INVESTIGATION OF HIPPURIC ACID IN VAPOUR EXPOSURE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AND COLORIMETRY

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ชื่อวิทยานิพนธ์ การตรวจพิสูจน์หากรดอิพพิวริกในกลุ่มผู้สัมผัสสารระเหย โดยวิธีโครมาโตกราฟีของเหลวแบบสมรรถนะสูง และการตรวจวัดสี

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บทคัดย่อ

ระดับกรดอิพพิวริกในปัสสาวะมีความสำคัญในการพิสูจน์เพื่อช่วยในการวินิจฉัยเกี่ยวกับภาวะของการได้รับสารระเหยหรือผู้สูดดมทินเนอร์ การใช้วิธีสำหรับวิเคราะห์ในห้องปฏิบัติการพิษวิทยาสามารถตรวจหาได้หลายวิธี จากการศึกษาโดยวิธีการตรวจวัดสี: วิธีใช้สารเบนซีน-ซัลโฟนิล คลอไรด์ (BSC), วิธีใช้สาร พารา-ไดเมทิลอะมิโน เบนซัลดีไฮด์ (DAB) เปรียบเทียบกับวิธี โครมาโตกราฟีของเหลว แบบสมรรถนะสูง (HPLC) โดยศึกษาในกลุ่มคนปกติ, คนในโรงงาน, และผู้สูดดมสารระเหย 2 กลุ่ม คือ กลุ่มของแก๊งวัยรุ่น และ กลุ่มของคนไข้ ในการศึกษากลุ่มคนปกติ พบว่าวิธี BSC และ DAB เมื่อเปรียบเทียบ กับวิธี HPLC มีความสัมพันธ์กันที่ค่าความสัมพันธ์ (r) = 0.96121 และ 0.97682 ตามลำดับ ส่วนกลุ่มผู้สูดดมสารระเหยเมื่อเปรียบเทียบกับกลุ่มคนปกติ พบว่ามีค่ากรดอิพพิวริกแตกต่างกันอย่างมีนัยสำคัญ ที่ $p<0.01$ ในขณะเดียวกัน การตรวจหากรดอิพพิวริกในคนปกติโดยวิธีการตรวจวัดสีด้วยสาร DAB เปรียบเทียบกับการวิเคราะห์โดยวิธี HPLC พบว่ามีค่าความสัมพันธ์ (r) = 0.97682 และมีค่าอ้างอิงปกติ 180-1,500 และ 150-1,400 ไมโครกรัม/มิลลิลิตร ตามลำดับ ตลอดจนมีค่าความสามารถในการตรวจพบสารโดยเฉลี่ยของวิธี DAB = 93.8% และของ วิธี HPLC = 105.4%

Thesis Title Investigation of Hippuric Acid in Vapour Exposure by High-Performance Liquid Chromatography and Colorimetry

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ABSTRACT

Urinary hippuric acid level is useful in the diagnosis of solvent or thinner exposure. Several methods can be used in Toxicology Laboratory. In this study, colorimetric methods: benzene-sulfonyl chloride (BSC) and p-dimethylamino benzaldehyde (DAB) methods were used and were compared to high-performance liquid chromatographic (HPLC) method, studied in non-exposed or normal subjects, industrial worker, and exposed persons. The exposed persons composed 2 groups: teenager gangsters and patients. In the normal group, correlation coefficient of BSC and DAB methods compared to HPLC method were 0.96121 and 0.97682, respectively. When compared hippuric acid levels in exposed and non-exposed group, there was a significantly different ($p<0.01$). Reference value in normal group determined by DAB and HPLC methods were 180-1,500 and 150- 1,400 μg/ml with the means of percent recovery 93.8 and 105.4, respectively.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Figure

Page

Chapter I

Introduction

Background of The Problem

In the recent year, Thailand is a developing country not only in agriculture but also in industry. Therefore in various industrial processes, solvent is used as a chemical intermediate.

There are 3 kinds of solvent as the following[145]:-

1. Volatile Hydrocarbon

This is the organic solvent obtained from petroleum and natural gas. It can volatile at room temperature and easily dry out.

2. Solvents

These solvents are used as composition of industrial and household products. List of products is shown in Table 1.[3]

Table 1. Solvent composition of products abused

Product	Chemical Constituents
Glue/adhesives	toluene , benzene , xylene , acetone , N-hexane trichloroethylene,ethyl acetate, tetrachloroethylene1,1,1-trichloroethane, carbon tetra chloride , isoamyl acetate
Gasoline (petrol)	hydrocarbons , tetraethyl lead , Naphtha
Aerosols	fluorocarbons
Lighter refills	butane
Acrylic paint	toluene
Paints , lacquers , varnishes	trichloroethylene , methylene chloride , toluene , xylene
Polystyrene cements	acetone , toluene , trichloroethylene , hexane
Dyes	acetone , methylene chloride
Nail polish remover	acetone , amyl acetate
Cleaning solution	benzene , trichloroethane

3. Aerosol

Aerosol is hydrocarbon or halocarbon compound composition of deodorant, hair spray, and pesticide which are in the spray form.

The Reasons of Solvent Abused

There are 2 main reasons that people become addicted by solvent abused.

1. Purposely used in order to relief the anxiousness. This group is found among the adolescences who have problems in the family.

The causes of inhaling solvent abused are as follows[143]:-

1.1 Introduced by friends who are being addicted.

1.2 Low cost. This group of solvents is cheaper than other drugs of abuse like heroin, morphrine, and etc.

1.3 Easy to acheive. These solvents are in the liquid form which are household products such as nail polish remover, laquer, thinner, glue, and paint sprays. They are sold in a wide range of shops.

1.4 Easy to keep and carry.

1.5 They have sedative effect.

1.6 The activity to relief anxiousness is faster than alcohol.

2. Accidentally addicted by solvents used in daily work. This is found in industrial workers.

Solvent abused or "glue sniffing" is a major problem throughout the world, especially among the teenagers. The problems presented to the forensic toxicologist are the fact that there are many possible solvents available for potential abuse. Identification, quantitation and subsequent diagnosis depend on the availability of a suitable methods of analysis. In 1979 Lush et al.[77] studied cases of suspected solvent abused during October 1977 and July 1979. In 21 months, there were a total of 82 referrals for suspected abuse (Fig 1.). Sixty of these referrals were found to have blood solvent levels. Four of these were fatal, one of which showed both

ethanol and toluene levels. Of the remaining 56 samples, 52 had toluene, 1 had toluene and ethanol, 1 trichloroethylene, and 2 ethanol alone. In conclusion 80% of the positive cases showed significant levels of toluene. In the 56 different abusers the average age was 14.5 years. It was interesting to find that only two (both of which were fatal) were over 18 years old. The male and female ratio was 5.7 to 1, which was lower than other studies where the ratio was given as 9 to 1 or even 20 to 1 (Watson 1974).

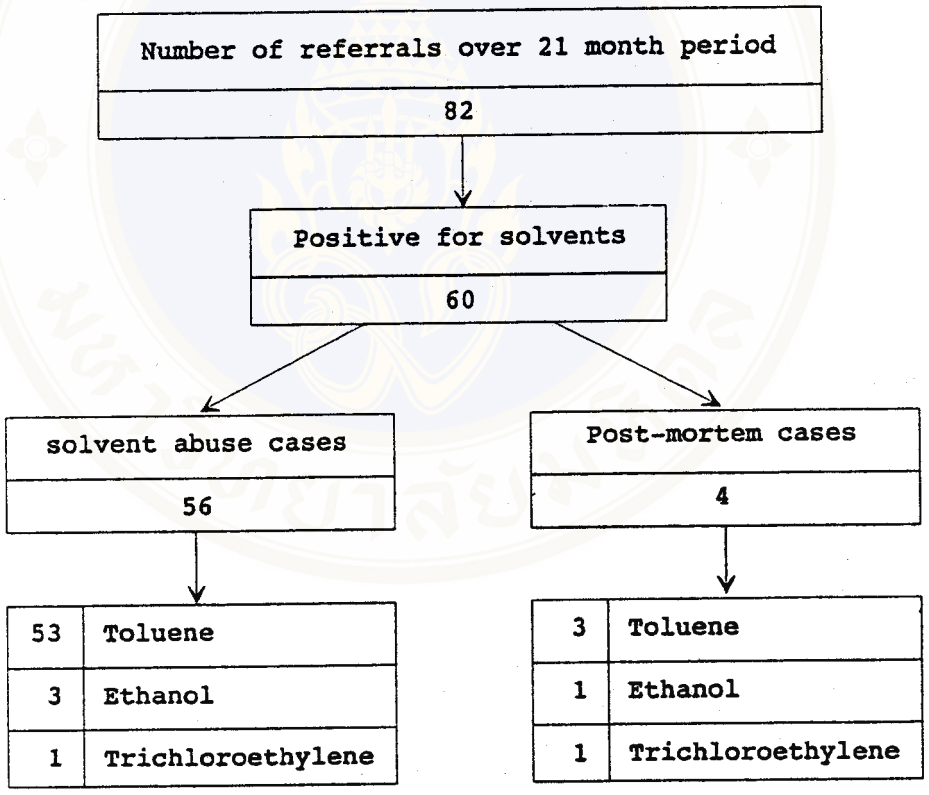


Fig 1. Referrals during period October 1977 to July 1979

In Thailand, addiction to volatile solvents such as 'Thinner' is one of drug abused problems. Although there is no scientific evidence proving its narcotic effect clearly, there is evidence that persons who inhale or sniff it to some extent need medical helps[139].

Since 1981, there have been many reports in Thai newspapers about juvenile delinquency and crimes in Bangkok metropolis that were caused by the narcotic and hallucinating effects of Thinner. One example was quoted from 'DAO SIAM' newspaper, vol. 2284, June 9,1981., a young gangster deceiving a girl student to his place and compelled the girl to inhale thinner vapor to neary unconscious before making sex crime with her. Moreover, he prepared to send her to a brothel but he was prior arrested by the policeman.

From a report of the Ministry of Health in 1981, there was evidence that volatile solvent sniffing became widespread in youngsters. The report showed that the volatile solvents i.e. benzene, and thinner can affect the respiratory, nervous, and hemopoietic systems.

In the document No. 03-2524 from the Division of Narcotic Drug Prevention, the Prime-Minister's Office[140], volatile solvents are included in the list of addictive substance. Although W.H.O. gave notices that thinner and plastic cement for toy-model caused offense to human health. These materials are illegal. There is no comment about usage and controls of these substance in the Thai's narcotic drug act. There was a survey among the volatile sniffers in 1978, showing that the inhalation of benzene and thinner vapor was widespread in the aldolescences, and girl students; 20% preferred to sniff some essential oils[140].

In 1984, There was a research of thinner abused in a group of student in the northern part of Thailand (Lampang, Lumpoon, Chiang Rai, Pitsanulok, Sukothai, Uttaradit, Prae, and Nan) and in the central part of Thailand (Kanjanaburi, Supanburi, and Bangkok). This was carried by The Center of Preventive Medicine of The Teacher's College in collaboration with the Narcotic assistant unit (N.A.U.) of the United State of America Embassy[141], with shown in Table 2.

Table 2. the results of research for thinner abused in 1984

Part	North			Central			Bangkok		
	No.	Use %	Unuse %	No.	Use %	Unuse %	No.	Use %	Unuse %
Student	3,838	1.3	98.7	999			2,963	1.1	98.9
Sex									
Male	1,992	1.4	98.6	584	5.0	95.0	1,754	1.5	98.5
Female	1,846	1.2	98.8	415	5.3	94.7	1,209	0.6	99.4
Age (years)									
13-15	1,210	1.2	98.8	341	3.2	96.8	948	0.5	99.5
16-18	1,775	1.2	98.8	444	7.2	92.8	1,033	1.5	98.5
19-21	692	1.7	98.3	184	3.2	96.8	865	1.6	98.4
22-24	114	-	100	25	4.0	96.0	88	-	100
> 25	47	-	100	5	20.0	80.0	28	-	100

Ingredients of Thinners

General formulation of thinner may contain the following solvents[54]:-

Acetone, Aliphatic petroleum solvents, Aromatic hydrocarbon solvents (xylene, toluene), Cellulosolve acetate, Diacetone alcohol, Ethyl alcohol, Ethyl amyl ketone, Isopropyl alcohol, Methyl ethyl ketone, Methyl isobutyl ketone, Naphthol, Naphthenes, Toluene, Xylene; and may including the following solvents:

Butanol or iso-Butanol Diisobutyl ketone, Esters, particularly butyl acetate, often ethyl acetate, rarely amyl acetate, also isopropyl acetate and isobutyl acetate, Nitromethane, Nitroethane, 2-Nitropropane.

Thinner could contain 0-100% of most common solvents listed above.

The following is an example of lacquer and paint thinner compositions which have toxicity rating about 4.[54]

ethyl alcohol	0-5 %
ethyl acetate	20-21 %
butyl alcohol	10-11 %
toluene	25-28 %
aliphatic hydrocarbons	16-20 %
butyl acetate	20-23 %

It may contain amyl acetate, isopropyl acetate, isopropyl alcohol, pigment, and xylene.

There are many formula for thinner of various grades and purposes in the Chemical Formulary, for examples:-

a) Lacquer solvent.

alcohol	13	parts
cellosolve	16	parts
butanol	16	parts
toluene	47	parts
ethyl acetate	8	parts

b) Optimum surface tension lacquer solvent.

butyl acetate	33	gallons
butyl alcohol	12	gallons
toluol	20	gallons
trolu oil	35	gallons

c) Brushing lacquer thinner.

Formula No. 1:

toluene	60	cc.
ethyl acetate	10	cc.
cellosolve	10	cc.
cellosolve acetate	10	cc.
butyl cellosolve	10	cc.

Formula No. 2:

toluene	60	cc.
ethyl acetate	10	cc.
cellosolve	10	cc.
amyl acetate	10	cc.
ethyl lactate	10	cc.

d) Thinner in rust-inhibiting primer for lacquer.

methyl ethyl ketone	106.72	parts (by weight)
methyl isobutyl ketone	35.48	parts
isopropyl alcohol	60.39	parts
" tolu-sol "	118.52	parts
toluene	51.89	parts

e) Solvent mixtures for lacquers.

Formula No. 1:

toluene	80-90 %
ethyl alcohol	10-20 %
xylene	0-10 %

Formula No. 2:

benzene	80-90 %
methanol	20-10 %

So that Curry[33] proposed that main composition of thinner was toluene, ethyl acetate, and xylene.

Estimation of Thinner

Methods for estimation of thinner are to estimate the main composition that are toluene, ethyl acetate, and xylene. The other methods are the analysis of the metabolite forms of those compositions of thinner. Summary of some metabolite forms of solvents is shown in Table 3.[5]

Table 3. Urinary metabolites used to monitor solvent abuse

Solvent	Urinary metabolite
Benzene	Phenol
Toluene	Hippuric acid
Xylene	Methylhippuric acid (Toluic acid)
Styrene	Mandelic acid and Phenylglyoxylic acid
Trichloroethylene	Trichloroacetic acid and Trichloroethanol

The fact that toluene is the main composition of thinner, analysis of toluene directly and metabolite form of toluene (hippuric acid) indirectly are performed. There are many methods for the analysis of hippuric acid with different specificity, sensitivity, and cost. For example: colorimetry, titration, thin-layer chromatography (TLC), gas chromatography (GC), and high-performance liquid chromatography (HPLC). This inspired me to carry out this study entitled " Investigation of hippuric acid in vapour exposure by high-performance liquid chromatography and colorimetry". In this study 3 methods: high-performance liquid chromatography (HPLC method) and two of colorimetric using Benzene-sulfonyl chloride reagent (BSC method) and p-dimethylamino benzaldehyde reagent (DAB method). The study was done in a group of normal persons for normal values in every methods, in persons who sniff thinner, and in workers who worked in a factory that used thinner, or toluene.

Chapter II

Toluene

Toluene is an aromatic hydrocarbon with a wide spread industrial use as organic solvent.[15] It is a colorless liquid used extensively as a solvent in the chemical, rubber, paint, varnish, glue, enamel, lacquer and pharmaceutical industries, as well as a chemical intermediat in the synthesis of organic compounds.

Since toluene is the best indicator for detection of "thinner" sniffing[33], its details are taken to reveal about its nature and roles in the body. This will help in assessing the analytical results.

Source of Toluene

Toluene is a commercially-important intermediate chemical produced throughout the world in enormous quantities (0.5-1x107 tonnes). It is produced both in the isolated form and as a componant of mixtures.

1. Production

1.1 From petroleum industry, by dehydrogenation of cycloparaffin (naphthene) fractions, or by cyclization and aromatization of paraffin (aliphatic) hydrocarbon.[20,97] This process was developed since 1941 due to the tremendous demand of toluene during World War II, giving a high yield of aromatics.[48] This process is its main source.[25]

1.2 As a product, from the gases and coal tar, of the coke-oven industry.[20] Toluene is present in coal tar and is found together with benzene in the first fraction on fractionally distilling the tar.[48]

1.3 The carbonization of coal was the major source of toluene and xylene.

1.4 By petrochemical processes.

2. Industrial uses[20]

2.1 As a solvent for gums, fats and rasins. Toluene is use extensively in chemical and drug industries.[97]

2.2 As a thinner for paints, vanishes, lacquers, glues and enamels, and as a paint remover.

2.3 As a starting material in the chemical industry, e.g. in the manufacturing of TNT (Trinitrotoluene) in the explosive industry.[97,118]

2.4 As a constituent of motor and aviation fuels.

2.5 In fabric and paper coating.

2.6 In the rubber industry, as a solvent for neoprene.

2.7 In the manufacture of artificial leather.

2.8 As an additive in cosmetic products.

3. Sources of exposure[29]

The primary man-made sources of toluene released into the environment are:

3.1 inadvertent sources (65%), i.e., emission from motor vehicles and aircraft exhaust, and losses during gasoline marketing activities, spills, and cigarette smoke.

3.2 processes in which toluene is used (33%).

3.3 toluene production (2%).

The general population is exposed to toluene mainly through inhalation of vapour in ambient air, cigarette smoking, and to a minor extent, by ingestion of food or water contaminated with toluene.

A special group exposed to toluene includes individuals who intentionally use abuse solvent mixtures containing toluene (e.g., "glue-sniffers") and those who are exposed to toluene accidentally. Solvent abused is a world-wide problem, and long-term abusers are routinely exposed to concentrations exceeding 3,750 mg/m^3.[29]

Fates of Toluene and Its Metabolites in The Body

1. Absorption

Absorption by inhalation is rapid.[20] Persons exposed to toluene vapors for 5 hours in atmospheric concentration of 271-1,177 μg/l of air were found to absorb during the period of inhalation between 41-63.5% (average 53.3%) of the toluene.[116] The unchanged compounds are mainly absorbed in fat tissue[118] with the concentration of more than 20 times high in peritoneal fat or bone marrow than in blood.[112] Since the solubility of toluene in water and blood is low, small amounts are present in blood[20], finding 7.3 mg/l in persons exposed to 300 ppm of toluene.[125]

Absorption through the skin also occurs at an appreciate rate.[43,110]

2. Excretion

2.1 Unchanged form

Excretion rate of toluene is at first rapid and later slow.[125] Moreover, it is increased with toluene concentration.[89] After 2 hours of exposure, small amounts are present in the blood.[20] The main route of excretion is lung elimination. During the desaturation period, 16 to 20% (or 16.3%) of absorbed toluene is eliminated in expired air.[115] Small amount (0.06%) may be found excreted in the urine.[115] The blood toluene concentration decreased rapidly after exposure, level of 100 μg/dl in person exposed to 100 ppm toluene in 2 hours would decrease to about 5 μg/dl within 5 hours[111], as seen in Fig 2.

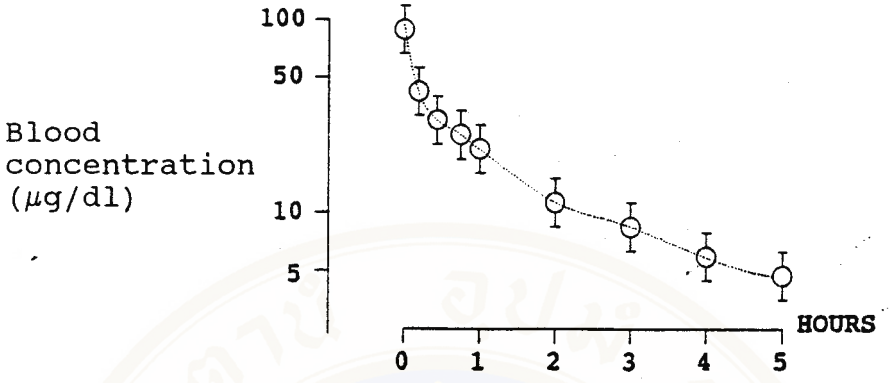


Fig 2. The time courses of blood toluene level in term of the mean and SD on men exposed to 100 ppm toluene in air for 2 hours.[111]

2.2 Metabolite forms

80 to 85% of absorbed toluene is oxidised to benzoic acid.[116,133] Some papers commented that the retained toluene is entirely converted to benzoic acid and eliminated within 24 hours.[48] Benzoic acid is conjugated with glycine in the liver and excreted in the urine as the water-solution hippuric acid. Therefore it is conjugated with glucuronic acid and excreted in the urine in form of benzoylglucoronic acid. Glucuronic acid conjugation would take place in the case of a large loading, namely in the case of heavy exposure in working environment or of liver function testing, as an alternative method of benzoic acid detoxication, but this is of much less importance. The biotransformation of toluene is shown in Fig 3.[29,117,133,137]

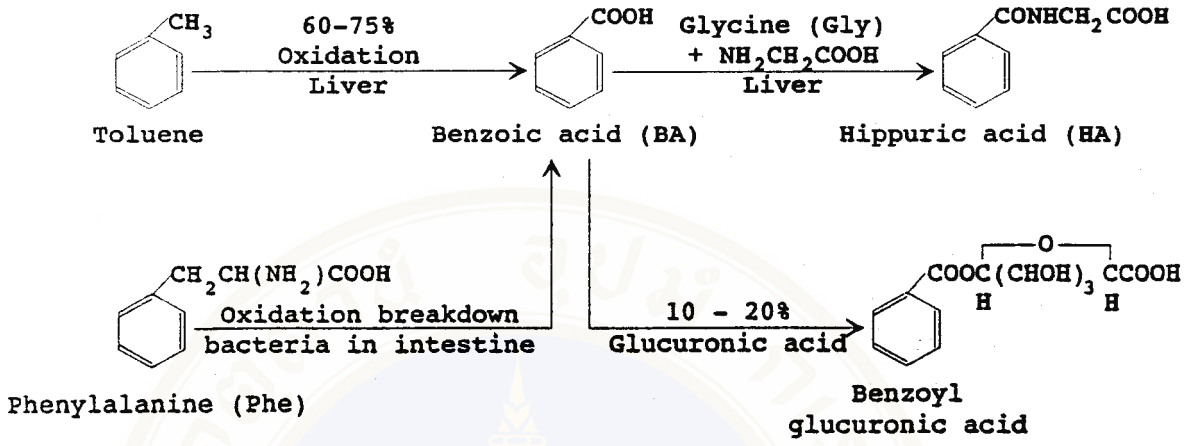


Fig. 3 Biotransformation of toluene

Elimination of toluene is almost entirely as hippuric acid.[46] It dose not form a phenol, and there is no increase in the excretion of ethereal sulfate, conjugated glucuronic acid or mercapturic acid in man.[133] Despite some investigators in the past believed that a small amount was excreted as a glucuronide[115], or partly excreted as benzoic acid in the urine.[116] It was proved by radiochemical technic that all benzoic acid administered in man and Rhesus monkey was excreted as hippuric acid, but excretion in other species was varied.[18] Hippuric acid excretion is practically completed in 16 hours after exposure.[20] Moreover, some recent studies have shown that after toluene exposure there are formation of cresols.[75] Using analytical methods, o- and p-cresol were detected in printing workers exposed to toluene[118] and further investigation o-, m- and p-cresol were found in an occupationally exposed group.[136] However, participation of p-cresol in toluene metabolism could not be shown clearly, but

the formation of o-cresol is due entirely to toluene metabolism. See Fig 4. This is only a minor metabolite since exposure to toluene in M.A.C. value for 4 hours showed only a little more than 1 mg/l of o-cresol in urine whilst hippuric acid excretion was more than 4 g/l.[135]

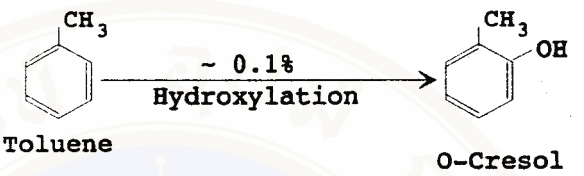


Fig. 4 Minor metabolite of toluene

Although benzoic acid is not detected in urine, ingestion of high doses benzoic acid yields some minor fraction of benzoylglucuronide which levels raise corresponding to benzoic acid ingested; eg. 2 g benzoic acid yield 95% of hippuric acid and 1.8% of benzoylglucuronide, 5 g benzoic acid yield 94% hippuric acid and 3.4% of benzoylglucuronide.[3]

Toxicity of Toluene

The toxic effects of toluene were reported as 3 groups.

1. The effects on the central nervous system, liver, heart, kidney, bone-marrow, lungs, the gastrointestinal system and skeletal muscles.

2. Chromosome changes.

3. The effects on endocrine function

3.1 The plasma level of follicular stimulating hormone (FSH) increases following toluene exposure.[82]

3.2 Toluene impacts on the cortisol metabolism and depresses thyroid functions.[82]

Certain industrial solvent mixtures containing toluene predomiinantly have been reported to cause bone marrow failure. It is now thought, that these mixtures were contaminated with benzene and that this contamination was the cause of the bone marrow depression. It is generally believed that toluene is much less toxic than benzene in chronic exposure.

Biological Monitoring of Toluene Exposure[29]

A number of biological tests have been investigated for evaluating human exposure to toluene: toluene in expired air and/or in blood and human breast milk; hippuric acid in urine and/or blood; and benzoic acid, and o-cresol in urine. The time of sampling of biological material is very critical in all cases, because of the rapid metabolism of toluene. In addition, the possibility that toluene metabolism might be modified by the presence of other chemicals must be considered.[127]

1. Blood, expired air, body fluids, and tissues

Toluene in blood has been determined by the GC analysis of headspace samples (detection limit: 10 μg/litre).[4,94,100,102] A direct injection method applicable to GC in the determination of toluene in whole blood has been reported by Aikawa et al.[1]

Cocheo et al.[30] have developed a purge and trap method for the detection of toluene in blood in which the detection limit is estimated to be less than 7.5 μg/litre. Bellanca et al.[12] described a similar method using GC-FID for detecting toluene and other organic compounds in tissues and body fluids.

The concentration of toluene in alveolar air samples, collected during exposure, is related to the intensity of the exposure.[7,8,21,22,26,27]

Under steady-state conditions, a constant relationship between the uptake rate of toluene and toluene concentrations in venous blood has been observed. Under non-steady-state conditions, however, no simple relation exists between uptake and the venous blood concentration of toluene.

Direct measurements confirmed a previous hypothesis that the concentration of toluene in arterial blood during and after exposure could be estimated from concentration in alveolar air.

While there is no unanimity, it can be concluded that analysis of expired air and/or blood reflects actual intake and may be a useful indicator of exposure to toluene.[69]

2. Urine

Toluene

Trace amounts of absorbed toluene, excreted in the urine, can be analysed by one of the three most commonly methods that used for the determination of toluene in aqueous media are the purge and trap.[13,14,40,57,76] The detection limit is generally 1 μg/litre, headspace. Toluene concentrations of the order of 0.1-1.0 μg/litre can be determined by this method[42,124], and sorption on solid sorbents, this method is rarely used.[108]

3. Metabolites of toluene

The major metabolite, hippuric acid, is eliminated in the urine. It can be determined by a number of methods including colorimetry, UV spectrometry, thin-layer chromatography (TLC), gas chromatography (GC), and high-performance liquid chromatography (HPLC). The topics of hippuric acid and method of analysis will described in the next chapter.

Sufficient data are not available to give an opinion about the measurement of other metabolites such as benzoic acid or o-cresol in urine to estimate exposure to toluene in the air.

Chapter III

Hippuric Acid

Hippuric acid is a glycine conjugated benzoic acid.[29,117] It is likely to be decomposed to benzoic acid and glycine in such drastic condition as in acidification or heating. It should be bear in mind that during such processes in aboratory procedure, some amounts of hippuric acid may be destroyed and benzoic acid yielded must be taken into accounts.

General Properties of Hippuric Acid

From the Merk Index and the Research Organics Inc 1990 Catalog, hippuric acid has the following properties:[2,154]

Synonyms	:	N-benzoylglycine, benzoylaminoacetic acid, benzamido acetic acid
Molecular formula	:	$C_9H_9NO_3$
Molecular structure	:	C_6H_5-CO-NH-CH_2-COOH
Molecular weight	:	179.17, 179.2
Formular weight	:	179.18
Melting point	:	188-191°c ; Crystal, 187-188°c
Source	:	Present in urine of herbivorous animals, also in small amounts in human urine.
Solubility	:	1 g dissolves in about 250 ml cold water ; 1,000 ml chloroform ; 400 ml ether ; 60 ml amyl alcohol. Slightly soluble in cold, freely in hot alcohol or hot water ; also soluble in aqueous solution of sodium phosphate ; practically insoluble in benzene, carbon disulfide, petroleum ether. Ammonium salt : freely soluble in water, soluble in alcohol.

Biosynthesis of Hippuric Acid

The synthesis of hippuric acid from benzoic acid and glycine was one of the earliest of the synthetic mechanism of the animal body to be recognized. In general, the aromatic carboxylic group may be excreted combining with glycine as a hippuric acid (aroyl glycine) or with glucoronic acid as an ester glucuronide. The quantities of these two main metabolites formed and excreted, however, depend on a number of factor including size of the dose, availability of glycine and the nature of substituents in the aromatic ring. Species differences have also been noted. In some species, other aromatic acids beside glycine may be involved.[133] Glycine conjugation of benzoic acid is shown in Fig 3.

Glycine utilized in the hippuric acid synthesis must be in only free form in the tissue. The rate at which glycine is mobilized for hippuric acid synthesis depends upon species. The rate in man is about 9 mg/kg body weight/hr.[133] The rate which benzoate in the body change to urinary hippurate is shown in Fig 5.[3]

Benzoate in the body ——R——> Hippurate in the body ——K——> Hippurate in urine

Fig. 5 Rate of benzoate excretion

$R = 10.5\ hr^{-1}$

$K = 2.7\ hr^{-1}$

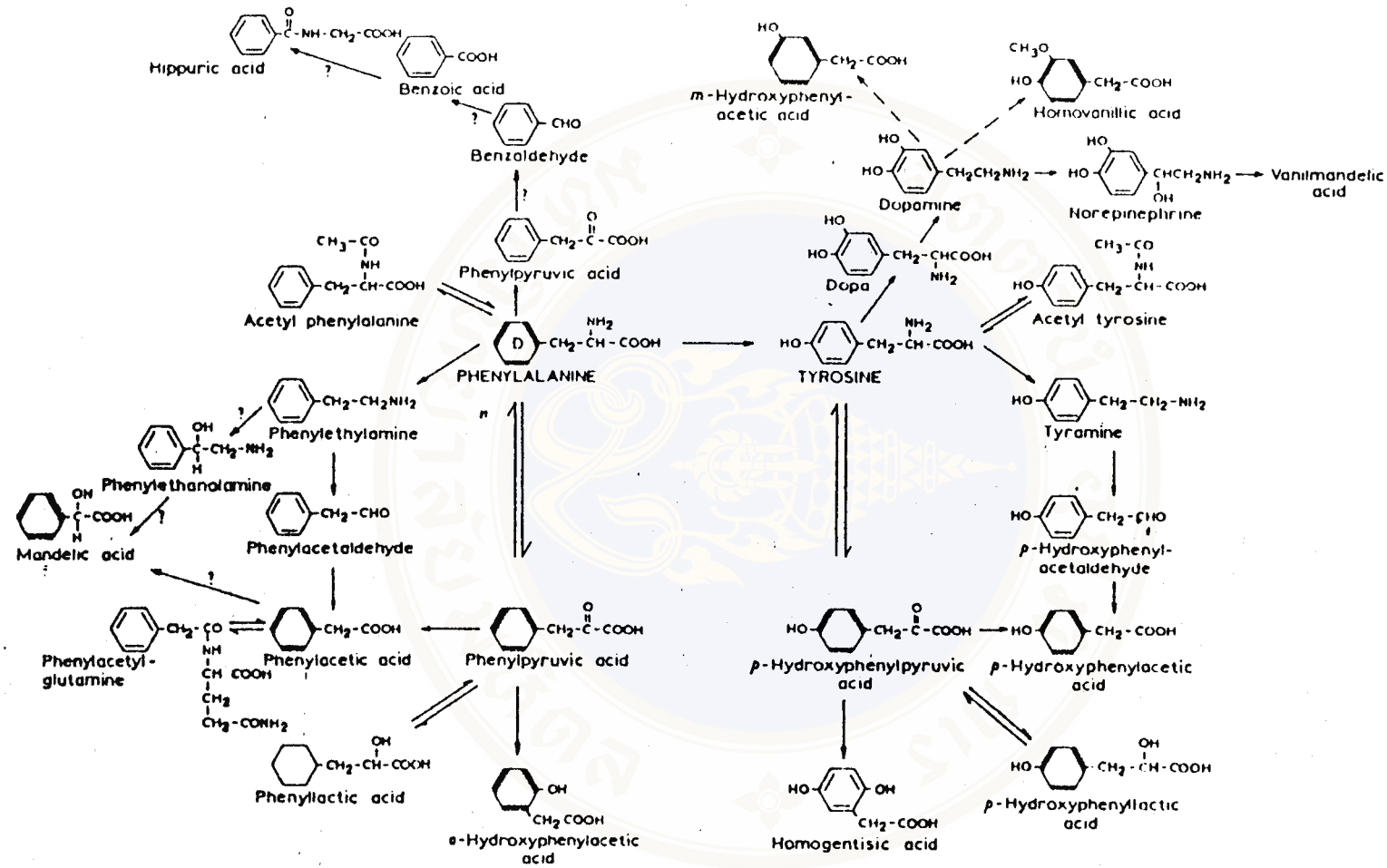
Value K is more than R, thus no benzoate be detected in urine unless benzoic acid presented in very high dose.[130]

Hippuric acid synthesis requires an activated form of benzoic acid which appears to be benzoyl-coenzyme A. Appearently benzoic acid is converted to benzoyl-Co A (the benzoyl thio-ester of coenzyme A) by means of ATP, probably via adenylbenzoate. Benzoyl-Co A is then to benzoylate glycine, in the presence of an enzyme to hippuric acid. The process can be represented as in Fig 6.[130]

Benzoic acid + ATP ——> Adenylbenzoate + Pyrophosphate
Adenylbenzoate + Co A ——> Benzoyl-Co A + Adenylic acid
Benzoyl-Co A + Glycine ——> Hippuric acid

Fig. 6 Process of hippuric acid synthesis.

According to Meister[81] and El Masty et.al.[46], hippuric acid is a normal urinary constituent naturally occurred or derived from food containing benzoate added as a preservative and is also formed from phenylalanine or other alkylbenzenes. Charles J. Umberger et.al.[121] stated that hippuric acid is an important metabolic product normally present in urine in amounts determined by the ingestion of benzoyl-containing foods and medicinals. Van Roosmalen et.al.[122] stated that hippuric acid arised from dietary sources such as quinic acid in prune and sodium benzoate in preserved food. Wilczok et.al.[130] also stated that variation in the physiological level of hippuric acid depended on fruit and vegetative consumptions. Pagnotto[96] noted that hippuric acid was present in urine as a normal constituent due to benzoic acid contained naturally in foodstuffs or by benzoate as a preservative added to foodstuff. It is known that coffee beans and certain kinds of vegetables and fruits, especially plums, cranberries, and prunes contain benzoate or quinic acid as precursors benzoic acid. It has been observed that when one pound of prunes is taken, over 10 g of hippuric acid is transformed. Japan's Food Sanitation Act allows the use of benzoic acid as a preservative for orange juice, an acid drinks. Kira[71] reported on an example of acetylsalicylic acid (Aspirin) administration with regard to the effect of medicine intake on the hippuric acid level in the urine. Ogata et.al.[89] believed that hippuric acid found in normal urine is due to benzoate in food. However, on studying a phenylalanine-tyrosine pathway of metabolism in man using isotopic technic by Curtius et. al.[34], they found that after giving deuterated phenylalanine, no deuterated hippuric acid was detected in urine. This showed that hippuric acid is synthesized mainly from alimentary benzoic acid and not through the above pathway. (See Fig 7.)



Normal subject-loading with deuterated phenylalanine (deuterated metabolites are marked).

FIG. 7 Pathway of phenylalanine-tyrosine metabolism

Thus, it can be concluded that hippuric acid formation in man is only from exogenous benzoic acid. Protein diets containing phenylalanine or derangement in the metabolism of the aromatic amino acids-phenylalanine and tyrosine which resulted in abnormal urinary phenolic acid[47] do not alter the amount of hippuric acid excretion.

Physiological Levels of Urinary Hippuric Acid

The amount of hippuric acid excreted in the urine varied with individuals, diets, medicines and with exposure to industrial organic solvent for example toluene and styrene. Many investigators have reported their values as are summerized in Table 4.[142]

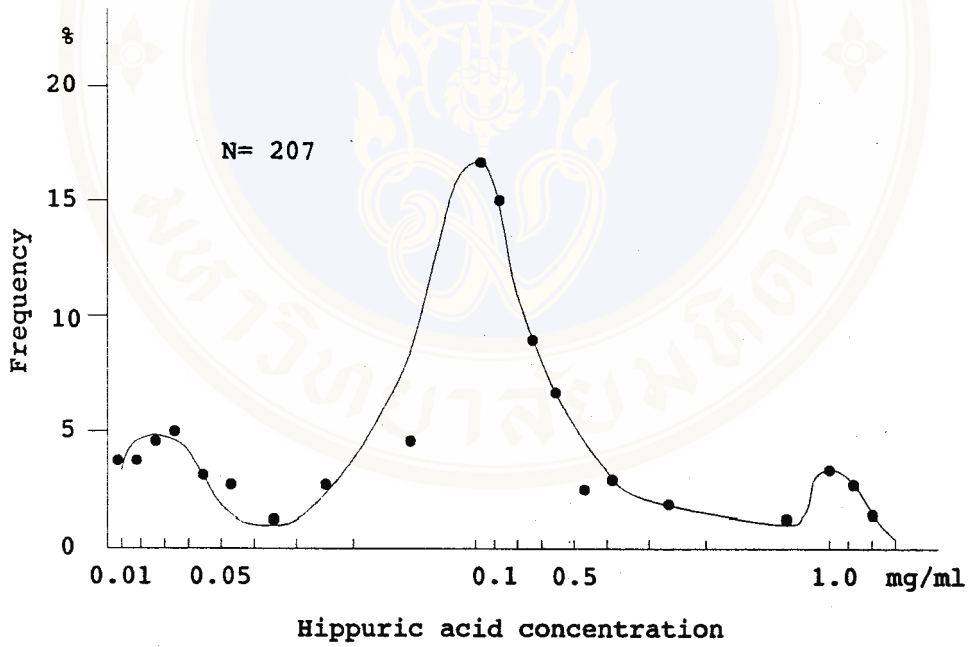


Fig. 8 Distribution of urinary hippuric acid in normal subjects (urinary SG corrected to 1.024)

N.B. 1. Minor peaks at low and high concentrations were caused by extraction method and effects of food and medicament.

2. The curve showed trend of logarithmic normal distribution with a range of 0.02-2.02 mg/ml

Table 4. Physiological levels of urinary hippuric acid

Condition	Hippuric acid level	Reference
24 hour-urine of normal persons	0.7 g/day	(77)
24 hour-urine of 6 normal persons used method of (77)	403 mg/day	(122)
Spot urine of 13 normal persons collect at 2 p.m.	184 ug/ml (range 35-414) 174 ug/min (range 70-495) 251 mg/day	(91)
Given 5 g benzoate in 3 healthy males	constant rate of 2.1 g/hr. equivalent to 1.4 g. benzoate per hour	(8)
31 males in factory no known exposure to industrial organic solvent	range 112-1084 mg/l geometric mean 350 mg/l cumulative variation 10%	(66)
Second morning urine at 10 a.m. of university students 36 males and 30 females	less than 1.4 g/l range 74-1266 110-1431 mg/l geometric mean 301 398 mg/l cumulative variation 11 11 % mean specific gravity 1.019 1.013 creatinine 1.32 0.89 g/l	(8)
31 persons with no occupational exposure to solvent	mean±SEM = 1.10±0.15 g/l or 0.8±0.08 g/g creatinine upper fiducial limit 97 mg% correct SG 1.016 = 105 mg% correct creatinine=0.88 g/g	(67,21)
207 male workers not expose to any solvent corrected for urinary SG to 1.024 (gas chromatography)	observed range0.01-3.6mg/ml arithmatic mean 0.34 mg/ml range ± 2 SD 0-1.08 mg/ml ± 1 SD 0.37 mg/ml upper rejection 1.38 mg/ml geometric mean 0.19 mg/ml range ± 2 SD 0.02-2.02mg/ml upper rejection 5.34 mg/ml #see distribution curve in Fig. 8	(142)
Peoples not exposed to toluene , xylene or styrene corrected for urinary SG to 1.024	0.5-1.2 g/l	(128)

Table 4. (continued)

Condition	Hippuric acid level	Reference
101 subjects not exposed occupationally to toluene and 80 subjects exposed occupationally but not toluene on the study day and the preceeding day.	excretion rate , mean ± SD = 20 ± 4.3 mg/hr. maximum rate (± 3 SD) = 33 mg/hr.	(135)
10 healthy men corrected for urine SG to 1.012	0.823 ± 0.518 g/l	(139)
27 male and 21 female volunteers, aged from 21-50 years , had not been toluene exposed. Corrected for urine SG to 1.024	geometric mean = 123 μg/ml # see distribuution curve in Fig.9	(74)

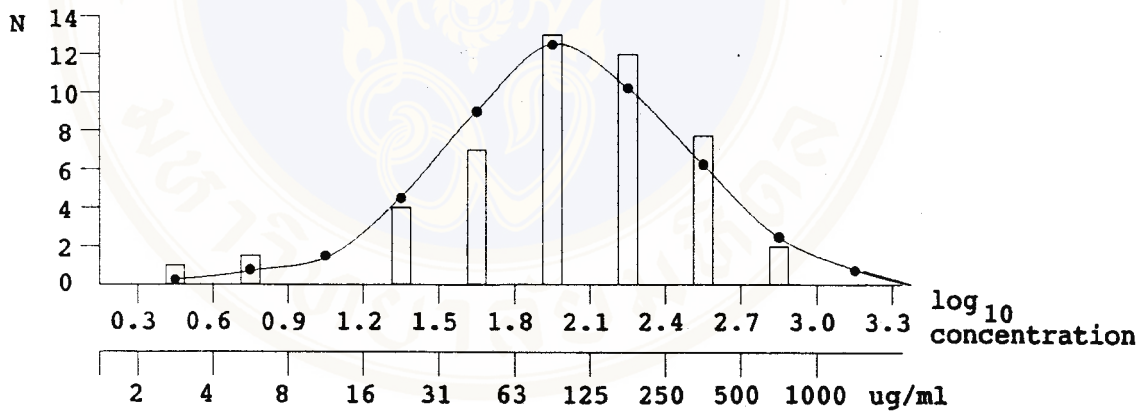


Fig. 9 Distribution of urinary hippuric acid levels in healthy subjects.
(urinary SG corrected to 1.024)

Hippuric acid is an endogenous metabolite common in human urine. Its formation in man in response to exogenous benzoic acid seems to be altered under certain conditions. Theoritically one might expect changes if the mobilization of glycine is altered, if coenzyme-A is deficient, or if the condensing enzyme is affected. Furthermore, damage to the liver, the main site of hippuric acid synthesis, or to the kidney which excreted hippuric acid, would also be expected to

affect hippuric acid formation and elimination.[133]

In old age, the amount of hippuric acid excreted in response to a given dose of benzoic acid is below normal. This may be due to decreased renal excretion of the acid rather than due to decreased formation. The hippuric acid excretion in many cases of catatonic schizophrenia is reduced. Hyperexcretion of hippuric acid has been observed in a condition known as 'free anxiety'. This state occurs in patients who have suffered long continued anxiety (e.g. in war). Hippuric acid excretion however, returns to normal after psychotherapy. The increased excretion of hippuric acid is believed to be due to an increased in the conjugating capacity of the liver which may be a physiological form of hepatic disease result in the hyperexcretion of hippuric acid.[133]

Thus, it can be concluded that the quantitative aspects of hippuric acid formation are of interest not only in the study of pharmacokinetics of salicylic acid[3] but also in the evaluation of liver function and renal function, to observe patients with the Lesch-Nyhan syndrome[11,67] and as a test of industrial exposure to toluene.

Moreover, there are a few conditions which were reported to alter the hippuric acid formation as follows:-

1. The intake of foodstuff, preservatives and medicaments containing benzoic acid and benzoate or quinic acid as precursors of benzoic acid, namely raisin, prune, plums and coffee beans, either benzoate in antipyretics or in liver function checking; resulted in remarkably increasing the urinary hippuric acid concentration. Their intake should be controlled at the time of a health check[137]; for example:-

a) The intake of 100g rasins make hippuric acid increase moderately and reach maximum concentration 3 mg/ml after the fifth hour.[96]

b) It has been observed that ingestion of one pound of prunes, over 10g of hippuric acid is transformed.[96]

C) The intake of sodium benzoate and caffein as used in the current study agreed with the peak of the hippuric acid itself; with a 0.2g administration (medical dosing), the concentration reached a level of 3 mg/ml after one hour.[71]

d) The ingestion of soft drinks, soysauce, syrup and caviar.[117] The maximun limits of benzoic acid contained in

soft drinks, soysauce, and syrup are 0.6 g/kg.[117]

2. After ingestion fruits and vegetables that benzoic acid is naturally present, especially orange, grape fruit, blackcurrent, cranberries, prunes, tomato juice, banana and coffee beans, analysis of urine found chance in excretion of hippuric acid and other phenolic acids.[113]

3. The formation of hippuric acid from benzoic acid due to glycine conjugation is capacity-limited process. It means that rate of hippuric acid formation increase corresponding to the amount of glycine available or be given. However, salicylic acid dose of 1-2 g orally which needs glycine conjugation to form salicyluric acid has no effect upon hippuric acid formation.[3]

As shown in Fig 3. not only glycine but phenylalanine is considered as factors which affect urinary hippuric acid excretion.[117]

It is thought that the intake of a large amount of protein and/or accelerated catabolism of protein may be the cause of urinary hippuric acid elevation.[117]

4. The intake of aspirin can elevate the urinary hippuric acid level because the chemical structures of aspirin and hippuric acid are similar.[117]

5. Concentration or dilution of urine affects urinary hippuric acid, therefore, the concentrations of chemicals in urine should be corrected with gravity of urine (GU) or urine creatinine concentration.[117]

6. Subjects having low or high diuresis at the rate of below or above 30 ml/hr give statistically highly significant variation in rate of hippuric acid excretion.[130]

Several investigators have commented on kinetics of hippuric acid excretion with toluene exposure as follows:

a) On maximum peak of hippuric acid concentration.

Ogata et.al.[92] stated that hippuric acid excretion increased rapidly for the first 2 hours of exposure and rose to a maximum before the 8th hour. This was similar to the observations of Piotrowski[99] and Dutkiewiez[43] who found the greatest excretion period of hippuric acid between the 6th and

8th hour of exposure. On daily exposure, Wilczok et.al.[130] observed that the maximum excretion period occurred in the final phase of exposure and concentration of hippuric acid in the last 4 hours of daily exposure was about 2.5 times higher than that of in the total 24 hours urine sample. And Sugita et.al.[117] reported that the urinary hippuric acid levels of an engineer who did not use toluene occupationally were elevated by toluene used privately.

b) On the disappearance of increased hippuric acid.

Von Oettingen et.al.[118] observed that after exposure to 50-800 ppm toluene in air, hippuric acid excretion is practically completed when 14 hours have elapsed from the termination of exposure. This agreed, withthe study of Wilczok et.al.[130] who indicated that hippuric acid concentration decreased to the physiological level 14 hours after the exposure time. On daily occupational exposure experimented by Ogata et.al.[92], the hippuric acid concentration fell nearly to the normal level by 18 hours later. Piotrowski[99] concluded that the result of hippuric acid determination reflected exclusively the degree of exposure on the day the sample was collected.

c) On the quantity of hippuric acid corresponding to toluene exposure.

Assume that all toluene absorbed is converted and excreted, it could be derived from the following formula[92]:-

$$\text{mg of excreted hippuric acid} = C.V.t.F.M_H/M_T$$

Whereas : C = toluene concentration in air, mg/l
V = minute volume
t = time of exposure
F = fraction of toluene retained by lungs
M_H = molecular weight of hippuric acid
M_T = molecular weight of toluene

d) On the correlation among absorbed toluene, toluene concentration in air, rate of hippuric acid excretion and concentration of hippuric acid in urine.

All of these show relationship to each other, e.g. rate of excretion depends on both quantity of absorbed toluene and toluene concentration.[90,130] Hippuric acid concentration depends on toluene concentration but with wide variability.[64] Ogata et.al.[92] proposed that measurement of excretion rate was much more reproducible than urinary concentration.

e) On reduction of individual variationin hippuric acid concentration.

Values of urinary specific gravity (SG) and urinary creatinine concentration must be taken into accounts[92,122] by correcting the specific gravity of each sample to the same value, say 1.024, or by expressing hippuric acid concentration in a unit of g/g creatinine. Formula for correcting observed hippuric acid concentration to a mean specific gravity of 1.024 is as follow[122]:-

$$\text{corrected value} = \frac{\text{observed value} \times 0.024}{\text{observed SG}-1}$$

On the contrary, Ikeda et.al.[64] found that correction of hippuric acid level with specific gravity and creatinine do not reduce the variation significantly. Both standard deviation (SD), range and fiducial range (P=0.05) increased at high hippuric acid concentration. De Rosa et.al.[37] found that correction of hippuric acid in urine end-of-work-shift values with specific gravity and g creatinine were significantly related to the mean daily environmental concentration of toluene. The correlation coefficients for hippuric acid after correction for specific gravity and g creatinine were 0.84 and 0.88 respectively.

Estimation of Urinary Hippuric Acid

The process requires mainly 3 steps:-

1. Isolation from the urine
2. Separation from related compounds
3. Detection

However, some older methods had only 2 steps e.g. isolation and quantitation, which was non-specific and lack of sensitivity[89].

There are 6 analytical technics available which were described below.

1. Crystallization-gravimetry

This method is considered to be the oldest technic introduced by Freidmann[52] in 1911

2. Crystallization-titration

This method involves acidification of urine, saturation with NaCl or $(NH_4)_2SO_4$, cool and add H_2SO_4 to crystallize the hippuric acid. Titration is done with NaOH using phenolphthalein as indicator.[73,118,128,138] Another modification is continuous extraction with ether,hydrolysis to benzoic acid and titration.[51,70,119] In 1926 Quick[101] used the above technic and estimated amino nitrogen in separated glycine by formal titration. Von Oettingen, Neal, and Donahue[125,132] found that this method was not sensitive enough for the quantitative analysis of urinary hippuric acid from workers exposed to small amounts of toluene.

3. Spectrometry

Spectrophotometric procedures were developed for the determination of hippuric acid by Gaffney, Schreier, Di Ferrante, and Altman in 1954[53]; Ogata, Sugiyama, and Moriyasu in 1962[89]; Pagnotto and Lieberman in 1967[96]; Ikeda and Ohtsuji in 1969[64]; and Ogata, Tomokuni, and Takatsuka in 1969.[91] Spectrofluorometry was described by Ellman et.al. in 1961.[45] The ultraviolet absorption method described by Pagnotto and

Lieberman in 1967[96] for hippuric acid measurement offered the advantage of simplicity but not specificity since it also had absorption to uric acid and other compounds.[91]

4. Radioactivity method

This method was introduced by Bridges et.al. in 1970.[26]

5. Colorimetry

a) In 1928 Nicholls[84] tried to develop color reaction by oxidising benzoic acid yielded from hydrolysis product with H_2O_2 to salicylic acid and measured with the color reaction of the latter, but this yielded only 10%.

b) In 1951 Dickens and Pearson[38] used colorimetric estimation microquantity by nitration the benzoicacid at room temperature.

c) In 1954 Gaffney et.al.[53] used continuous extraction of urine with ethyl acetate for 24 hours, evaporated, dissolved in ethanol and separated with paper chromatography. Color reaction was brought about by reacting with p-dimethylamino benzaldehyde in acetic anhydride to colored azlactone. The quantity was measured by elution of the colored spot on the paper and compared with standard color. This method was not reliable because the color intensity depended on type of paper used. Therefore, standard solutions had to be spotted to each paper.[91]

In 1962 Ogata et.al.[89] modified the above method and used for mass screening by saturating urine with salt, acidifying, and extracting with ether-alcohol (8:2). Spot the residue on a piece of filter paper, cover with another piece of filter paper impregnated with the color reagent, and press with heated iron for 1 minute. The developed color was then compared with standard color.

d) In 1963 Umberger and Fiorese[121] used the reaction between hippuric acid and pyridine-benzenesulfonyl chloride. The red-orange color from the reaction was measured spectrophotometrically at wavelength 380 nm.

e) In 1972 Burkiewicz and Zielinska[24] extracted urine with chloroform, dried and reacted with acetic anhydride in

the presence of pyridine. The colored complex was measured at wavelength 430 nm.

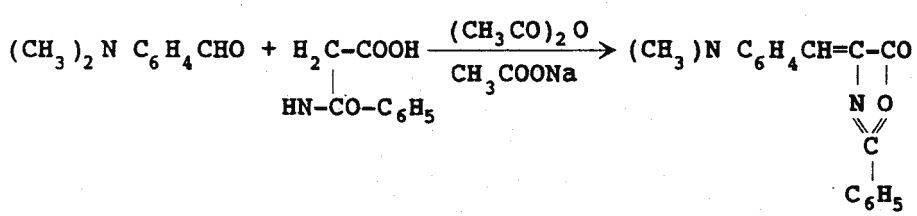
f) In 1977 Ohmori et.al.[93] applied colorimetric methods of Umberger which was later modified by Ogata[91] for the determination of hippuric acid. This method could be used for mass screening by saturating urine with salt, acidifying, and extracting with ethyl-acetate, drying and reacting with acetic anhydride and 0.5% DAB solution in pyridine at 40°c for 1 hour. The colored complex was measured by spectrophotometry at wavelength 458 nm.

6. Chromatography

This technic is used for separation of hippuric acid from m- and p-toluic acid which gives the same color reaction.[91] It also enhances the specificity of the method.

a) Paper chromatography (PG)

It was first introduced for estimation of hippuric acid by Gaffney et.al.[53] The extract of hippuric acid was prepared with ether alcohol and spotted on the filter paper. The system was carried out on filter paper strips (Toyo filter paper, No. 50) in the solvent system : n-butanol-glacial acetic acid-water (4:1:1). R_f value of hippuric acid found in this system was 0.81. After developed the strips were completely dried by a fan and sprayed with 4% p-dimethylaminobenzaldehyde (DAB) solution mixed in acetic anhydride saturated sodium acetate. Then it was made to react in a drying oven for one minute at 135°c, producing azlactone of hippuric acid. This coloration reaction was expressed as follows:-



p-dimethylamino benzaldehyde | hippuric acid (HA) | azlactone of hippuric acid

The azlactone of hippuric acid was detected as a orange spot in around as small as 1 μg. The quantitation was done by eluting the spot with methanol and carried out the colorimetric determination as soon as possible since the color was unstable. The absorbance was measured at 460 nm, compared with standard color. In case of sufficient amounts of hippuric acid, urine might be applied directly to the paper strip.

Ogata et.al.[89] also used this method but modified by extracting the color with ethanol. This modification was used by many investigators.[18,64,91,92]

Orlowski[95] modified the method of Ogata by using paper chromatography. The accuracy of Orlowski's method of hippuric acid and m-hippuric acid estimation was +3%, yet the development of the chromatogram was time consuming.

b) Ion-exchange chromatography (IEC)

It was carried out in 1957 by Elliot.[44] He separated hippuric acid fraction from urine by Ion-exchange chromatography and measured with spectrometry.

c) Gel chromatography

It was used by Sinha and Gabrieli in 1968[114], using Sephadex gel in pH 7 buffer for separation and detection by ultraviolet spectrophotometry at wavelength 232 nm. This method simultaneously detected both benzoic acid and hippuric acid in separated fraction. The maximum absorptivity of hippuric acid was at 228 nm and benzoic acid was at 225 nm.

d) Thin-layer chromatography (TLC)

It came in place of paper chromatographic since thin-layer chromatographic is simpler and faster than the corresponding paper technic. Ersser et.al.[47] have developed the TLC method for investigation of abnormalities in urinary phenolic acid excretion using thin-layer of cellulose or silica gel on aluminium foil. The paper showed comparison among various solvent systems and locating agents. R_f values of hippuric acid and related compounds were also included. This technic was used for rapid screening of abnormalities in urinary phenolic acid and useful for further experiments.

TLC-colorimetric determination of hippuric acid and m-methyl hippuric acid in urine after mixed exposure to toluene and xylene was described in 1981 by Bienick and

Wilezok.[16] Chloroform was used for urine extraction according by Burkiewicz and Zielinska.[24] Satisfactory separation of these metabolites was obtained on TLC plates covered with silica gels and developed in chloroform-acetic acid-water (4:1:1) ; p-dimethylamino benzaldehyde in acetic anhydride was applied to develop the color was done in similar way to that of Ogata et.al.[91] and Orlowski.[95] The sensitivity of this method was 6 μg hippuuric acid per 1 ml urine and the recovery was 100% (±1).

In 1982 Bienick, Palys and Wilczok[15] used thin-layer chromatography to determine the concentration of hippuric acid, mandelic acid and phenylglyoxyline present in the urine after occupational mixed exposure to toluene and styrene. This procedures were proposed to separate the metabolites as follows:-

1) separation of hippuric acid from mandelic acid

2) separation of mandelic acid from phenyl glyoxylic acid

3) separation of hippuric acid and mandelic acid from phenyl glyoxylic acid

The developing reagent, p-dimethylaminobenzaldehyde in acetic anhydride was used after separation on Kiesel gel and Silica gel. The sensitivity of this method was 6 μg of hippuric acid, 10 μg of mandelic acid, and 7 μg of phenylglyoxylic acid with an average recovery of 94%.

e) Gas chromatography (GC)

This method was applied to the determination of urinary glycine conjugates, hippuric, o-, m- and p-methylhippuric acids. The gas chromatography is an instrument commonly used for routine work. The analysis method developed by Buchet[23], Caperos[25], Kira et.al.[71] demonstrated excellent separation capabilities. This procedure was as specific and sensitive as Ogata's method and offered the advantage of being more rapid. Principle of this technic involved the extraction of the acid from urine, making derivative compounds by either esterification or silylation for render thermal stability and lower polarity of the compounds, then gas chromatograph separation in the non-polar packed column such as silicone gum, SE-30. Urine extraction methods were similar to the previous technics, using ethyl acetate[25,34,59,62,65,71,122,131,135] or

ether[31,32,47] extraction from salt saturated urine. Derivatizations might be methylation with ethereal diazomethane[23,35,126] or with 3-methyl-1-p-tolyl triazine[25] or pyrolysis of tetramethylammonium or trimethylanilinium salt.[9,19,55,63,74,106] But yield of esters depended on column temperature. Another esterification was formation of isopropyl ester.[135] Silylation was said to be the most common used among others. Use of silylation-esterification was also reported.[32,62,68]

Derivatization using methylation by diazomethane method for hippuric acid determination was found to be the best method but reagent was easily exploded if handled carelessly. In quantitation, errors from incomplete extraction and loss in process were eliminated by using internal standard technic. The internal standard used for this purpose was heptadecanoic acid[65,71] or tridecanoic acid.[25] Gas chromatography instrumentation generally used in toxicology laboratory comprises of injection port, isothermal column oven, hydrogen flame ionization detector, and chart recorder. Quantitation is done by comparing peak area of unknown and that of standard hippuric acid.

f) High-performance liquid chromatography (HPLC)

This technic was recently developed. The procedure is similar to gas chromatographic method but not require derivatization. Only extraction and injection of the aqueous solution into the high-performance liquid chromatography will result in both peak identification and estimation. In 1977 Matsui et.al.[79] and Ogata et.al.[91] used this technic for the separation and quantitation of hippuric acid and m-methylhippuric acid in urine after extraction and redissolved in water. The materials used were reverse-phase HPLC column, ultraviolet spectrophotometric detector, isocratic pump and methanol-buffered water a mobile phase. They found linear relationship between peak height and quantity of hippuric acid or m-methylhippuric acid.

Yoshida et.al.[137] used this technic without extraction of urine but direct introduced of urine sample. In this case, the solvent peak disappeared but several minor peak groups were observed due to other urine constituents. Separation showed excellent results but took rather longer times than gas

chromatographic method. The direct-introducing of urine has one advantage that it required no internal standard addition. The measured levels showed directly the exact amount of urinary hippuric acid without any loss.

In 1982 Hansen and Martin[58] used a chromatographic system based on dynamically modified silica for the determination of hippuric acid and o-cresol in urine, as indices of toluene exposure. The detection limits were found to be 0.05 mg/ml and 0.05 μg/ml of urine for hippuric acid and o-cresol, respectively. When using ultraviolet detection at 254 nm. the recovery for hippuric acid was about 100% and for o-cresol 33-36%

In 1983 Sakai et.al.[109] described the use of cyclodextrin as a component of the mobile phase in reversed-phase. High-performance liquid chromatography leaded to effective separation of isomers of methylhippuric acid, and simultaneous determination of hippuric acid and o-, m-, and p-methylhippuric acid in urine served as a useful index of solvent exposure. The detection limits for hippuric acid and each methylhippuric acid were found to be 50 mg/l urine and 10 mg/l urine, respectively. However, Fujimura et.al.[109] pointed out problems often encountered with this method, such as contamination of solutes in the effluent with cyclodextrins, the loss of cyclodextrins, which were expensive, or partial plugging of the mobile phase delivery line.

In 1989 Matsui and Sekiya[80] reported a high-performance liquid chromatographic method for the simultaneous determination of hippuric acid and all three isomers of methylhippuric acid in urine using a ß-cyclodextrin-bonded column. The detection limits of the four metabolites in urine ranged from 0.03 to 0.04 mg/ml. Analytical recoveries for hippuric acid and methylhippuric acid over the concentration range 0.2-1.0 mg/ml were within 100 +5% for the twenty samples.

Chapter IV

Purposes of the Study

This study is mainly devoted to find evidence of solvent exposure. As mentioned in the previous chapters, thinner is the common solvent. The major ingredient of it is toluene which urinary metabolite is hippuric acid. Urinary samples are biological material which are easily collected. There fore, measurement of hippuric acid levels in urine is the most suitable method. However, one problem arises which is the presence of hippuric acid in normal urine due to interference or substances which reaction as same as hippuric acid.

The objectives of the study are:-

1. Estimation of urinary hippuric acid levels in normal persons, toluene exposed subjects and the workers who work in a paint factory.
2. Measurement of hippuric acid by colorimetry by Benzene-sulfonyl chloride (BSC method), colorimetry by p-Diamino benzaldehyde method (DAB method), and High-performance liquid chromatography methods (HPLC method).
3. After comparing the colorimetric method with the reference method (HPLC), it will be expected to find a suitable colorimetric method for using in routine service.

Chapter V

Materials and Methods

5.1. Collection and Treatment of Urine Samples.

Urine was kept unpreserved in plastic containers not less than 5 ml. The urinary specific gravity (SG) of fresh urine was measured with a refractometer (Atago-Uricon).

The urine was kept frozen in a refrigerator for 7 days and bring to room temperature before analysis. If urine became cloudy, it was centrifuged and the clear portion was used.

5.1.1. Normal Urine.

Single-voided urine samples from the volunteer non exposed to toluene or any solvent, was collected before breakfast between 8.00-9.00 a.m.

21 males and 73 females: nurse students of The Red Cross Nursing College, Medical Technology students of Mahidol University and staff of central laboratory of Chulalongkorn hospital not using toluene.

5.1.2. Urine of Thinner-exposure.

Single-voided urine samples were randomly collected from three sources:-

a) 54 samples of teenager gangster who sniffed thinner and were arrested by the policemen. Urine samples were collected between 1-12 hrs in jail.

b) 21 samples of patient from Thunyaruck Hospital who were 'Chronic Thinner Abuse'.

c) 4 samples of thinner inhalated patients who admitted in Siriraj Hospital. Each sample was collected in 3 consecutive in the morning.

5.1.3. Urine of Paint industry workers.

31 samples of single-voided urine were collected randomly.

5.2. Quality Control

a) Pooled frozen urine 100 ml. Pooled normal urine were aliquoted 2 ml-vials and kept frozen in a refrigerator. Each vial was analyzed along with the test samples.

b) Commercial control urine: Lyphocheck urine metals control(C-405-25) Level 2. Lot No. 44502, Bio Rad ECS, Div. Anaheim Ca, USA.

5.3. Urine Treatment.

Hippuric acid in urine was extracted into organic solvent phase by the procedure of Ogata et.al.[91,121]

a) Chemical Reagent.

Conc HCl (AR grade)
NaCl (AR grade)
Ethyl acetate (AR grade)

b) Equipments.

Test tube (15x150)
Stoppered tube
Vortex mixer
Water bath
Centrifuge

c) Method.

If the urine was cloudy, it was centrifuged and the clear portion was used. One milliliter of urine was placed in a stoppered tube, its pH was adjusted to about 2.0 with conc HCl, and saturated with 3 mg of NaCl, to increase the extraction rate, 4 ml of ethyl acetate was added and mix about 1 minute on vertex mixer. Hippuric acid was extracted into ethyl acetate layer, 0.2 ml of the extract was transferred to two test tubes (tube A, and B) for the estimation of hippuric acid by BSC and HPLC methods, and 0.1 ml of the extract to one

test tube (tube C) for the analysis of hippuric acid by DAB method. Dry the extract in these three tubes at 70°c in a water bath.

Hippuric acid standard, control urine and blank were treated in the same way.

5.4 Determination of Urinary Hippuric Acid.

After extraction urine was performed by 3 methods: Benzene-sulfonyl chloride method (BSC), p-Dimethylamino benzaldehyde method (DAB), and High-performance liquid chromatography (HPLC).

5.4.1 Colorimetry by Benzene-sulfonyl Chloride.[121]

Hippuric acid dissolved in pyridine produced a red-orange color upon the addition of benzene-sulfonyl chloride. The color had a stable absorbance at 380 nm. It was sensitive in microgram quantities of hippuric acid and followed the Beer-Lambert Law up to 1 mg/ml.[119]

a) Chemical Reagents.
   Stock standard hippuric acid (3,000 μg/ml)
   Pyridine
   Benzene-sulfonyl chloride
   Chloroform

b) Equipments.
   Spectrophotometry (Shimadzu UV-Visible spectrophotometer)
   Vortex mixer

c) Method.

0.5 ml of pyridine solution, and 0.2 ml of benzene-sulfonyl chloride were added to the dry extract (tube A) and mix thoroughly. The mixture was allowed to stand for 30 minutes at room temperature. It was then diluted with 4.3 ml chloroform and the absorbance was read at 380 nm. against a pyridine-benzene-sulfonyl chloride blank.

d) Standard Curve Preparation.

1. Hippuric acid was diluted from stock solution (3,000 μg/ml) to 3,000, 2,500, 2,000, 1,500, 1,000, 500, 200, 100 μg/ml with distilled water.

2. Standard solutions were treated in the same way as urine samples as described in 5.3.

3. Color development of standards was performed by benzene-sulfonyl chloride as in 5.4.1.c.

4. The absorbances VS hippuric acid were plotted concentrations on an ordinary graph paper.

5.4.2 Colorimetry by p-Dimethylamino Benzaldehyde.[53][91,98]

Hippuric acid produced a yellow-orange color upon the addition of p-dimethylamino benzaldehyde in acetic anhydride. The color intensity was measured at 458 nm. This method was in good agreement with Beer's law up to 2 mg/ml.[88]

a) Chemical Reagents.

Stock standard hippuric acid (3,000 μg/ml)
Acetic anhydride
p-Dimethylamino benzaldehyde
Pyridine

b) Equipment.

Spectrophotometer (Shimadzu UV-Visible spectrophotometer)
Vertex mixer
Water bath

c) Method.

The dried extract containing hippuric acid from 5.3.3 (tube C) was taken up in 1.0 ml of acetic anhydride and 2.0 ml of 0.5% p-dimethylamino benzaldehyde (DAB) solution in pyridine were added, and the solution was kept at 40°c for 1 hr after thorough mixing. The absorbance was then determined at 458 nm against a blank containing acetic anhydride, DAB, and pyridine.

d) Standard Preparation.

1. Hippuric acid dilutions was prepared from stock solution (3,000 μg/ml) to 3,000, 2,500, 2,000, 1,500, 1,000, 500, 200, 100 μg/ml in distilled water.

2. Standard solutions were treated in the same way as urine in 5.3.c.

3. Color development of standards was performed by DAB in acetic anhydride as in 5.4.2.c.

4. The absorbances and concentrations were plotted on an ordinary graph paper.

Note : If the concentrations of hippuric acid was off scale (more than 3,000 μg/ml), it would be better to dilute the urine with distilled water (1:20) before treatment.

5.4.3 High-performance Liquid Chromatographic Method.[137]

Urine extract from 5.3.c (tube B) was dissolved in 0.2 ml mobile phase and mix well. 10 μl of this sample was injected into the HPLC instrument. The identified hippuric acid peak was calculated for its concentration against the concentration of the standard solution.

a) Chemical Reagents.

- Mobile phase was the mixture of 200 ml of acetonitrile, 800 ml distilled water, 15 ml of acetic acid. The solution was degassing with ultrasonic bath about 5 minutes until no bubble evolved.

Note : Acetonitrile was of HPLC grade and other chemicals were of analytical grade.

- Standard Hippuric Acid Solution Dissolve 100 mg of hippuric acid, A.R. in 100 ml distilled water.

b) Equipments.

- High-performance liquid chromatograph.
  Shimadzu liquid chromatography (LC-6A series, Shimadzu, Kyoto, Japan)
  Ultraviolet-wavelength spectrophotometric

detector (SPD-6A Module)
Integrator (Chromatopac C-R6A)
- 50 μl Microsyringe.

c) Principle.

Hippuric acid in urine was separated by the C_{18}-bonded silica gel in reverse phase HPLC system using acetonitrile : distilled water (200:800) added 15 ml of acetic acid as an isocratic eluting solvent. The separated fractions were detected spectrophotometrically by UV detector at wavelength 272 nm and were recorded on strip chart. The peak of hippuric acid in urine was identified by comparison with the retention time obtained from standard hippuric acid solution. Quantitation was done by measuring the peak area and calculating from the concentration of hippuric acid standard.

d) Method.

1. Instrument condition.

Column oven (CLC-ODS)	: Shim-pack CLC-ODS (0.15m x 6.0ϕ)
Guard column (G-ODS)	: Shim-pack G-ODS (4.0mmϕ x 1cm)
Automatic sample injection (SIL-6A)	
Pump control (LC-6A)	: Constant flow rate
Solvent flow rate	: 1.5 ml/minute
Pressure	: 2.5 ppm.
Detector (SPD-6)	: UV spectrophotometer at 272 nm
Attenuation	: 1
Sensitivity	: 0.08
Slop	: 100
Chart speed	: 5 minute/inch
Minimum area	: 500
Calibration	: one point (Mode 101)
Print level	: + 2000

2. Identification of hippuric acid.

2.1 Set the HPLC condition as in 5.4.3.d.1, absorbance to zero and chart pen to base line.

2.2 Inject 10 μl of standard hippuric acid solution (1,000 μg/ml) and note the retention time of hippuric

acid peak for this system.

2.3 One point calibration. Use mode 101 to find factor of standard solution concentration at hippuric acid retention time. This factor was used for automatic calculation by Integrator (C-R6A).

2.4 Confirmation of hippuric acid difficult peaks from urine: dried extract containing hippuric acid from 5.3.c (tube B) dissolved with 0.2 ml mobile phase and mix well. 10 µl of this sample was injected in the chromatography and the chromatogram was observed. The integrator calculated and reported the concentrations of the peak of hippuric acid at the retention time of hippuric acid that calibrated as described in 5.4.3.d.2.3.

3. Standard Preparation.

Dilute hippuric acid standard was performed by diluting the stock hippuric acid standard (3,000 µg/ml) to 1,000 µg/ml in distilled water.

Note : If the peak of hippuric acid was off scale, reselected the higher attenuation and injected again. But if the concentration was higher than 3,000 µg/ml, it would be better to dilute the urine with water before treatment. If the peak of hippuric acid was so small, that the integrator couldn't calculate, reselected the lower attenuation and lower minimum area (about 200) and injected again.

5.5 Percentage of Recovery.

Recoveries of hippuric acid added to urine determined by the following procedures.

5.5.1 Method.

Hippuric acid concentrations of 200, 500, 1,000, 1,500, 2,000, 2,500, 3,000 were added into a pooled urine with known hippuric acid concentration (1:1). The samples were treated in the same manner as the urine samples as described in 5.3.4. The concentrations of hippuric acid were estimated by BSC, DAB, and HPLC methods.

Chapter VI

Results

1. Colorimetric methods.

The responses in both absorbance and concentration of a serial standard hippuric acid solution by BSC and DAB methods were summarized in Table 5.

Calibration curve by BSC and DAB methods were constructed from data in Table 5. It was showed in Fig 10.

Table 5. Absorbance of hippuric acid solution by BSC and DAB method

Concentration (μg/ml)	BSC (OD)	DAB (OD)
100	0.009	0.039
200	0.031	0.070
500	0.081	0.176
1,000	0.167	0.471
1,500	0.260	0.736
2,000	0.353	1.018
2,500	0.458	1.308
3,000	0.568	1.573

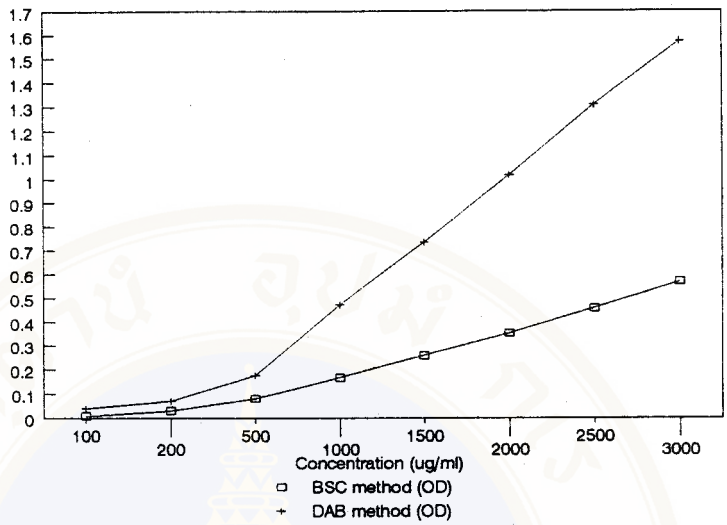


Fig 10. Standard curve of hippuric acid by BSC and DAB methods.

2. High-performance liquid chromatographic method.

The result of analysis the hippuric acid by using high-Performance Liquid Chromatography (HPLC Method).

The peak characteristic of hippuric acid in the described system shows a peak at retention time = 4.188 minutes. The peak shape is symmetric, acute, and no tailing (Fig 11.). The calibration by the described system of Chromatopac C-R6A used one calibration to find factor that used to calculated concentration of hippuric acid in samples. For Example.

-Injected standard hippuric acid solution concentration 1,000 μg/ml in HPLC system press start.

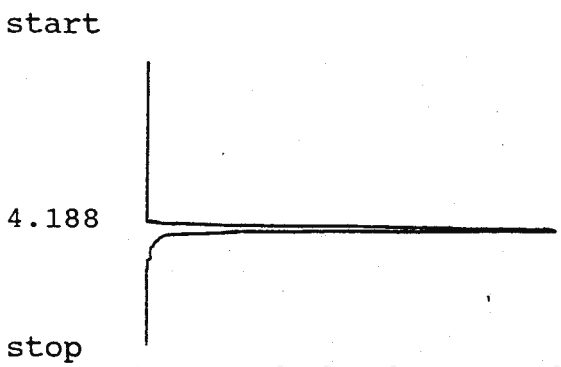


Fig 11.

Pk No	Time	Area	conc	Name	ID No
1	4.188	5931	1000	HA	1
Total		5931			

Calibration made in identification

ID No	Name	Time	factor	conc
1	HA	4.188	0.168618	1000

-Injected Sample. press start

start

stop

Pk No	Time	Area	ID No	conc	Name
1	2.600	536	-	-	-
2	4.188	1713	1	288.8	HA
Total		2249		288.8	

3. Recovery method.

The percent recovery of hippuric acid were procedure by added serial standard solution in known value of control urine. Then found the value of hippuric acid by these three method. The percent recovery was found by used this equation.[146]

$$\% \text{ recovery} = \frac{C_T - C_C}{C_S} \times 100$$

whereas, C_T = Corrected concentration
C_C = Control concentration
C_S = Standard concentration

For example:-

Control urine conc	=	220 µg/ml
Standard conc	=	1,000 µg/ml
Control urine : Standard 1,000	=	1:1
C_C (220/2)	=	110 µg/ml
C_S (1,000/2)	=	500 µg/ml
Control urine : Standard 1,000	=	1:1
analyzed by colorimetry BSC method		
C_T	=	600 µg/ml

$$\% \text{ recovery} = \frac{600-110}{500} \times 100$$
$$= 98\ \%$$

Table 6. Percent recovery

standard conc (μg/ml)	BSC (%)	DAB (%)	HPLC (%)
200	13	37	96
500	45	63	95
1,000	75	77	106
1,500	90	92	102
2,000	89	93	110
2,500	95	97	106
3,000	90	90	103

4. Urinary Hippuric Acid in Non-exposured or Normal Subjects.

Urinary specific gravity and urinary hippuric acid in three methods including corrected value of hippuric acid concentration using are shown in Table 7. The SG corrected valued are performed as in Chapter III(E), correcting to value 1.024 (which hippuric acid level in Thai normal urine 200 mg/l was correcting by SG 1.024[148]).

5. Urinary Hippuric Acid in Person Who Exposed to thinner.

5.1 Urine samples in groups of paint industry workers (5.1.3), teenager gangster (5.1.2a) and patients from Thunyaruck Hospital (5.1.2b) are analyzed in the same way as normal group. Urinary SG, hippuric acid by three methods and corrected values using SG value 1.024 are recorded in Table 8., Table 9., and Table 10.

5.2 Urine sample of thinner inhalated patients from Sliriraj Hospital are test by only DAB method and corrected value with SG value 1.024 are recorded in Table 11.

Table 7. Hippuric acid concentration (μg/ml) of 94 normal sample by Three methods.

No.	BSC	DAB	HPLC	No.	BSC	DAB	HPLC
1	994	514	493	41	600	252	184
2	1644	1056	1062	42	693	213	259
3	480	188	145	43	570	240	255
4	758	429	415	44	1256	889	601
5	908	566	630	45	508	155	96
6	438	125	121	46	288	48	50
7	590	420	444	47	600	216	252
8	456	120	142	48	593	282	265
9	1032	816	818	49	976	448	406
10	457	206	249	50	823	394	411
11	832	640	625	51	1960	1450	1213
12	655	425	401	52	540	180	246
13	747	613	594	53	1560	1200	1054
14	1366	997	1130	54	1056	624	664
15	933	653	757	55	528	144	122
16	560	320	340	56	576	288	268
17	1333	907	992	57	1080	660	609
18	3360	2348	2396	58	1920	1244	1070
19	330	150	147	59	1505	877	1041
20	607	310	400	60	1260	580	733
21	240	155	182	61	2898	2364	2256
22	408	288	264	62	1005	293	103
23	1237	868	950	63	2662	2084	2043
24	480	266	329	64	3258	2458	2390
25	852	715	717	65	632	278	276
26	480	218	213	66	2272	1232	1531
27	897	366	429	67	2460	1635	1672
28	450	225	264	68	789	171	322
29	650	310	365	69	784	288	369
30	371	196	273	70	605	259	302
31	584	271	309	71	1047	578	801
32	820	340	446	72	2640	720	1200
33	103	68	61	73	754	206	380
34	832	488	564	74	2194	1577	1668
35	1517	778	1090	75	1030	640	602
36	1532	1191	1302	76	840	347	442
37	741	407	515	77	1720	950	999
38	1410	1000	1133	78	2920	2080	2080
39	1440	960	878	79	499	125	236
40	819	452	482	80	703	206	382

Table 7. (continue)

No.	BSC	DAB	HPLC	No.	BSC	DAB	HPLC
81	1030	510	509	88	1000	620	718
82	2160	1360	1020	89	823	411	462
83	650	220	283	90	2304	1920	1564
84	672	240	276	91	1008	528	633
85	1013	560	589	92	640	293	401
86	754	206	226	93	1040	672	798
87	756	360	392	94	647	313	390

Table 8. Hippuric acid concentration (μg/ml) in 31 workers from paint industry.

No.	BSC	DAB	HPLC	No.	BSC	DAB	HPLC
1	928	504	367	17	1457	1022	650
2	1760	1360	664	18	1312	800	506
3	1460	657	532	19	1272	704	581
4	1565	845	666	20	3120	2400	1334
5	1296	710	396	21	1482	891	794
6	1875	1140	906	22	1152	624	417
7	2112	1512	1399	23	1176	624	494
8	1632	792	346	24	2577	1955	2034
9	2310	1275	1053	25	860	680	176
10	2400	1440	420	26	1496	1044	640
11	1232	760	539	27	1456	720	314
12	1310	830	454	28	2560	2160	2258
13	1209	720	389	29	1524	1355	584
14	960	720	150	30	763	412	255
15	975	675	314	31	1883	1606	1490
16	706	422	204				

Table 9. Hippuric acid concentration (μg/ml) of 54 teenagers gangster who sniffed thinner.

No.	BSC	DAB	HPLC	No.	BSC	DAB	HPLC
1	1680	860	732	28	5600	4700	2947
2	11286	8054	7792	29	680	260	176
3	1200	930	584	30	768	336	220
4	2430	2150	1288	31	540	120	100
5	1160	690	392	32	576	216	240
6	1800	1140	640	33	6104	5165	5347
7	9380	8290	7413	34	7333	6933	6190
8	1680	1030	583	35	996	468	463
9	2680	2240	1183	36	1089	618	780
10	1110	570	264	37	789	274	286
11	10770	9460	9910	38	23143	23143	18531
12	5040	4600	3216	39	2291	1135	1132
13	680	400	190	40	12000	9312	8577
14	7360	6600	6496	41	6545	5236	5415
15	1760	1530	1174	42	1120	560	505
16	3440	3000	2482	43	21800	15500	15946
17	3700	3000	2900	44	3840	2784	2608
18	3870	3220	2630	45	26100	23700	23227
19	2240	2000	1529	46	4560	3120	2640
20	5540	4690	5085	47	896	496	361
21	2570	2090	1640	48	19800	14400	13956
22	10210	9690	9365	49	2373	1787	1301
23	3189	2811	2314	50	2580	1880	1720
24	9943	8229	7907	51	1160	600	395
25	8592	7248	7536	52	8817	5635	5400
26	8000	7636	5616	53	1843	1114	1109
27	2736	1824	1416	54	2942	975	1114

Table 10. Hippuric acid concentration (μg/ml) in 21 patients from Thunyaruck Hopital.

No.	BSC	DAB	HPLC	No.	BSC	DAB	HPLC
1	1890	1820	1793	12	570	240	264
2	30500	27200	19063	13	14400	10970	10594
3	6600	4900	4036	14	36630	24000	29308
4	18600	15300	13656	15	15500	14400	14275
5	21500	20700	16121	16	980	610	449
6	26400	11900	18084	17	624	460	264
7	21800	20400	16490	18	17470	15470	15560
8	990	830	717	19	32230	30860	22429
9	30750	19500	21375	20	20160	17120	17512
10	23500	5600	8606	21	1060	850	592
11	34150	26250	24309				

Table 11. Hippuric acid concentration (μg/ml) of 4 patients from Sliriraj Hospital.

Time / No.	1	2	3
1	2080	1970	540
2	16600	7100	3600
3	2120	1440	340
4	6156	1280	700

6. Statistical Analysis of Data from Urinary Determination.

6.1 Frequency Distribution in Normal Samples.

Frequency distribution of urinary hippuric acid that corrected by SG (1.024) from normal subjects by three methods BSC, DAB, and HPLC methods are presented in Table 12. Frequency polygon constructed from the distribution tables above are also shown in Fig 12., Fig 13., and Fig 14. respectively.

Table 12. Frequency distribution of SG corrected urinary hippuric acid in normal subjects.

Concentration Range (μg/ml)	BSC (persons)	DAB (persons)	HPLC (persons)
< 200	1	13	11
200 - 400	5	31	28
400 - 600	19	15	17
600 - 800	19	11	12
800 -1000	14	9	6
1000 -1200	10	3	9
1200 -1400	5	3	2
1400 -1600	6	2	2
1600 -1800	2	1	2
1800 -2000	2	1	0
>2000	11	5	5

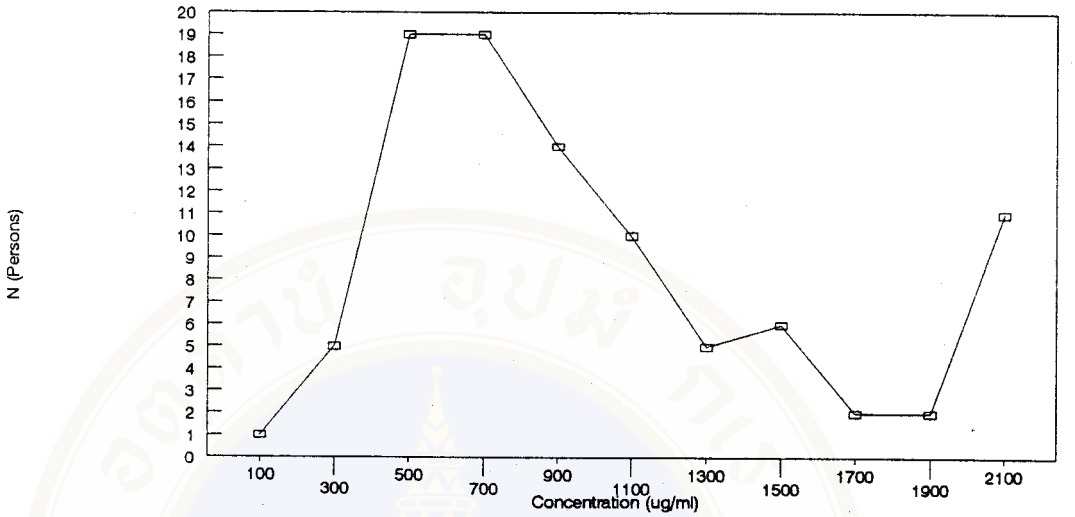


Fig 12. BSC

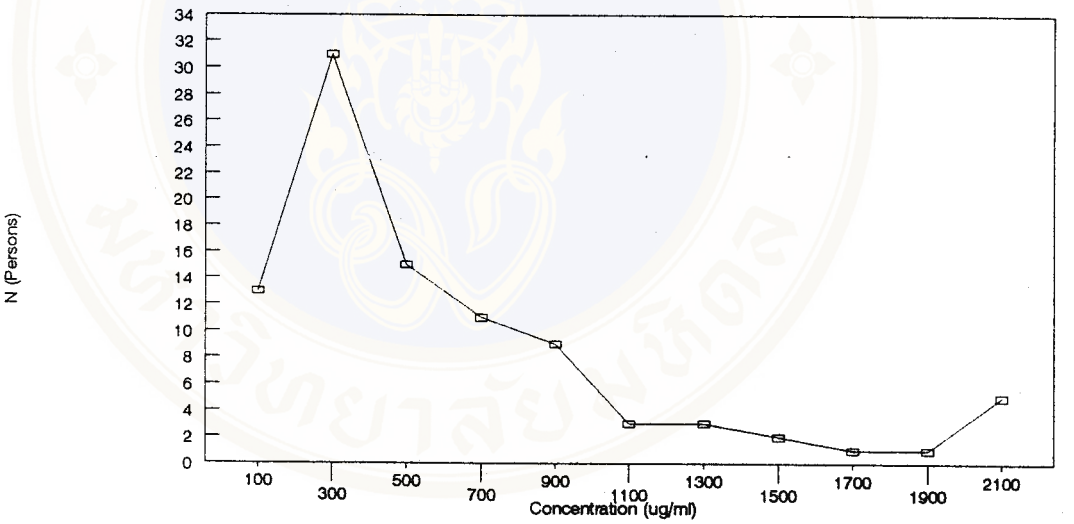


Fig 13. DAB

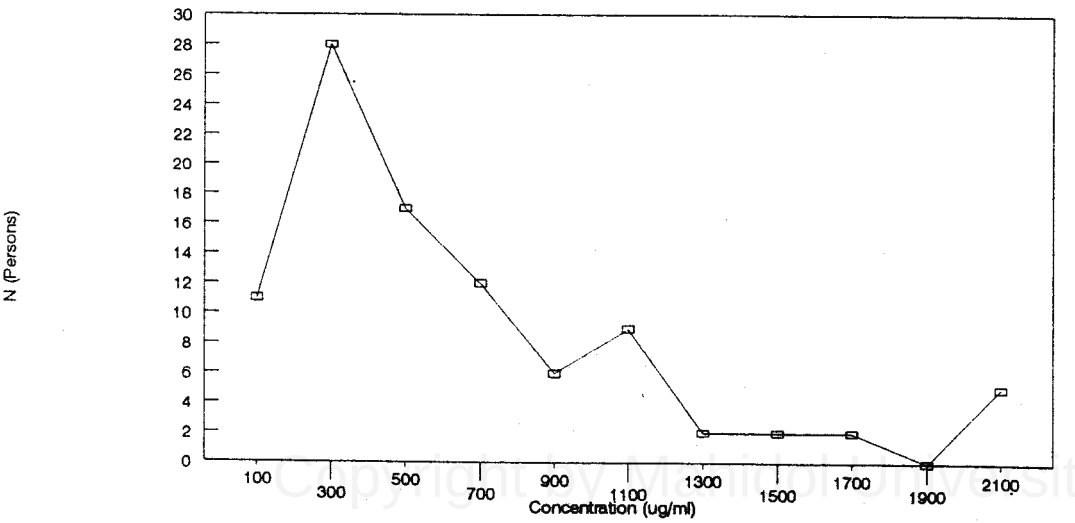


Fig 14. HPLC

6.2 Frequency Distribution in Comparison of Normal Subjects, Industrial Worker, and Thinner Exposure.

Frequency distribution of SG corrected urinary hippuric acid that determined by BSC, DAB, and HPLC method from normal subjects, industrial worker, and thinner sniffers' urine in two sourses are presented in Table 13. Frequency polygon constructed from the distribution tables above are also shown in Fig 15., 16., and 17. respectively.

Table 13. Frequency distribution of SG corrected urinary hippuric acid by three methods from normal subjects, industrial worker, teenager thinner-sniffer, and thinner-sniffer patient.

Range	Normal			Worker			Teenager			Patient		
	BSC	DAB	HPLC	BSC	DAB	HPLC	BSC	DAB	HPLC	BSC	DAB	HPLC
0-500	15	55	54	-	6	45	-	14	20	-	9	14
501-1000	47	29	25	19	55	36	15	14	11	19	14	9
1001-1500	18	8	12	42	23	13	11	7	14	5	-	-
1501-2000	9	3	4	19	10	-	8	8	6	5	5	5
2001-2500	5	5	5	10	6	6	7	6	4	-	-	-
2501-3000	4	-	-	7	-	-	8	7	8	-	-	-
3001-3500	2	-	-	3	-	-	4	4	2	-	-	-
3501-4000	-	-	-	-	-	-	6	-	-	-	-	-
4001-4500	-	-	-	-	-	-	-	-	-	-	-	5
4501-5000	-	-	-	-	-	-	2	6	-	-	5	-
5001-5500	-	-	-	-	-	-	2	4	7	-	-	-
5501-6000	-	-	-	-	-	-	4	2	2	-	5	-
6001-6500	-	-	-	-	-	-	2	-	4	-	-	-
6501-7000	-	-	-	-	-	-	2	4	-	5	-	-
7001-7500	-	-	-	-	-	-	4	2	2	-	-	-
7501-8000	-	-	-	-	-	-	2	2	6	-	-	-
8001-8500	-	-	-	-	-	-	-	6	-	-	-	-
8501-9000	-	-	-	-	-	-	4	-	2	-	-	5
9001-9500	-	-	-	-	-	-	2	4	2	-	-	-
9501-10000	-	-	-	-	-	-	2	2	2	-	-	-
10001-15000	-	-	-	-	-	-	7	2	2	9	14	14
15001-20000	-	-	-	-	-	-	2	2	4	9	23	29
20001-25000	-	-	-	-	-	-	4	4	2	19	14	14
25001-30000	-	-	-	-	-	-	2	-	-	5	6	5
>30001	-	-	-	-	-	-	-	-	-	24	5	-

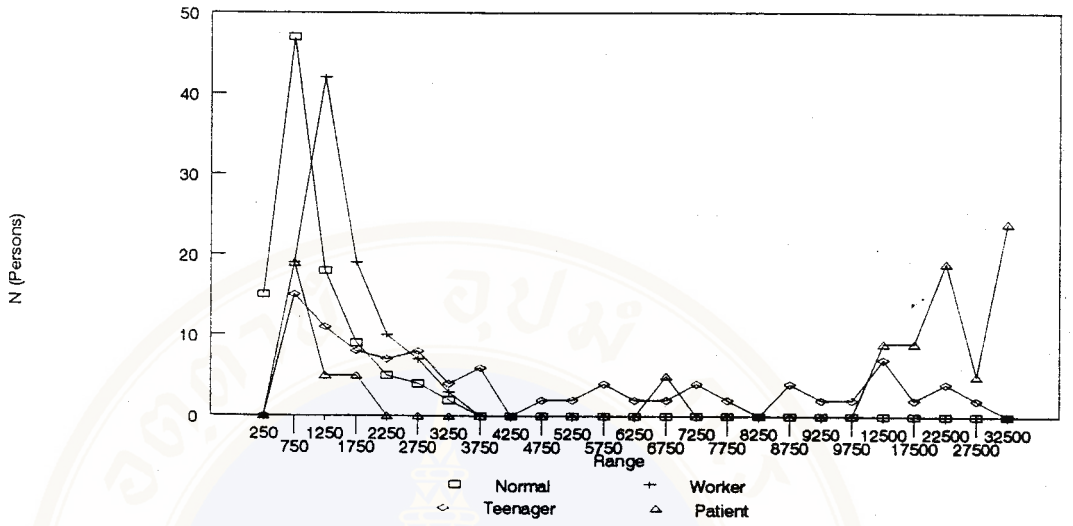


Fig 15. BSC

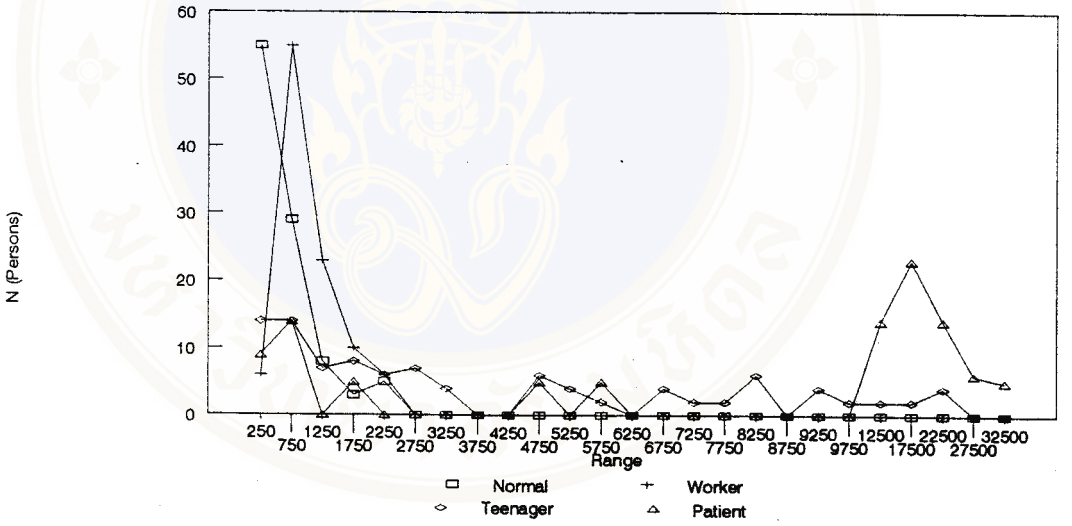


Fig 16. DAB

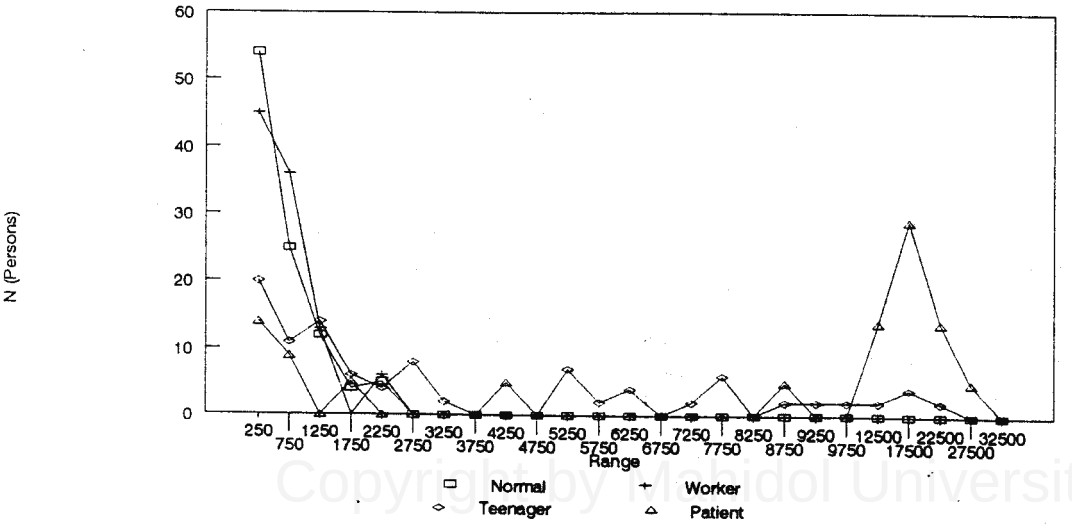


Fig 17. HPLC

6.3 Measure of Central Tendency and Dispersion.

The SG corrected urinary hippuric acid of normal subject, industrial worker, thinner-sniffer teenager, and thinner-sniffer patient as shown in Table 7., 8., 9., and 10. are statistical measured for central tendency and dispersion in term of arithmetic mean (X), range, standard derivation (SD), and standard error of arithmetic mean (SEM) for further discussion.

These measurements are shown in Table 14., 15. and 16. for BSC, DAB, and HPLC method respectively and SG corrected urinary hippuric acid in all groups from three method are shown in Fig 18.

Table 14. Statistical measurements of BSC method.

Sample group	n	x	Range	SD	SEM
Normal	94	1057.9	103-3360	698.97	2.727
Worker	31	1541.3	706-3120	581.85	4.332
Teenager	54	5376.5	540-26100	5931.96	10.481
Patient	21	16966.9	570-36630	12490.02	24.388

Table 15. Statistical measurements of DAB method.

Sample group	n	x	Range	SD	SEM
Normal	94	621.8	48-2458	550.34	2.420
Worker	31	1011.6	412-2400	501.22	4.021
Teenager	54	4341.6	120-23700	5223.99	9.836
Patient	21	12827.6	240-30860	10057.81	21.885

Table 16. Statistical measurements of HPLC method.

Sample group	n	x	Range	SD	SEM
Normal	94	650.9	50-2396	534.18	2.384
Worker	31	691.2	150-2258	516.57	4.082
Teenager	54	3949.3	100-23227	4938.03	9.563
Patient	21	12380.8	264-29308	9150.81	20.875

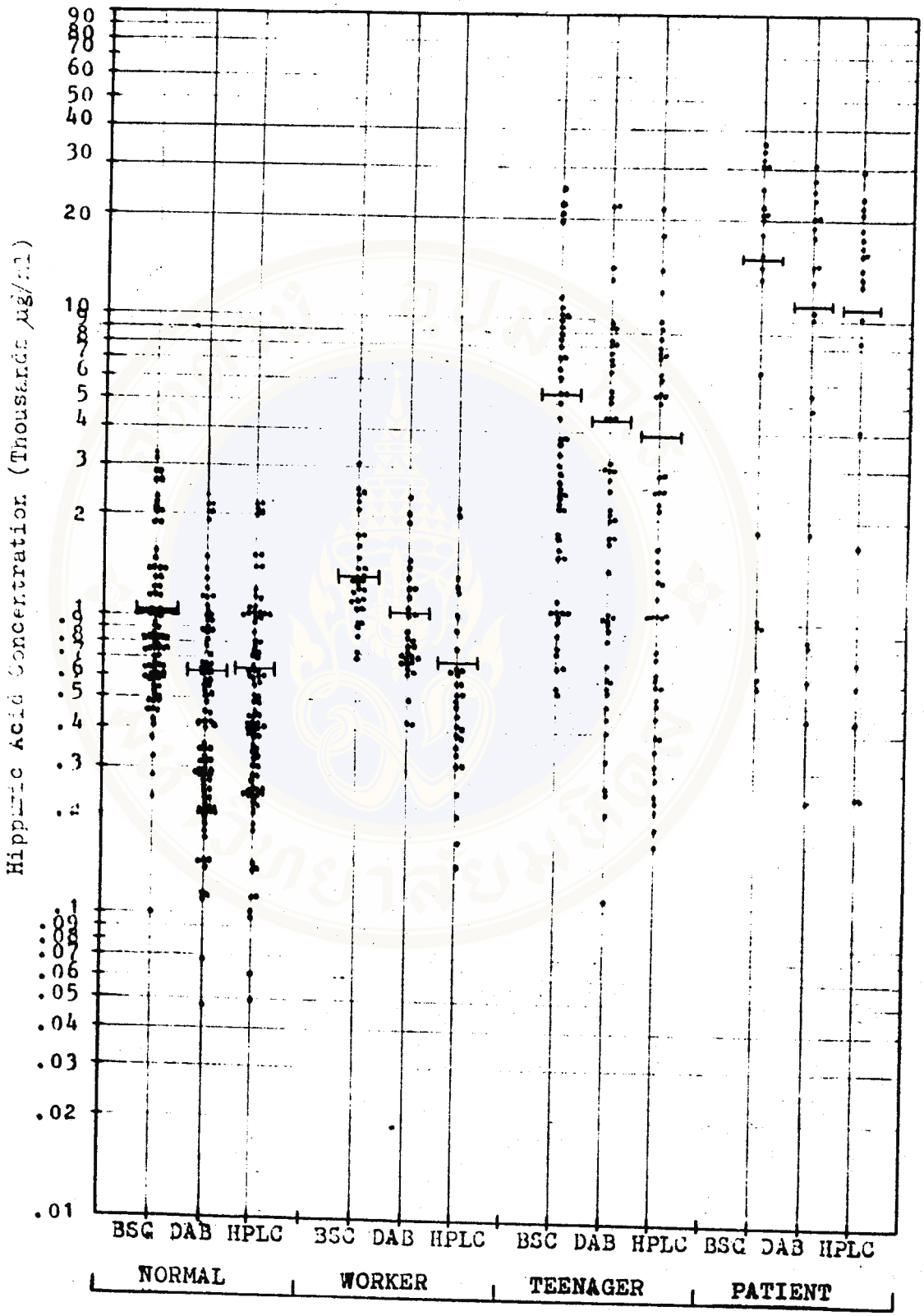


Fig 18.

6.4 Regression Analysis.

The SG corrected urinary hippuric acid of normal subjects, industrial worker, thinner-sniffer teenager gangsters, and thinner-sniffer patients determined by BSC, DAB, and HPLC method are analysis between BSC with HPLC method and DAB with HPLC method. Intercept (b), Slope (a), and correlation coefficient (r) are shown in Table 17., and 18. respectively.

Relationship between BSC and DAB with HPLC method in normal subjects, worker, thinner-sniffer teenager gangster, and thinner-sniffer patient are present in Fig 19., and 20.

Table 17. Regression value between BSC and HPLC methods.

Sample group	Intercept (b)	Slope (a)	Correlation (r)
Normal	239.30347	1.25773	0.96121
Worker	922.51123	0.89527	0.79482
Teenager	682.87681	1.18847	0.98933
Patient	929.08006	1.31819	0.96429

Table 18. Regression value between DAB and HPLC methods.

Sample group	Intercept (b)	Slope (a)	Correlation (r)
Normal	-33.16820	1.00637	0.97682
Worker	439.35006	0.82793	0.85328
Teenager	203.42586	1.04783	0.99047
Patient	287.42827	1.03072	0.93633

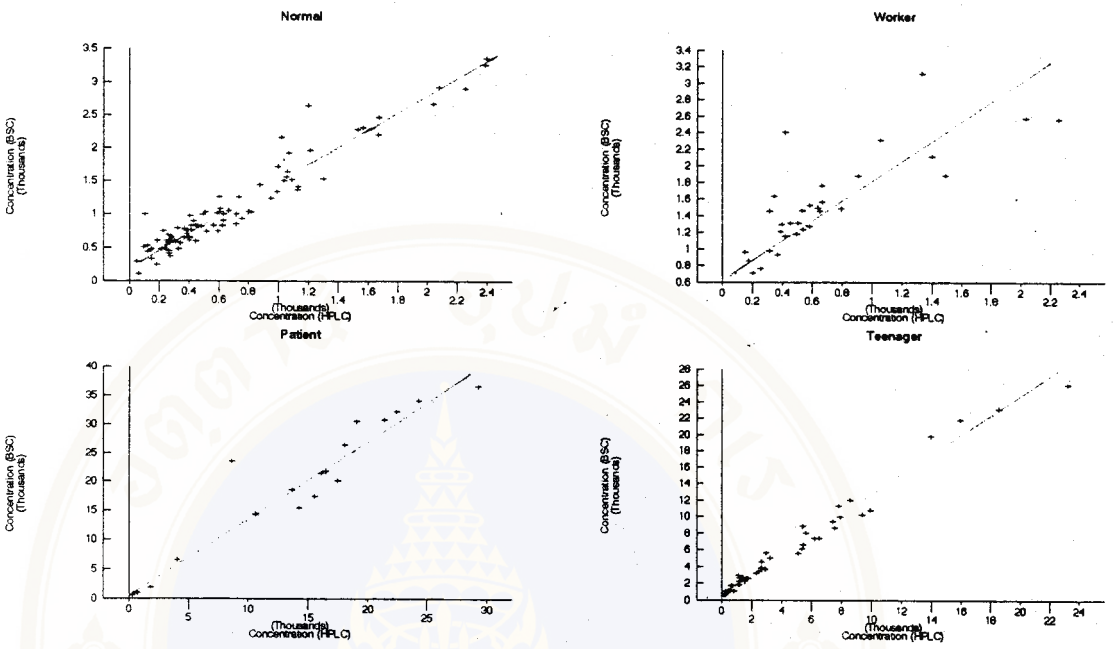


Fig 19. Relationship between BSC and HPLC methods.

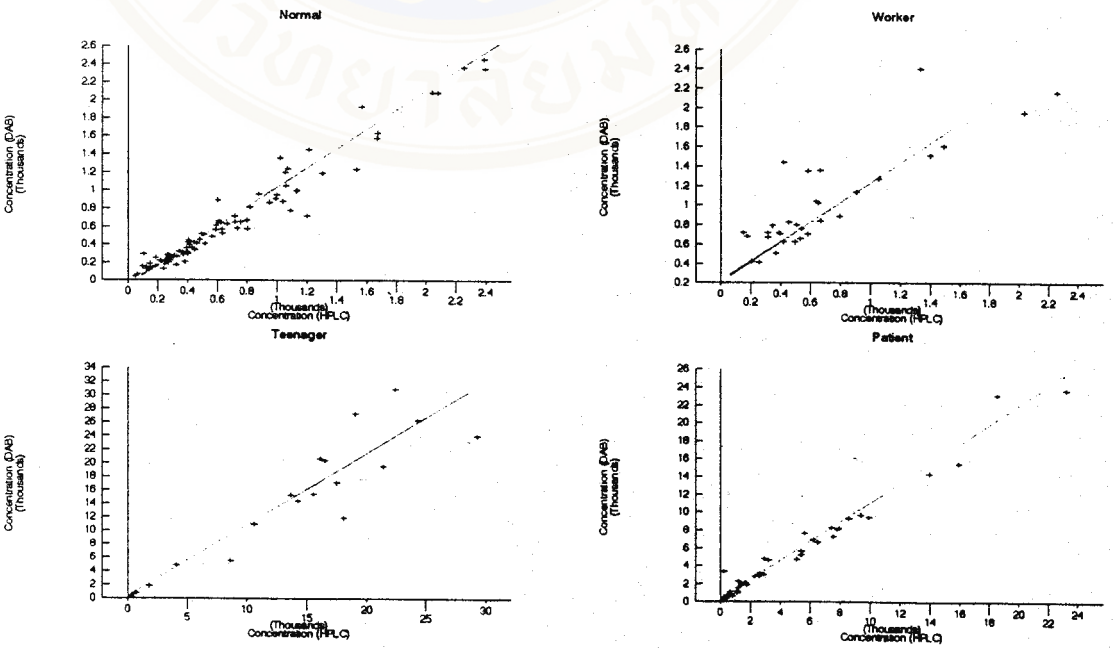


Fig 20. Relationship between DAB and HPLC methods.

6.8 Pair t-test.

Pair t-test of SG corrected urinary hippuric acid between BSC and DAB compared with HPLC method of normal subjects, industrial worker, thinner-sniffer teenager gangster, and thinner-sniffer patient are shown in Table 19., and 20. (95% confidence level (p=0.05))

Table 19. Comparation between BSC with HPLC methods.

Sample group	n	diff mean	SD	t-value	2-Tail Prob	ϕ
Normal	94	407.0532	236.892	16.66	0.000	93
Worker	31	850.1290	357.214	13.25	0.000	30
Teenager	54	1427.1852	1269.988	8.26	0.000	53
Patient	21	4586.0476	4561.404	4.61	0.000	20

Table 20. Comparation between DAB with HPLC methods.

Sample group	n	diff mean	SD	t-value	2-Tail Prob	ϕ
Normal	94	-29.0213	117.853	-2.39	0.019	93
Worker	31	320.4194	276.061	6.46	0.000	30
Teenager	54	392.3333	757.118	3.81	0.000	53
Patient	21	446.8000	3542.730	0.58	0.575	20

Chapter VII

Discussion

Urinary hippuric acid was analysed by 3 methods: colorimetry by using BSC, colorimetry by using DAB in acetic anhydride, and high-performance liquid chromatography methods. Advantages and disadvantages of those methods are shown in Table 21.

Statistic analysis for normal range of urinary hippuric acid. By using three methods for analysis hippuric acid found that the DAB method with used to analysis hippuric acid by colorimetric method is most reliable method when compared with HPLC method from the data shown in Table 19. and Table 20., normal group of both colorimetry are compared with HPLC method and found that the BSC method is significant different from HPLC method at $p<0.01$ which shown in Table 19.

From the freguency distribution of 94 normal samples in Fig 12, 13, and 14. It can be seen that the distributions curve of urinary hippuric acid concentration in corrected values are not normal but have much positve skews. In Fig 12, 13, and 14 ; the frequency distribution of urinary hippuric acid that determined by DAB and HPLC methods are almost the same. The normal value from all data is determined by using 10 and 90 percentile according to Herrena(146). It is found that the reference value of BSC, DAB, and HPLC methods are 450-2200 μg/ml, 180-1500 μg/ml, and 150-1400 μg/ml, respectively. It can be seen that the normal range of DAB is almost the same as that of HPLC more than the normal range of BSC method.

Reliability of selected the method for using in toxicology laboratory. In this experiment, hippuric acid was determined by using 2 colorimetric methods compared with HPLC which was used as reference method. Urine samples were collected from 3 group subjects; normal sample, workers who were supposed to expose to solvent in daily life, and thinner sniffers.

Table 21. Advantages and disadvantages of BSC, DAB, and HPLC methods

	BSC	DAB	HPLC
1.% Recovery	low when HA concentration is low	low when HA concentration is low but higher than BSC	96-110%
2.Interference	Many substances can interfere this method, namely, methyl hippuric acid, phenol, mandelic acid, caffeine, salicylic acid derivatives, and some amino acids.	Interfering substances are less than BSC method. Caffeine itself dose not interfere this method. Some amino acids do not react with DAB.	Only HA at retention time of standard HA will be determined This retention time is 4.188.
3.Instrument cost	UV spectro-photometer is cheaper than HPLC and is available in most laboratories.	UV spectro-photometer is cheaper than HPLC and is available in most laboratories.	Instrument are expensive and complicate.
4. Methodology	Easy	Easy	Difficult
5. Time	Thirty minutes for incubation.	Sixty minutes for incubation.	Retention time 4.188 mins.
6. Chemical substances	-Analytical grade. -Carcinogeni-city. -Forbidden BSC is not allowed to be imported.	-Analytical grade. -Carcinogeni-city. -Forbidden substance. It is used for producing heroin.	-HPLC grade.

From Table 17, 18 and Fig 19, 20 ; it is seen that, in normal group, correlation coefficients of BSC and DAB compared to HPLC were almost the same, that is, 0.96121 and 0.97682, respectively. When compare DAB and HPLC, the correlation coefficient was near 1.0 than when compare BSC and HPLC. The slope were 1.00637 and 1.25773 respectively. This means that DAB method is correlated with HPLC method more than BSC method. Moreover, intercept were 239.30347 and -33.16820, respectively. It showed that the intercept of BSC method is higher than that of DAB method. This indicates that not only hippuric acid will be detected but the interference will also be detected. For DAB and HPLC method, the intercept has negative charge. This means that when the amount of urinary hippuric acid is small, DAB will react with the interference less than BSC. And due to the % recovery of DAB method is low, in normal samples, therefore the hippuric acid concentration detected by DAB method is lower than HPLC method.

From the industrial worker group, correlation coefficients of BSC and DAB compared to HPLC methods are 0.79482 and 0.85328, respectively. The slope of these 2 methods are almost the same, that is 0.89527 and 0.82793. But the intercept of BSC method is higher than that of DAB method obviously (922.51123>439.35006). This indicates that BSC method has interference more than DAB methods.

For the solvent exposed group, from 2 sources, correlation coefficient, slope, and intercept are not different. When compare BSC and DAB with HPLC methods by using pair t-test, means of BSC and HPLC methods in all groups are different significantly ($p<0.01$) Means of normal group and patients from Thunyaruck hospital determined by DAB method are not different from means of those by HPLC method significantly ($p<0.01$). This means that urine samples of both groups have very little interferences. But for workers and teenager gangsters, who expose glue, means of these 2 groups are different significantly between DAB and HPLC methods. This might be due to they received other solvents, for example, benzene, xylene, or styrene of which metabolites can react with DAB.

The criteria of decision in non-solvent exposure and solvent exposure to the difference of urinary hippuric acid. In Table 14, 15, and 16 shows mean of urinary hippuric acid of

normal, industrial worker, and solvent exposure groups. It can be seen that mean of urinary hippuric acid in normal group is lower than mean of in solvent exposure group significantly ($p<0.01$) when it was determined by BSC, DAB, and HPLC methods. But mean of normal urinary hippuric acid is lower than mean of industrial worker urinary hippuric acid significantly ($p<0.01$) when it was determined by BSC and DAB. But for HPLC method, means of urinary hippuric acid in normal and industrial worker groups are not different significantly at $p=0.05$. However, range of urinary hippuric acid in normal group is wider than that of in worker group by all methods.

If we see the range of urinary hippuric acid of 2 solvents exposed groups examined by 3 methods, most of hippuric acid concentrations fall from normal up to high value. Furthermore, the urine samples were collected randomly. They might be collected after the subjects exposed solvent for a long time. Normally, hippuric acid is high in 12 hours after exposing. After this period, hippuric acid will be decreased. As we see from Table 11, urinary hippuric acid is high in the first day after exposing. In the second day, urinary hippuric acid is already decreased. And it is in normal range in the third day. Accuracy and precision of analysis for hippuric acid from the occupational worker must be collected urine before start works and after the end of the work-shift.

Chapter VIII

Conclusion

Solvent exposure is a common problem found throughout country. It affects economics and health of exposed persons. The most common solvents used for inhaling are glue and thinner. Both of them compose of toluene. Hippuric acid is the major metabolite which excrete in urine so urinary hippuric acid determination is the easiest method for detection metabolite of toluene in solvent exposed persons. Because urine is easy for being collected. And hippuric acid is stable. It dose not evaporate easily. Although hippuric acid can be detected in normal urine. Because of hippuric acid is the end product of benzoic acid, quinic acid or some amino acid found in some foods, fruits, and drugs. However, in this study, the normal range of hippuric acid from 94 urine samples detected by 3 methods is corrected with SG=1.024. The normal ranges got by this experiment by BSC, DAB, and HPLC methods are 450-2200, 180-1500, and 150-1400 μg/ml, respectively. In addition, mean of normal urinary hippuric acid is different significantly from that of exposed urinary hippuric acid ($p<0.01$).

In this study, urinary hippuric acid was determined by using 2 colorimetric and HPLC methods. HPLC was used as reference method because it has high sensitivity, specificity, precision, and accuracy. BSC method was correlated with HPLC method. The correlation coefficient is 0.9843 which is so high. But urinary hippuric acid determined by BSC method is higher than that of HPLC. This probably due to other substances can interfere reaction. These interferences are methyl-hippuric acid, phenol, and mandelic acid which are metabolic form of xylene, benzene, and styrene, respectively. Those are component of glue and thinner which usually use in many industries. Furthermore, BSC can react with some foods and drugs directly, for example, caffeine, paracetamol which are commonly used. From this study, mean values of hippuric acid detected by BSC method are higher than those of HPLC in

all groups ($p<0.01$).

DAB method also correlated with HPLC method. The correlation coefficient is 0.9770. But this method has less interferences than BSC. This experiment showed that mean values of urinary hippuric acid in group of normal samples and group of thinner exposed from Thunyaruck hospital when detected with DAB and HPLC methods were not significantly different at $p=0.05$. For industrial workers and teenager gangsters, mean values of urinary hippuric acid detected by DAB method is higher than that of urinary hippuric acid detected by HPLC method significantly ($p<0.01$). This means that industries where they works were using many solvents. For the gangsters, very few information could be got from the sources, that is, they were accused because of sniffing glue. Another information we could not know. So that the range of hippuric acid is quite broad. However, mean value is higher than that of normal group in all methods.

From this study, we might say that DAB is a good method for urinary hippuric acid determination used in place of HPLC especially in toxicology laboratories which have no HPLC instruments but already have UV spectrophotometer. And the other reasons are HPLC procedure is complicated, the instrument are expensive. In contrast, DAB method is not expensive, and correlated well with HPLC method, also the reagents used are available.

For the industrial workers, we found that most industries have safety systems. The urinary hippuric acid of them were not so high as we expected. So the workers have little risk in exposuring to solvent. The intercepts of urinary hippuric acid by colorimetric methods are high, but hippuric acid determined by HPLC method is almost the same as that of normal group. So in these information indicates that the industries not only using the toluene but mixed of the other solvent are expected etc: benzene, xylene, styrene, or toluene.

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