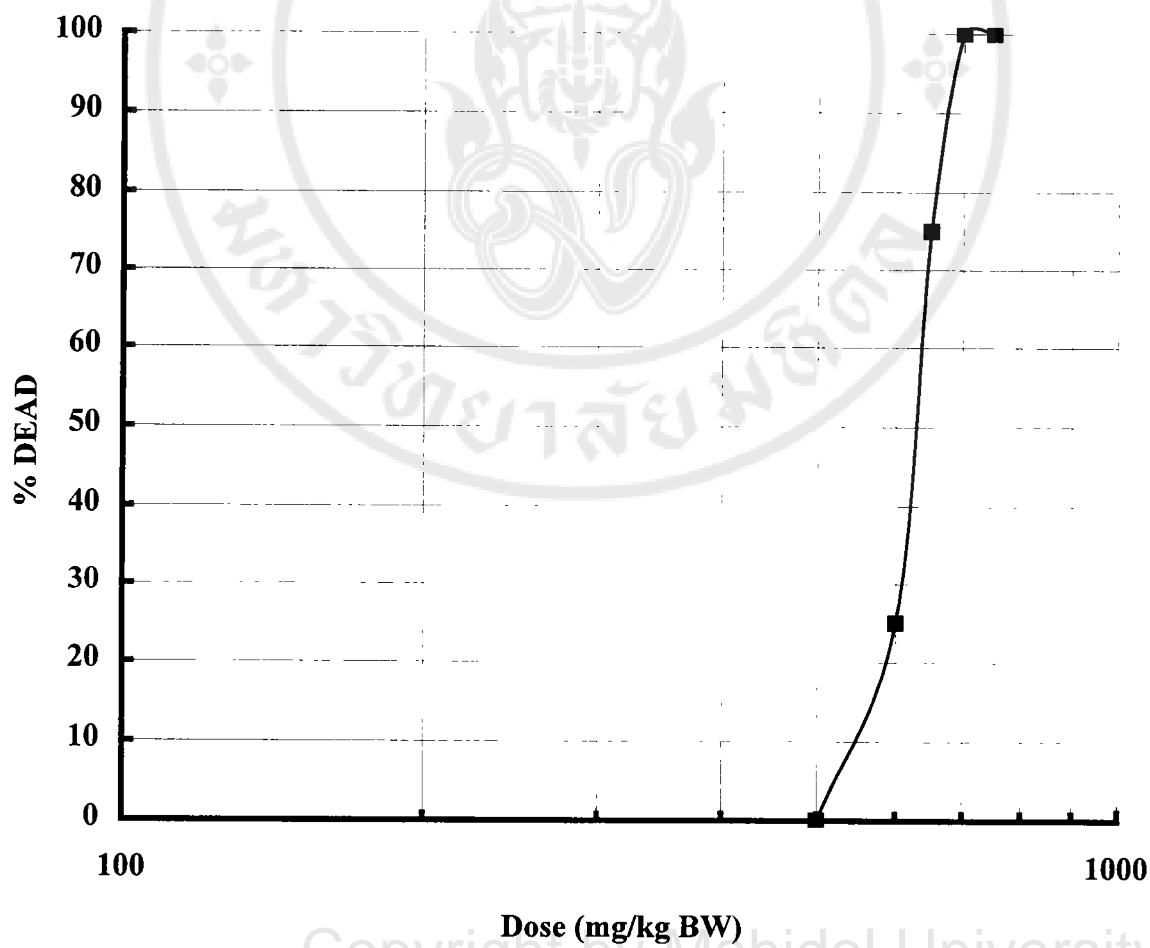
APPENDIX VII

LD_{50} of 2,4,6-trihydroxyacetophenone in male Wistar rat (i.p.)

Dose (mg/kg BW)	% Dead
750	100
700	100
650	75
600	25
500	0



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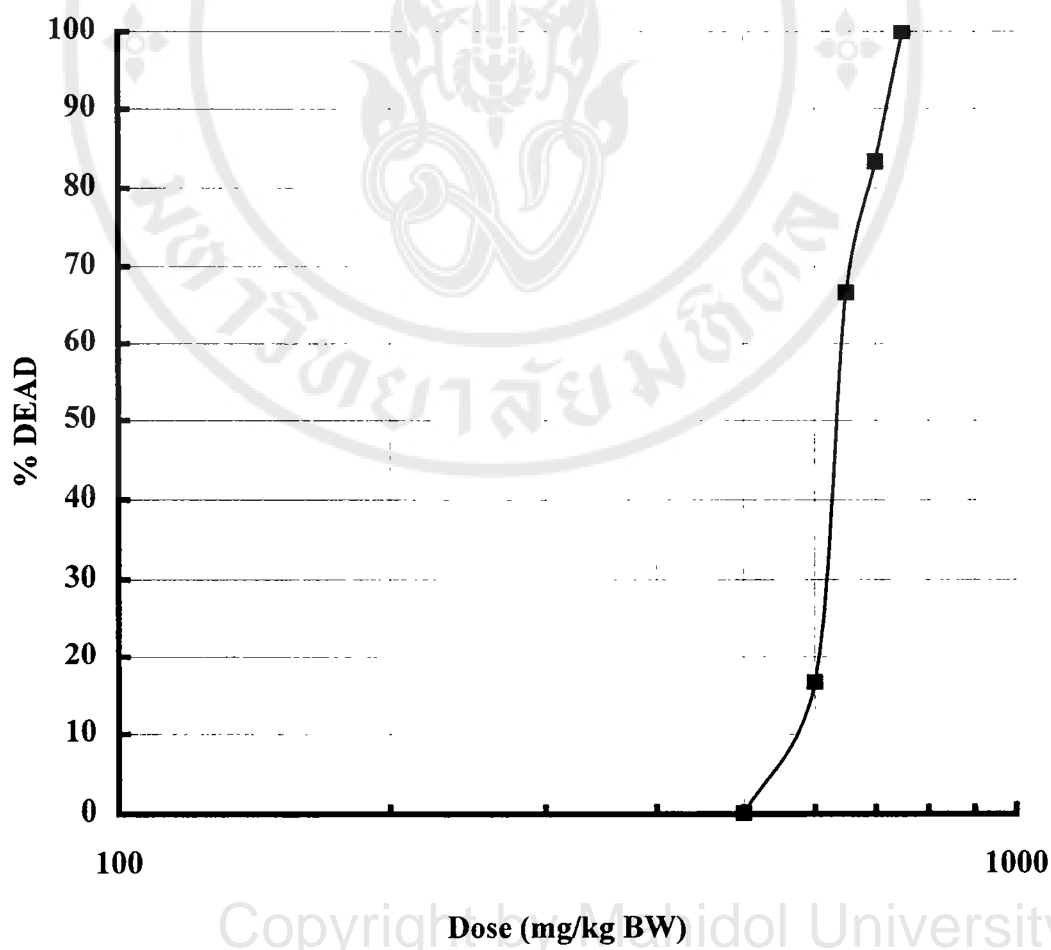
LD_{50} = 620 mg/kg BW

APPENDIX VIII

LD_{50} of 2,4,6-trihydroxyacetophenone in female Wistar rat (i.p.)

Dose (mg/kg BW)	% Dead
750	100
700	83.33
650	66.66
600	16.66
500	0



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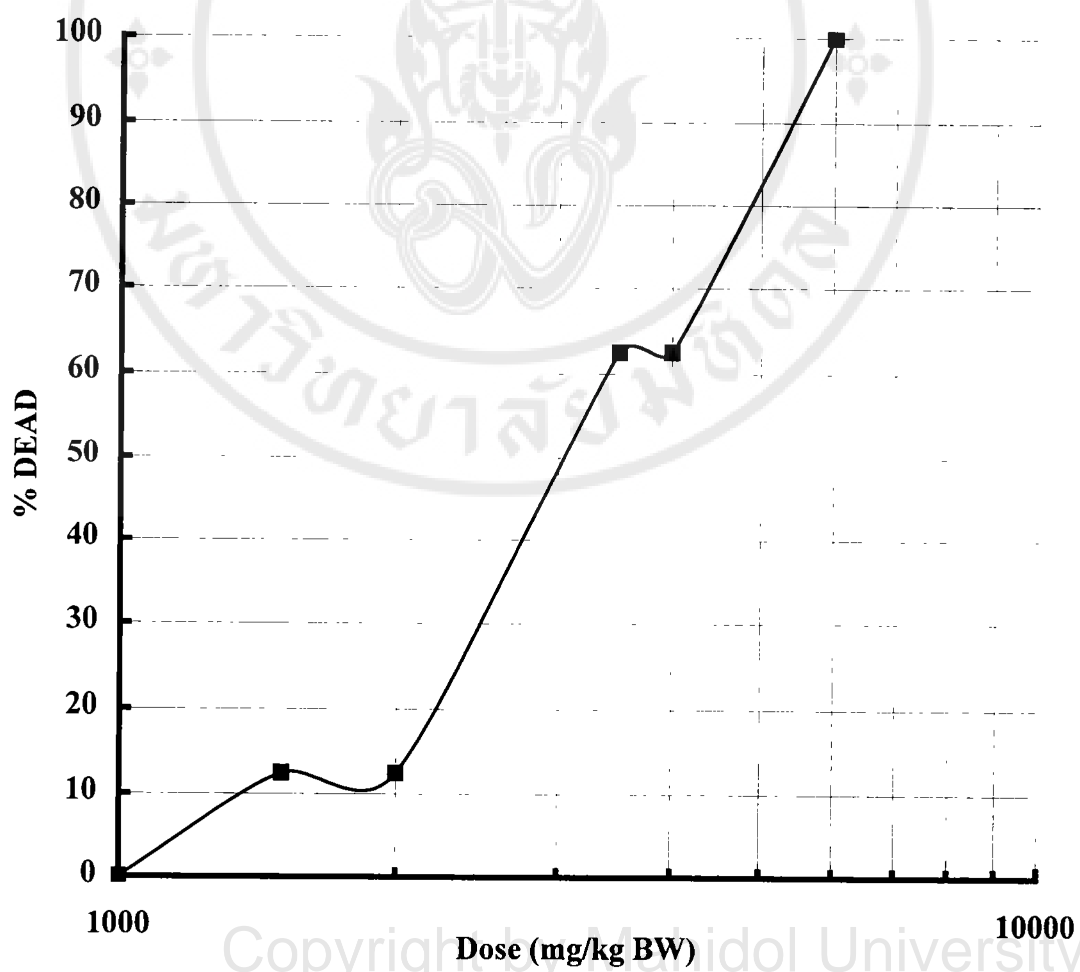
LD_{50} = 660 mg/kg BW

APPENDIX IX

LD_{50} of 2,4,6-trihydroxyacetophenone in male Swiss albino mice (i.g.)

Dose (mg/kg BW)	% Dead
6000	100
4000	62.5
3500	62.5
2000	12.5
1500	12.5
1000	0



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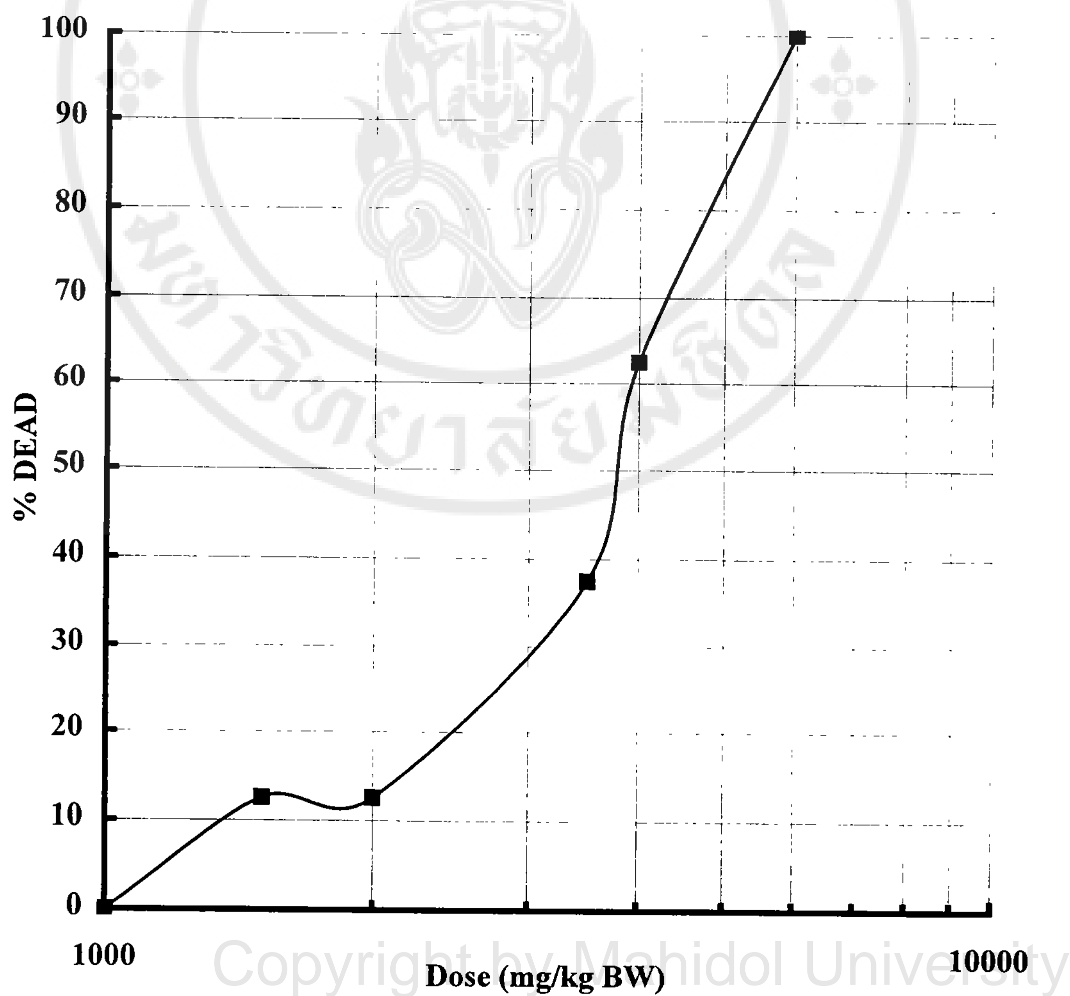
LD_{50} = 3.2 g/kg BW

APPENDIX X

LD_{50} of 2,4,6-trihydroxyacetophenone in female Swiss albino mice (i.g.)

Dose (mg/kg BW)	% Dead
6000	100
4000	62.5
3500	37.5
2000	12.5
1500	12.5
1000	0



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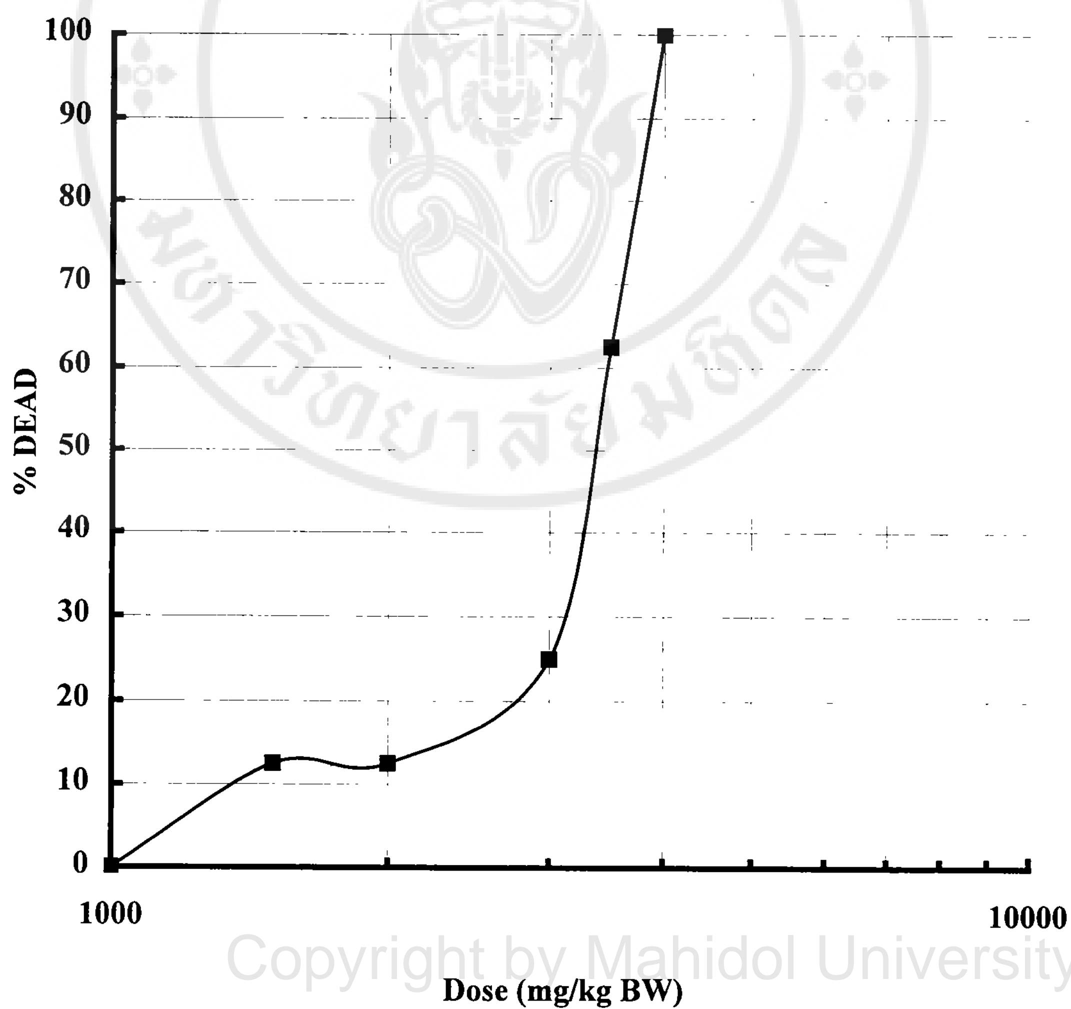
LD_{50} = 3.4 g/kg BW

APPENDIX XI

LD_{50} of 2,4,6-trihydroxyacetophenone in male Syrian golden hamster (i.g.)

Dose (mg/kgBW)	%Dead
4000	100
3500	62.5
3000	25
2000	12.5
1500	12.5
1000	0



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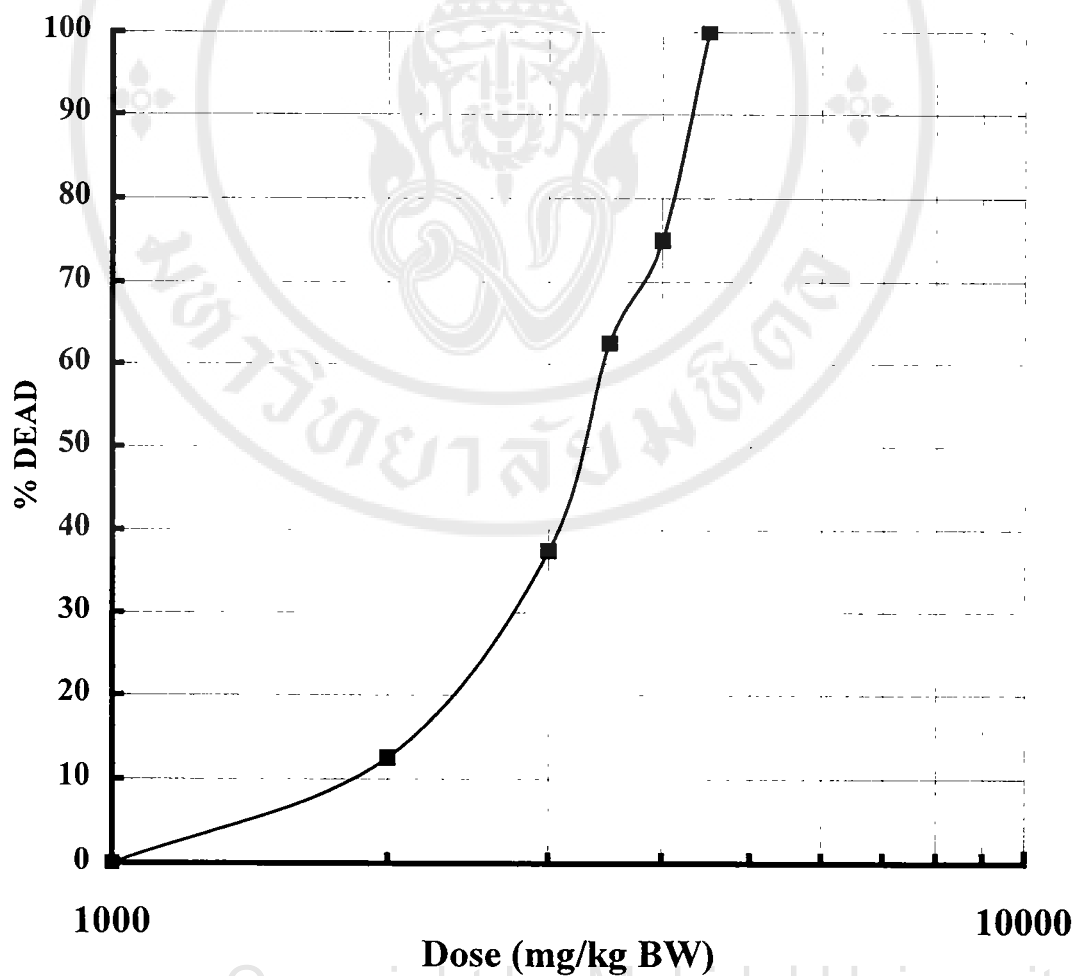
LD_{50} = 2.95 g/kg BW

APPENDIX XII

LD_{50} of 2,4,6-trihydroxyacetophenone in female Syrian golden hamster (i.g.)

Dose (mg/kg BW)	%Dead
4500	100
4000	75
3500	62.5
3000	37.5
2000	12.5
1000	0



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LD_{50} = 3.1 g/kg BW

APPENDIX XIII

Effect of 2,4,6-trihydroxyacetophenone (THA) treatment on biochemical parameters in adult male mice. Animals were intragastrically received the compound once a day for 30 consecutive days.

Body weight (g)

Animal No.	Control	2,4,6-Trihydroxyacetophenone (mg/kg BW)				
		0	37.5	75	150	300
1	25.16	25.44	26.55	25.58	25.45	33.23
2	26.72	27.66	28.26	27.04	27.08	34.96
3	28.74	29.2	28.93	29.57	29.72	35.13
4	30.4	30.98	32.11	31.14	31.25	37.17
5	31.12	31.98	32.42	31.63	31.48	38.87
6	33.64	34.86	33.21	33.08	34.13	
7	33.88	35.06	34.76	32.76	34.75	
8	36.08	39.06	37.75	37.62	38.28	
9	35.66	37.1	36.2	35.91	35.66	
N	9	9	9	9	9	5
MEAN	31.27	32.37	32.24	31.59	31.98	35.87
SEM	1.22	1.42	1.18	1.22	1.31	0.97

Liver weight (g/ 100 g BW)

Animal No.	Control	2,4,6-Trihydroxyacetophenone (mg/kg BW)				
		0	37.5	75	150	300
1	4.75	4.96	4.85	4.94	4.87	5.1
2	4.96	4.85	5.1	4.93	4.86	5.12
3	4.85	5.07	4.85	4.79	4.54	4.99
4	4.79	4.82	4.96	4.98	5.14	5.36
5		4.83	4.85	4.58	4.86	5.31
6		4.62	4.86	4.98	5.13	
7		4.87	4.96	5.32	4.67	
8			4.64	4.86	4.88	
9				4.96	4.67	
N	4	7	8	9	9	5
MEAN	4.84	4.86	4.88	4.93	4.85	5.18
SEM	0.05	0.04	0.04	0.03	0.08	0.07

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Plasma alanine aminotransferase (Unit/ml)

Animal No.	Control	2,4,6-Trihydroxyacetophenone (mg/kg BW)				
		0	37.5	75	150	300
1	33.32	43.28	50.52	55.56	61.84	74.24
2	38.24	59.24	47.92	55.72	61.24	74.08
3	43.28	52.24	51.36	56.16	60.24	77
4	45.36	58.36	49.44	58.84	73.32	76.92
5	47.92	53.12	56.6	59.2	78	78.32
6	40.96	49.84	57.92	59.44	81.8	77.84
7	45	47.52	56.36	61.84	75.64	80.64
8	45	46.64	60.08	61.12	79.44	82.28
9	41.08	44.96	55.8	61.24	89.12	86.2
10	44.96	42.24	54.24	61.32	86.2	90.36
N	10	10	10	10	10	10
MEAN	42.51	49.74	54.02	59.04	74.68	79.79
SEM	1.35	1.88	1.27	0.77	3.3	1.65

Plasma aspartate aminotransferase (Unit/ml)

Animal No.	Control	2,4,6-Trihydroxyacetophenone (mg/kg BW)				
		0	37.5	75	150	300
1	58.92	74.08	85.04	92.64	102.68	138.28
2	66.48	98.2	81.16	92.92	101.2	138.36
3	74.08	90.92	86.36	95.76	102.96	129.84
4	77.28	96.88	90.32	97.6	119.16	133
5	81.16	88.96	94.24	98.12	126.12	134.08
6	74.6	88.56	96.2	98.48	131.72	128.52
7	78.24	80.52	93.88	101	122.64	133.84
8	86.6	79.24	99.48	101.2	128.2	135.48
9	70.24	88.64	93.04	101.84	129.84	133.84
10	74.24	91.52	94.6	101.32	138.08	141.84
N	10	10	10	10	10	10
MEAN	74.18	87.75	91.43	98.09	120.26	134.71
SEM	2.44	2.43	1.79	1.08	4.24	1.27

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Total bilirubin (mg %)

Animal No.	Control	2,4,6-Trihydroxyacetophenone (mg/kg BW)				
		0	37.5	75	150	300
1	0.76	0.91	1.11	1.21	1.37	1.6
2	0.95	0.83	1.19	1.24	1.35	1.57
3	0.9	0.99	1.16	1.26	1.32	1.57
4	0.91	0.99	1.19	1.25	1.34	1.56
5	0.86	0.95	1.13	1.22	1.37	1.56
6	0.87	0.86	1.12	1.23	1.38	1.59
7	0.83	0.88	1.15	1.24	1.39	1.59
8	0.76	0.88	1.19	1.25	1.34	1.55
9	0.78	0.89	1.16	1.26	1.31	1.58
10	0.9	0.78	1.16	1.27	1.33	1.56
N	10	10	10	10	10	10
MEAN	0.85	0.88	1.16	1.24	1.35	1.57
SEM	0.02	0.02	0.01	0.01	0.01	0.01

Blood urea nitrogen (mg %)

Animal No.	Control	2,4,6-Trihydroxyacetophenone (mg/kg BW)				
		0	37.5	75	150	300
1	6.97	7.85	9.26	9.75	10.96	11.97
2	7.94	7.96	9.76	9.84	10.67	11.98
3	7.69	8.12	9.56	10.69	10.61	11.69
4	6.31	8.54	9.18	10.67	10.79	12.58
5	7.44	7.69	9.46	10.45	11.45	11.46
6	7.89	8.64	9.78	10.67	10.69	11.57
7	7.96	8.95	9.75	10.63	11.57	11.59
8	7.39	8.63	9.56	11.56	11.69	11.57
9	7.59	7.94	9.82	10.69	11.97	12.97
10	7.96	8.46	9.81	10.93	11.49	12.59
N	10	10	10	10	10	10
MEAN	7.51	8.28	9.59	10.59	11.19	12
SEM	0.17	0.13	0.07	0.16	0.16	0.17

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Liver triglyceride (mg/ g liver)

Animal No.	Control	2,4,6-Trihydroxyacetophenone (mg/kg BW)				
		0	37.5	75	150	300
1	10.36	10.71	12.89	14.92	15.35	18.97
2	10.23	11.55	12.99	15.30	15.67	18.56
3	10.26	10.24	12.91	15.53	16.01	18.47
4	10.24	11.25	13.14	15.58	16.26	19.20
5		11.52	13.65	15.81	16.34	19.64
6		11.65	13.72	16.21	16.95	
7		10.94	12.82	16.34	17.32	
8			12.97	16.36	18.08	
9				16.9	18.42	
10						
N	4	7	8	9	9	5
MEAN	10.27	10.94	12.98	15.33	15.82	18.80
SEM	0.03	0.2	0.12	0.21	0.35	0.21

Liver glutathione content (μmol/ g wet weight)

Animal No.	Control	2,4,6-Trihydroxyacetophenone (mg/kg BW)				
		0	37.5	75	150	300
1	0.20	0.28	0.53	0.42	0.39	0.17
2	0.24	0.16	0.42	0.36	0.36	0.17
3	0.20	0.19	0.46	0.30	0.28	0.17
4	0.23	0.23	0.50	0.42	0.34	0.25
5		0.21	0.59	0.34	0.33	0.24
6		0.20	0.42	0.41	0.33	
7		0.22	0.47	0.39	0.27	
8			0.48	0.35	0.26	
9				0.46	0.52	
N	4	7	8	9	9	5
MEAN	0.22	0.21	0.48	0.38	0.33	0.20
SEM	0.22	0.25	0.51	0.35	0.25	0.56

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APPENDIX XIV

DETERMINATION OF TRANSAMINASE (AST OR PGOT)

(Reitman and Frankel method)

PRINCIPLE

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

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

1. SGOT substrate (α-ketoglutarate-aspartate substrate)

   Consists of α-ketoglutarate and L-aspatate at pH 7.5
2. Phynylhydrazine (2,4-dinitrophynylhydrazine in HCL)
3. 0.4 N NaOH
4. Standard pyruvate solution: Store all reagents at 2-8 °C

PROCEDURE

1. Add the solutions into each tube as follows:

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Tube No.	Test (ml)
1. SGOT substrate	0.25
Incubate at 37 °C, 5 min	
2. Serum	0.05
Mix, incubate at 60 °C, 60 min exactly	
3. Phenylhydrazine	0.25
Mix, stand at room temperature for 20 min	
4. NaOH	2.5

2. Mix well and stand at room temperature for 5 min.

3. Read optical density at 505 nm by MILTON ROY SPECTRONIC GENESYS 5 Spectrophotometer against distilled water blank.

4. The activity of AST is determined from the calibration curve.

CALIBRATION CURVE

Tube No.	**Blank**	**1**	**2**	**3**	**4**	**5**
(Units of SGOT)	(0)	(20)	(55)	(95)	(216)	-
1. Standard pyruvate (ml)	-	0.025	0.050	0.075	0.100	0.125
2. SGOT substrate (ml)	0.250	0.225	0.200	0.175	0.150	0.125
3. Distilled water (ml)	0.05	0.05	0.05	0.05	0.05	0.05
4. Phynylhydrazine (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

Mix well and exactly 5 min later, read absorbance at 505 nm by Spectrophoto meter, using water at the blank.

Plot a calibration curve of O.D. corresponding units of SGOT.

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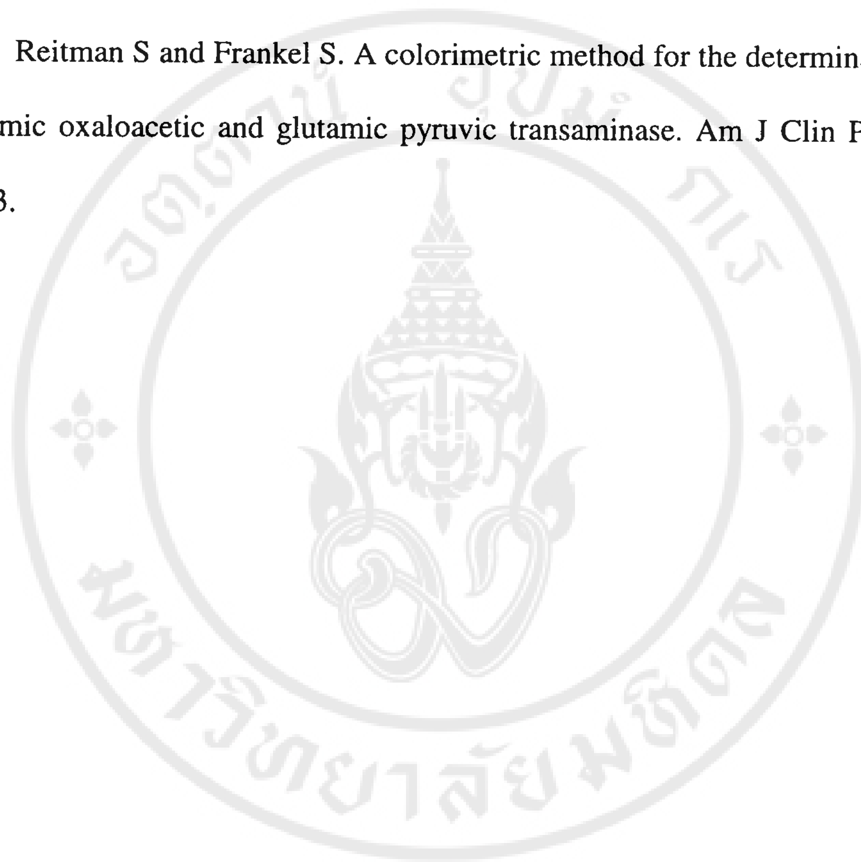
NOTE: If the value of SGPT is higher than 216 IU, dilute the sample by distilled water.

REFERENCE

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



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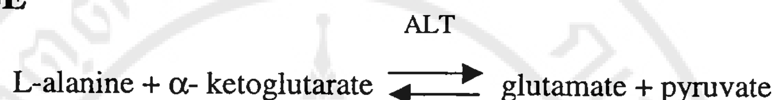
APPENDIX XV

DETERMINATION OF TRANSAMINASE (ALT OR SGPT)

(Reitman and Frankel method)

PRINCIPLE



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

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

1. SGPT substrate (α-ketoglutarate-alanine substrate)

   Consists of α-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 °C

PROCEDURE

1. Add the solutions into each tube as follows:

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Tube No.	Test (ml)
1. SGPT substrate Incubate at 37 °C, 5 min	0.25
2. Serum Mix, incubate at 60 °C, 60 min exactly	0.05
3. Phenylhydrazine Mix, stand at room temperature for 20 min	0.25
4. NaOH	2.5

2. Mix well and stand at room temperature for 5 min.

3. Read optical density at 505 nm by MILTON ROY SPECTRONIC GENESYS 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**
(Units of SGPT)	(0)	(23)	(50)	(83)	(125)	-
1. Standard pyruvate (ml)	-	0.025	0.050	0.075	0.100	0.125
2. SGPT substrate (ml)	0.250	0.225	0.200	0.175	0.150	0.125
3. Distilled water (ml)	0.05	0.05	0.05	0.05	0.05	0.05
4. Phynylhydrazine (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

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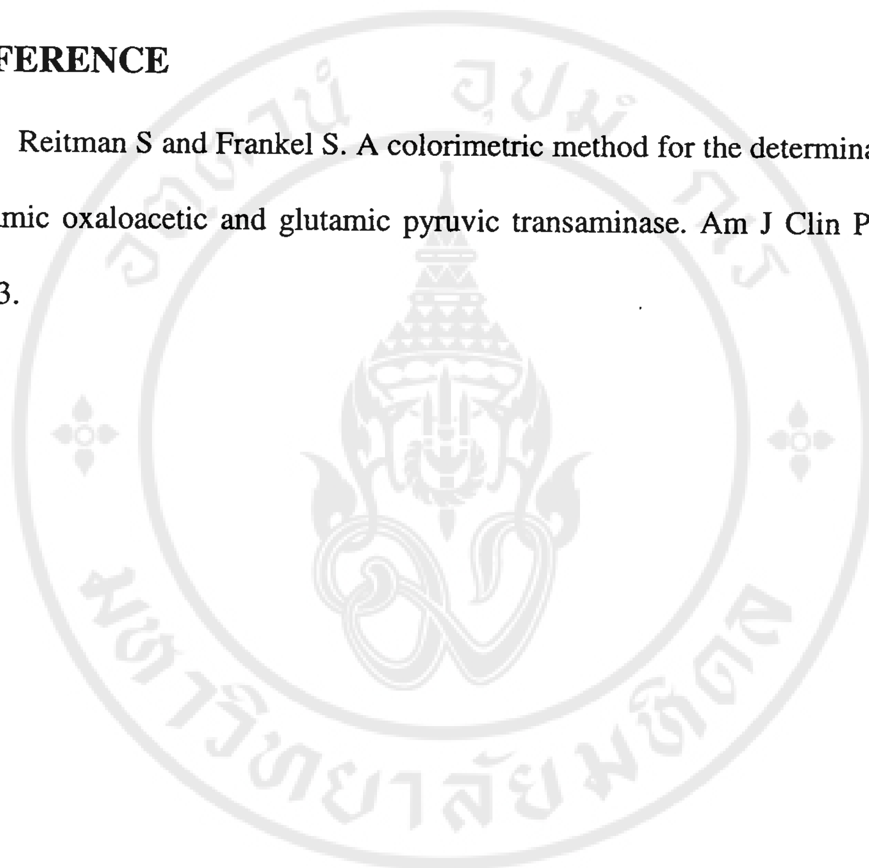
Mix well and exactly 5 min later, read absorbance at 505 nm by Spectrophoto meter, using water at the blank.

Plot a calibration curve of O.D. corresponding units of SGPT.

NOTE: If the value of SGPT is higher than 125 IU, dilute the sample by distilled water.

REFERENCE

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



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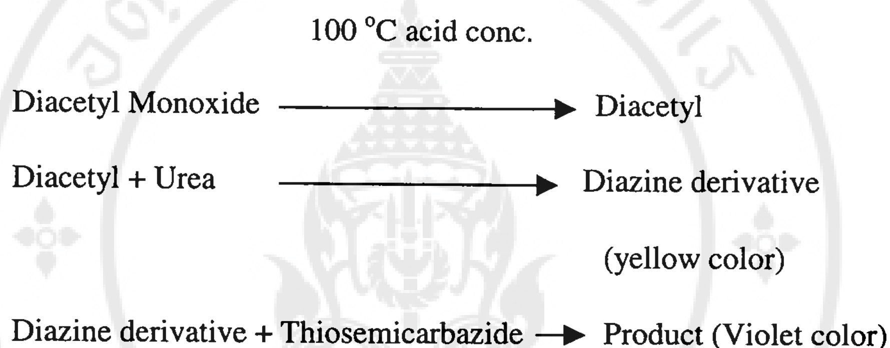
APPENDIX XVI

DETERMINATION BLOOD UREA NITROGEN (BUN)

(Diacetyl Monoxime method)

PRINCIPLE



REAGENTS

1. BUN (A) is Thiosemicarbazide in H_2PO_4 and H_3PO_4 solution.
2. BUN (B) is Diacetyl Monoxide solution.

1 and 2 stored at 2-8 °C

PROCEDURES

Tube No.	Test	Blank
1. BUN (A) (ml)	1.5	1.5
2. BUN (B) (ml)	1.5	1.5
3. Serum (ml)	0.01	-

Mix, boiling in water 100 °C for 10 min, after cooling for 5 min.

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Read O.D. at 540 nm, set zero by blank.

*Standard Calibration curve from BUN standard 3 levels: 20 mg/dl, 40 mg/dl and 60 mg/dl, stored at 2-8 °C.

STANDARD CALIBRATION CURVE

Tube No.	1 (20 mg/dl)	2 (40 mg/dl)	3 (60 mg/dl)	4 (Blank)
1. BUN (A) (ml)	1.5	1.5	1.5	1.5
2. BUN (B) (ml)	1.5	1.5	1.5	1.5
3. Standard urea (ml)	0.01	0.01	0.01	-

Mix, boiling in water 100 °C for 10 min, after cooling for 5 min.

Read O.D. at 540 nm, set zero by blank.

REFERENCES

1. Wybenga DR, Di Giorgio, and Pileggi VJ. Clin Chem 1971; 17: 891.
2. Read AH, Cannon DC, Winkelman JW, Bahasin YP, Henry RT, and Pileggi VJ. Clin Chem 1972; 18: 57.

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APPENDIX XVII

DETERMINATION OF PROTEIN

(Modified Lowry method)

PRINCIPLE

The first step of protein assay is the reaction of protein with cupric ions (Cu^{++}) in alkaline medium, lead to reduction of cupric ions (Cu^{++}) to cuprous ions (Cu^{+}). The second step is reduction of Folin ciocateu's phenol reagent with the alkaline copper-protein complex, giving a characteristic blue reaction color. Absorbance was measured with a spectronic 301 at 650 nm.

REAGENTS

1. Solution A: Alkaline tartrate reagent

Na_2CO_3	10.0 g
$Na_2C_4H_4O_8$ H_2O	0.1 g
NaOH	1.2 g

   Dissolve these chemicals in distilled water and make up to 500 ml.

2. Solution B: 0.5% copper sulfate

   Dissolve 0.5 g $CuSO_4$ $5H_2O$ in 100 ml distilled water.

3. Solution C

   Mix 50 ml of solution A with 1 ml of solution B, use immediately.

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4. Solution D: 1 N folin phenol reagent

Dilute 2 N folin ciocalteu's phenol reagent 1:1 with distilled water and use immediately.

5. Standard protein solution

Bovine serum albumin (BSA) 80 mg% is used as standard protein.

PROCEDURES

1. Add solution into each tube as follow:

Solution	Blank (ml)	Standard (ml)	Unknown (ml)
Distilled water	0.1	-	-
Standard protein	-	0.1	-
Diluted sample	-	-	0.1
Solution C	5.0	5.0	5.0

Mix well and stand at room temperature for 10 min.

2. Mix throughly with 0.5 ml of solution D and let stand for 60 min at room temperature.

3. Read optical density at 650 nm by Spectronic-20 against the reagent blank.

CALCULATION

$$\text{Protein concentration (mg\%)} = \frac{\text{O.D. Unknown}}{\text{O.D. Standard}} \times \text{Conc. of standard} \times \text{Dilution factor}$$

REFERENCE

Lowry OH, Rosenbrough NJ, Farr AL, and Randall RT. Protein measurement with the folin phenol reagent. J Biol Chem 1951; 193: 265-257.

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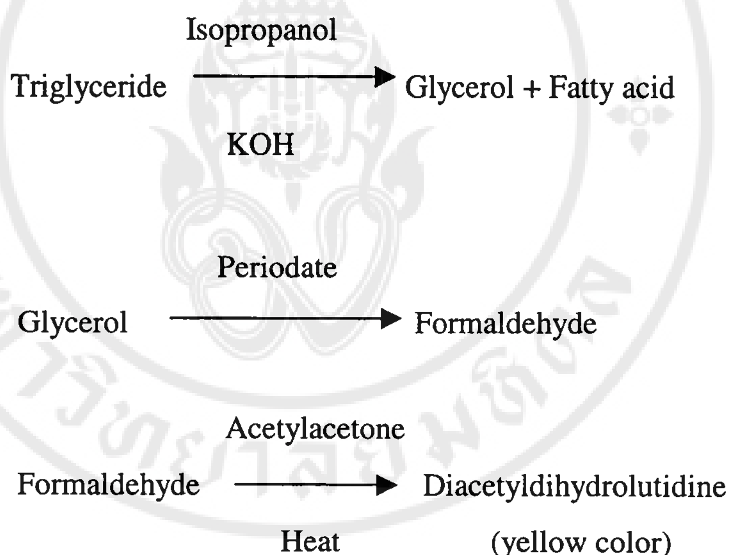
APPENDIX XVIII

DETERMINATION OF LIVER TRIGLYCERIDE CONCENTRATION

(Modification of Mendez et al.,1975)

PRINCIPLE



REAGENTS

1. Saponification reagent: Potassium hydroxide (KOH) 10% in isopropanol 25% (v/v). This solution is stable for at least 2 months at room temperature in a brown glass bottle.

2. Sodium metaperiodate reagent: Dissolve 77 g of anhydrous ammonium acetate in 700 ml of distilled water. Add 60 ml of glacial acetic acid and 650 mg of sodium metaperiodate. Dilute to 1 litre with water and mix throughly. The resulting

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solution is stable for at least 2 months at room temperature is stored in a brown glass bottle.

3. Acetylacetone reagent: Add 0.4 ml of 2,4-pentanedione to 100 ml of isopropanol. This reagent is stable for at least 2 months at room temperature in a brown glass bottle.

4. Triolein standard solution (Sigma stock No. 405-10): Triolein (glycerol trioleate) 300 mg% of isopropanol.

5. Triolein working standards: Working standards of 100, 200 and 300 mg% are prepared by diluting stock standard with isopropanol.

6. Phosphate buffer 0.067 M, pH 7: Mix 0.067 M Na_2HPO_4 solution and 0.067 M KH_2PO_4 solution until pH 7.

PROCEDURES

1. Add 4.0 ml-phosphate buffer to 1.0 g of liver. Mince with scissors and homogenize in glass homoginizer with Teflonpestle at medium speed (10 strokes).

2. Extraction: Use glass tubes, 13x100 mm screw-capped tubes.

	Blank	Standard	Unknown
Liver Homogenates	-	-	0.5ml
Water	0.5ml	0.5ml	-
Standards	-	0.5ml	-
Heptane	2.0ml	2.0ml	2.0ml
Isopropanol	3.5ml	3.0ml	3.5ml
Sulfuric acid	1.0ml	1.0ml	1.0ml

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Mix with a vortex-type mixer for 30 second and allow the phases to separate without centrifugation.

3. To 0.2 ml of heptane (upper) layer from the extraction procedure, add 2.0 ml of isopropanol and 0.6 ml of Saponification reagent. Mix and allow standing at room temperature for at least 5 min.

4. Add 1.5 ml of metaperiodate reagent and mix.

5. Add 1.5 ml of acetylacetone reagent and mix.

6. Cap each tube and place all tubes and allow then cooling to room temperature.

7. Read the absorbance within 45 min at 415 nm. A blank set at zero absorbance with spectrophotometer.

CALIBRATION CURVE

The standard curve is prepared by plotting the concentration of standard (100, 200, 300 mg%) VS absorbance. The calibration curve is linear to 300 mg/dl.

REFERENCE

Mendez J, Franklin B and Gahagan H. Simple manual procedure for determination of serum triglycerides. Clin Chem 1975; 21(6): 768-770.

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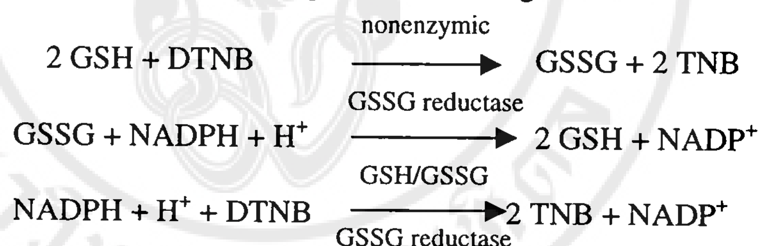
APPENDIX XIX

DETERMINATION OF LIVER GLUTATHIONE

(Theodorus Method)

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:



$$2\ GSH + DTNB \xrightarrow{\text{nonenzymic}} GSSG + 2\ TNB$$

$$GSSG + NADPH + H^+ \xrightarrow{\text{GSSG reductase}} 2\ GSH + NADP^+$$

$$NADPH + H^+ + DTNB \xrightarrow[\text{GSSG reductase}]{\text{GSH/GSSG}} 2\ TNB + NADP^+$$

The reaction rate is proportional to the concentration of glutathione at values up to about 2 μM. The formation of 5-thio-2-nitrobenzoate (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 0.001 M EDTA at pH 7.0; prepare daily from stock solutions of 0.1 M KH_2PO_4 and 0.1 M K_2HPO_4, each containing 1 mM EDTA.

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2. 5,5′-Dithiobis (2-nitrobenzoic acid) (DTNB), 1.5 mg/ml: Prepare fresh each day; dissolve DTNB in 0.5% $NaHCO_3$ and store in the dark.

3. NADPH, 4 mg/ml: Dissolve NADPH in 0.5% $NaHCO_3$ and store at 4°C.

4. Glutathione reductase, 6 units/ml: Dilute commercial enzyme in buffer each day.

5. Glutathione disulfide (GSSG), 10 μM; Prepare standard daily from stock solution (1 mM).

PROCEDURE

1. Liver tissues (200-250 mg) are immediately removed and homogenized in 2 ml of 0.25 M $HClO_4$ and 2 ml of 0.1 M phosphate buffer, pH 7.0 (For bile sample dilute by 0.25 M $HClO_4$ 1:5 before assay).

2. The mixture is centrifuged at approximately 8000 rpm for 5 min.

3. The supernatant is assayed for GSH by DTNB method.

4. Pipet into cuvette: 1.0 ml of buffer, sample, 100 μl containing 0.5-2 nmol glutathione, 50 μl NADPH, 20 μl DTNB, 20 μl glutathione reductase. After mixing the contents of the cuvette, record the linear increase in absorbance at 412 nm. A blank assay, without glutathione, is run separately. For calibration, the procedure is repeated using 100 μl GSSG (10 μM) instead of sample. Temperature is controlled at 25°C.

5. The amount of glutathione level is expressed as μmole/g wet weight.

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CALIBRATION CURVE

1. Prepare series of tubes containing GSSG in the following concentrations: 1 µmol, 2 µmol, 4 µmol, 6 µmol.
2. Perform the procedure as in step 4.
3. Determine the optical density at 412 nm.
4. Plot a calibration curve of optical density versus µg of GSH.

REFERENCE

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

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APPENDIX XX

DETERMINATION OF TOTAL BILIRUBIN

(Jendrassik and Grof Method)

PRINCIPLE

Bilirubin in the serum is coupled with diazotized sulfanilic acid to from azobilirubin with purple color. Intensity of the purple color is proportional to the bilirubin concentration in the serum. The conjugated glucuronide bilirubin or direct bilirubin reacts with the diazo reagent in aqueous solution to from the color, which occurs within 1 minute. The subsequent addition of alcohol accelerates the reaction of all forms of bilirubin in the serum in which a value obtained derived from total bilirubin. The reaction takes about 30 minutes. The total bilirubin value presents the sum of the bilirubin glucuronide (direct) and the unconjugated (indirect) bilirubin.

Noted that the azobilirubin color has indicator properties. In strongly acid or strongly alkaline solution the color is blue. In the method of Jendrassik and Grof the diazotization reaction is accelerated by caffeine and sodium and the final blue color is developed at a strongly alkaline pH.

REAGENTS

Kit of total bilirubin test from BM-Lab Bangkok consisted of the following solutions;

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1. Sulfanilic acid

2. Sodium nitrite solution. Sodium nitrite will react with sulfanilic acid and change it into beta-benzenediazonium sulfonate.

3. Accelerator is a solution of caffeine and sodium acetate, which drives nonreaction bilirubin into reaction.

4. Alkaline tartrate buffer, a solution, which enhances reaction mixture to be alkaline. The standard kits of total bilirubin were 6, 4.5, 3, 1.5 mg/dl which were dissolved in distilled water 3 ml.

PROCEDURE

Tube No.	Test	Blank
1. Sulfanilic Acid (ml)	1.0	1.0
2. Sodium Nitrite (ml)	0.05	-
3. Serum (ml)	0.2	0.2
4. Accelerator (ml)	1.0	1.0
Mixed and left at room temperature for 5 min.		
5. Alkaline Tartrate Buffer (ml)	1.0	1.0

Mixed and left for 5 min. The optical density was react at 600 nm within 30 min. Standard curve was constructed and the unknown were read off the standard curve in mg/dl.

REFERENCES

1. Jendrassik L and Grof P. Biochem Z. 1938 ; 297 : 81-89.

2. Gambio R. Standard Method in Clinical Chemistry. New York: Academic Press, 1965. p.55.

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APPENDIX XXI

DETERMINATION OF ALKALINE PHOSPHATASE (AP)

(Phenolphthalein Monophosphate Method)

PRINCIPLE

AP hydrolyses phenophthalein monophosphate in alkaline solution to produce phenophthalein (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 °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 indicated below:

	Test	Blank
AP substrate (drop)	1.0	1.0
Distilled water (ml)	1.0	1.1
Incubate at 37°C, 5 min		
Serum or plasma (ml)	0.1	-
Incubate at 37°C, 20 min exactly		
AP buffer (ml)	10	10

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

STANDARD CURVE

Tube No.	1	2	3	4
Units of AP	25	50	75	100
AP buffer (ml)	3	3	3	3
Standard (ml)	0.05	0.05	0.05	0.05

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 1966; 12: 482.

2. Fubr J and Stary EA. Lab 1969; 15: 55.

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APPENDIX XXII

PARAFFIN SECTION

PREPARATION OF 10% NEUTRAL BUFFERED FORMALIN

Formaldehyde (37%)	340	ml
NaH_2PO_4	13.78	g
Na_2HPO_4	22.12	g
Water up to	1.0	gallon

PREPARATION OF PARAFFIN SECTIONS

1. The organs were fixed in 10% buffered formalin.
2. The fixed tissues were trimmed.
3. The trimmed tissue were dehydrated, cleared, and embedded with the following solutions for 30 min per step.

    a. 70% Ethanol

    b. 80% Ethanol

    c. 90% Ethanol

    d. Absolute ethanol, twice

    e. Absolute ethanol and xylene (1:1, V:V), once

    f. Xylene, twice

    g. Immersed in soft, medium hard and hard paraffins, respectively at 60 °C under vacuum.

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h. The tissues were embedded with paraffin in plastic holders.

4. The blocks of tissues were cut to provide sections 5 microns in thickness.
5. The sections were mounted on glass slides by standard warm water technique and dried at room temperature overnight.

HEMATOXYLIN AND EOSIN STAINING

1. The mounted sections were held in the following solutions for the indicated times:
   a. Absolute ethanol, 2 min
   b. 95% Ethanol, 2 min
   c. 80% Ethanol, 2 min
   d. 70% Ethanol, 2 min
   e. Distilled water, 2 min
   f. Harris hematoxylin, 8 min
   g. Distilled water, 2 min
   h. 80% Ethanol, 2 min
   i. Eosin, 5 min
   j. 95% Ethanol, 2 min
   k. Absolute ethanol, 2 min, twice
   l. Xylene, 2 min, twice
2. Mounted with permount and dried overnight at room temperature.

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APPENDIX XXIII

Laboratory Test	Reference Range
Alanine aminotransferase (ALT)	5 to 35 IU/L
Alkaline phosphatase	30 to 120 IU/L
Aspartate aminotransferase (AST)	5 to 40 IU/L
Total bilirubin	0.1 to 1.2 mg/dl
Blood urea nitrogen (BUN)	5 to 25 mg/dl
Triglycerides	< 200 mg/dl, normal

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BIOGRAPHY

NAME	**Miss Chatsuda Tubtim**
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RESEARCH GRANT	**Partially supported by a grant from**
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