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High Performance Liquid Chromatography Determination of Urinary Hippuric Acid and Benzoic Acid as Indices for Glue Sniffer Urine

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***Abstract*—**A simple method for the simultaneous determination of hippuric acid and benzoic acid in urine using reversed-phase high performance liquid chromatography was described. Chromatography was performed on a Nova-Pak C_{18} (3.9 x 150 mm) column with a mobile phase of mixed solution methanol: water: acetic acid (20:80:0.2) and UV detection at 254 nm. The calibration curve was linear within concentration range at 0.125 to 6.0 mg/ml of hippuric acid and benzoic acid. The recovery, accuracy and coefficient variance of hippuric acid were 104.54%, 0.2% and 0.2% respectively and for benzoic acid were 98.48%, 1.25% and 0.60% respectively. The detection limit of this method was 0.01ng/l for hippuric acid and 0.06ng/l for benzoic acid. This method has been applied to the analysis of urine samples from the suspected of toluene abuser or glue sniffer among secondary school students at Johor Bahru.

***Keywords*—**Glue sniffer, High Performance Liquid Chromatography, Hippuric Acid, Toluene, Urine.

I. Introduction

TOLUENE is also known as methyl benzene or phenyl methane. It is a clear and water insoluble liquid with a typical smell and redolent of sweet smell derived from benzene compound. This chemical is available and used by industry for chemical synthesis or use as solvent in paint, print ink and adhesive [1]. It is also the common solvent in glue and thinner and because of that, this organic solvent was used as a drug for glue sniffer. Toluene is the major compound in the glue samples was entered in glue sniffer bodies through the nose. This statement has been verified by Yacob *et al.*, (2009) in his paperwork the characterization of local glue [2].

Toluene is the solvent with the most documentation of abuse, possibly because of its relative low risk in sudden death or ease of detection in blood for glue abuser. Many aromatic compounds such as toluene and benzoic acid are taken internally. They are converted to hippuric acid by the reaction with amino acid glycine present in blood [3]. The principal metabolite is benzoic acid conjugated with the glycine to form hippuric acid and excreted in urine with half-life of 2-3 hours [4]. Besides that, toluene also can be absorbed into the blood from the lung, gastrointestinal tract and through the skin. Fat rich tissue such as brain and liver serve as the most extensive reservoir for toluene deposits [1].

Approximately 60 – 75% of absorbed toluene is metabolite to benzoic acid [4, 5]. The initial step involves side chain oxidation to benzyl alcohol by cytochrome P450 enzymes in liver. Less than 1% of absorbed toluene undergoes ring hydroxylation to form o-, and p-cresol, which are excreted in the urine as glucuronide or sulphate conjugates [6,7,8]. The metabolite of toluene is shown in Fig. 1.

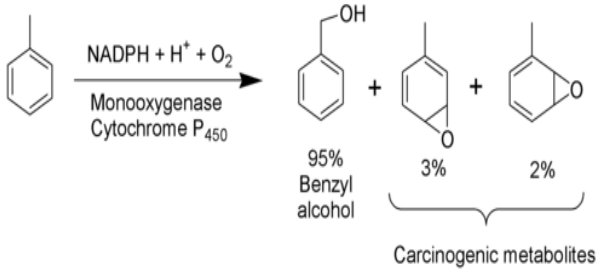

Fig. 1 First step of toluene metabolite

Benzyl alcohol is then oxidized to benzoic acid by alcohol dehydrogenase and aldehyde dehydrogenase. Benzoic acid is subsequently reacted with glycine to produced hippuric acid [4]. Benzoic acid may also be reacted with glucuronic acid to form benzoyl glucuronide in the urine shown in Fig. 2.

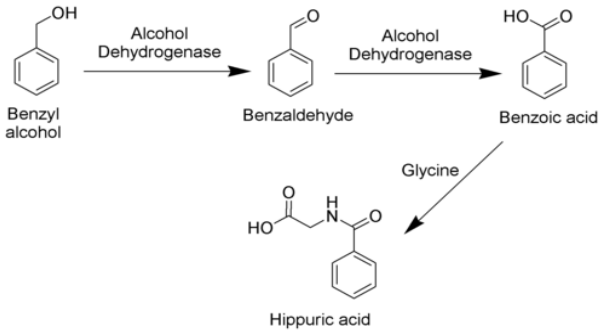

Fig. 2 Second step of toluene metabolite

Hippuric acid is the normally main composition of none protein nitrogen in urine and it is an important metabolic intermediate or end product for protein or nucleic acid. Among the metabolites, hippuric acid and benzoic acid are the

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traditional marker in the biological monitoring of people exposed to toluene [8,9].

Many methods have been introduced for qualitative and quantitative analysis of urinary hippuric acid. Colorimeter determination after extraction with ethyl ether and color production by the addition of benzenesulphonylchloride was studied by Manabu *et al.,* (2005) [10]. Besides that, Yacob *et al.,* (2009) in the study used spectrophotometer for qualitatively determination of hippuric acid [11]. This method however was successful for the screening of but not in quantitative determination. For thin-layer analysis and paper chromatographic methods which are elaborate and time consuming, it is difficult to measure a large number of samples quickly and accurately. Gas chromatographic techniques on the other hand require pretreatment such as methylation of the extracts. Kiyoshi *et al.,* (1988) study the determination of benzoic acid and hippuric acid in human plasma and urine by High Performance Liquid Chromatography. This paper is for biomedical application showing HPLC is the good and accurate equipment for the determination of benzoic acid and hippuric acid in urine [12].

This paper demonstrates a rapid, simple high performance liquid chromatographic assay for the simultaneous determination of hippuric acid and benzoic acid in urine. A single buffer use as the mobile phase along with a simple sample preparation makes this method applicable to numerous clinical samples without any difficulties. It can detect qualitatively and quantitatively hippuric acid and benzoic acid in urine sample extracted from glue sniffer and toluene abuser.

II. Experimental

A. Reagents

Hippuric acid 99.7%, Benzoic acid 99.7%, Methanol 99.7% and Sodium hydroxide 99.7% were purchased by Merck. Distilled water and buffer solution contain mixed solution, methanol: water: acetic acid (20:80:0.2).

B. Instrument

High Performance Liquid Chromatography brand Agilent Technologies, Nova-Pak C_{18} (3.9 x 150 mm) column brand Agilent and Head space Hamilton syringe 50 µl brand Agilent Technologies. The mobile phase was mixed solution of methanol: water: acetic acid (20: 80: 0.2); flow rate 0.8 ml/min; detection wavelength 254 nm; sensitivity 0.1 AUFS. The chromatography was performed at room temperature. A mobile phase solution was adjusted to pH 6.7 with 10 M sodium hydroxide. The mobile phase was passed under reduced pressure through a 0.45 µm filter and degassed in an ultrasonic bath.

C. Determination of Hippuric Acid

1. Preparation of Calibration Standards of Hippuric Acid

12.5 mg, 25.0 mg, 50.0 mg, 100 mg, 150 mg, 200 mg, 300 mg and 600 mg of hippuric acid was dissolved into eight different 100 ml volumetric flasks. The volumetric flask was labelled as standard HA 0.125, HA 0.25, HA 0.5, HA 1.0, HA 1.5, HA 2.0, HA 3.0 and HA 6.0. The volumetric flask was marked up with methanol. All the standards were sonicated in the ultrasonic sonic bath and stored in the dark at 4°C. The working solution was prepared by taking 500 µl of hippuric acid standards and diluting to 500 µl with mobile phase.

2. Preparation of Calibration Standards of Benzoic Acid

Step II.C.1 was repeated with benzoic acid and label as standard BA 0.125, BA 0.25, BA 0.5, BA 1.0, BA 1.5, BA 2.0, BA 3.0 and BA 6.0. All the standards were sonicated in the ultrasonic sonic bath and stored in the dark at 4°C. The working solution was prepared by taking 500 µl of hippuric acid standards and diluting to 500 µl with mobile phase.

3. Sample Collection and Preparation

Urine samples were collected from secondary school around Johor Bahru, Malaysia in collaboration with the National Anti Drug Agency to screen the drug abuser and glue sniffer. The urine specimens were stored at 0-5°C. 1 ml of urine specimen was placed in a 50 ml beaker, 0.2 ml Hydrochloric Acid was be added and extracted with 4 ml Diethyl ether/methanol (9:1 by volume). One ml of each extract was transferred to another test tube. After drying, 0.5 ml of methanol was added and the solution was injected into the HPLC.

III. Result and Discussion

A. Result Determination of Hippuric Acid Standard

TABLE I
Concentration versus Peak Area using High Performance Liquid Chromatography for the Determination of Hippuric Acid Standards

Concentration of Hippuric Acid	Peak Area
0.000	0.00000
0.125	1242.08704
0.250	2002.78905
0.500	2960.46729
1.000	5081.78296
1.500	7836.77832
2.000	10127.15000
3.000	14612.45000
6.000	29890.37000

Determination of hippuric acid was prepared using standard samples containing 0.125, 0.25, 0.50, 1.0, 1.50, 2.0, 3.0 and 6.0 mg/ml hippuric acid. Table I shows the concentration of hippuric acid versus peak area using High Performance Liquid Chromatography with UV detector at 254 nm. Based on the table, this method was successfully used to determine the present of hippuric acid at different concentration, subsequently this results proved that this method might be applied to detect hippuric acid in urine samples.

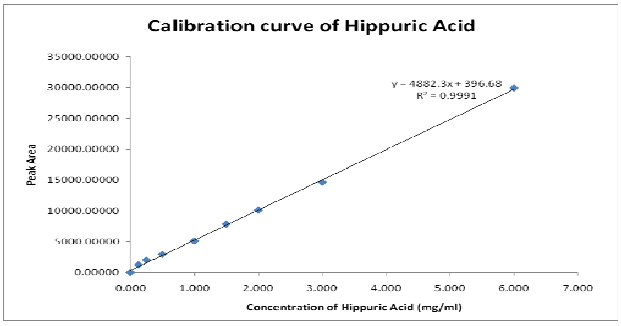

Fig. 3 Calibration curve of hippuric acid using High Performance Liquid Chromatography

The linearity of the concentration (mg/ml) versus response (peak area) was verified shown in the Fig. 3. The regression equation of the calibration curve for hippuric acid was y = 4882.3x + 396.68 with correlation coefficients of 0.9991. Calibration curve was linear up to 6.0 mg/ml for hippuric acid. The limit of detection, at the signal to noise ratio of 2, was 0.01 ng/l for hippuric acid.

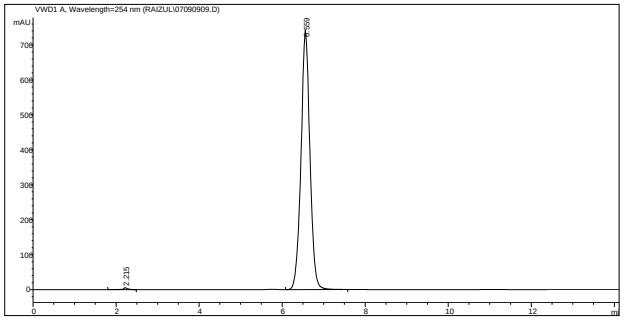

Fig. 4 High Performance Liquid Chromatography spectrum of 2 mg/ml hippuric acid standard

Fig. 4 shows the spectrum of 2 mg/ml hippuric acid standard as a calibrated standard for the High Performance Liquid Chromatography. This standard is the reference to know identify the possibility of toluene abuser and glue sniffer.

B. Result Determination of Benzoic Acid Standard

Benzoic acid standards were prepared with the concentrations of 0.125 to 6.0 mg/ml. The concentration of benzoic acid versus peak area is tabulated in Table II. From the table, it can be clarify that the present of benzoic acid at different concentration have significant with the response from the HPLC.

TABLE II
CONCENTRATION VERSUS PEAK AREA USING HIGH PERFORMANCE LIQUID CHROMATOGRAPHY FOR THE DETERMINATION OF BENZOIC ACID STANDARDS

Concentration of Benzoic Acid	Peak Area
0.000	0.00000
0.125	309.37129
0.250	571.74191
0.500	1051.87390
1.000	2054.58203
1.500	2997.57117
2.000	3895.10230
3.000	5579.99292
6.000	10934.29175

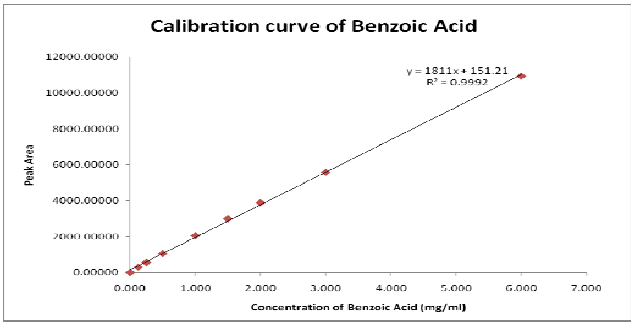

Fig. 5 Calibration curve of benzoic acid using High Performance Liquid Chromatography

Calibration curve of benzoic acid standard was verified in Fig. 5. The regression equation of the calibration curve for benzoic acid was y = 1811x + 151.21 with correlation coefficients of 0.9992. Calibration curve was linear up to 6.0 mg/ml for benzoic acid. The limit of detection, at the signal to noise ratio of 2, was 0.10 ng/l for benzoic acid.

Fig. 6 demonstrated the spectrum of 2 mg/ml benzoic acid standard was used as calibration standard using HPLC. This standard was cutting limit of toluene abuser or glue sniffer.

C. Result of Urine Sample

Calibration curve for both metabolite of toluene which is benzoic acid and hippuric acid 2 mg/ml was tabulated at Fig. 7.

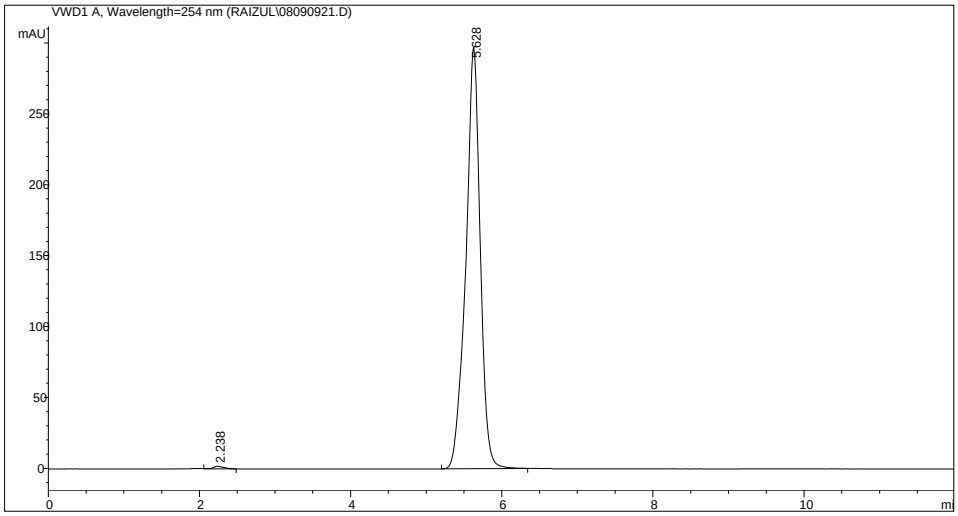

Fig. 6 High Performance Liquid Chromatography spectrum of 2 mg/ml benzoic acid standard

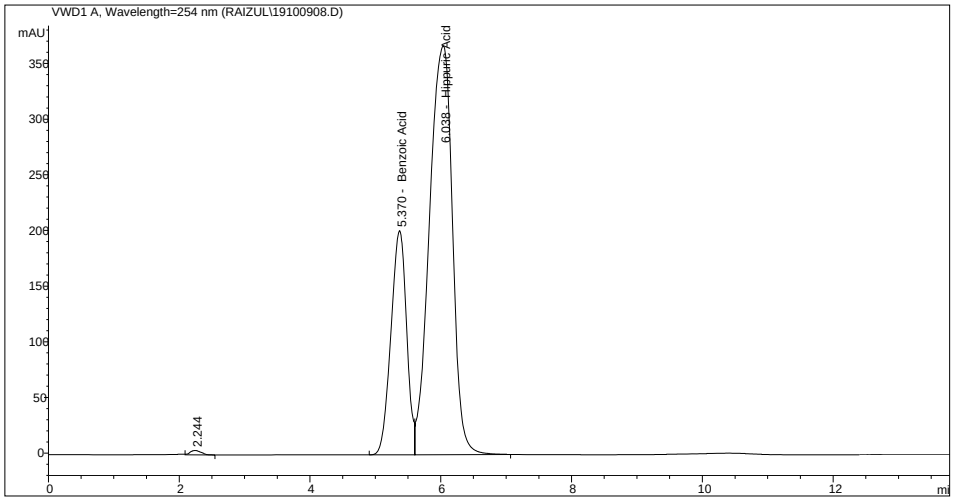

Fig. 7 High Performance Liquid Chromatography spectrum of mixed standard 2 mg/ml benzoic acid standard and 2 mg/ml hippuric acid standard

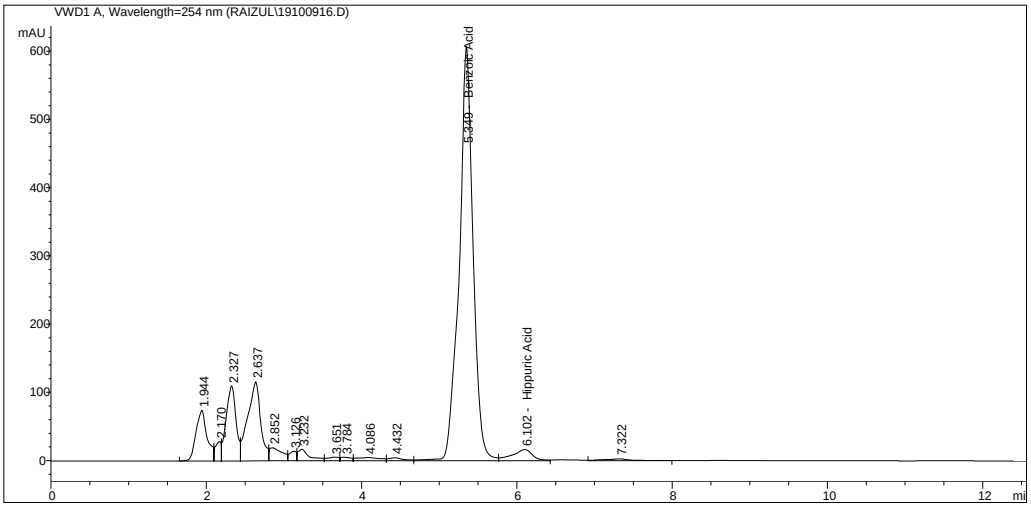

Fig. 8 High Performance Liquid Chromatography spectrum of urine sample 11

The mixed standard was used as calibrated standard of HPLC to determine the amount of hippuric acid and benzoic acid in urine samples.

C. Linearity and Recovery

TABLE III
ANALYTICAL LINEARITY AND RECOVERY OF HIPPURIC ACID

Linearity and Recovery	Mean	Standard Deviation (std dev)	Coefficient of Variation (CV)	% Error	% Recovery
Hippuric Acid	1.996	0.008	0.20	0.20	104.54
Benzoic Acid	1.975	0.012	0.60	1.25	98.48

The results of hippuric acid and benzoic acid show the good accuracy and precision. Within run precision of the method was obtained by processing 10 aliquots of pooled urine. The concentration of hippuric acid and benzoic acid were established at 2.0 ± 0.008 and 2.0 ± 0.012. The coefficient of variation both metabolites were 0.20% and 0.60%. The analytical recoveries of hippuric acid and benzoic acid were determined by adding known quantities of hippuric acid and benzoic acid to urine and analyzing. Recoveries illustrated in Table III ranged between 104% for hippuric acid and 98% for benzoic acid.

D. Case Study of Screening Urine Sample of Secondary school

44 students were involved in the screening urine test at Secondary school. A number of 4 students gave positive results for hippuric acid in urine sample while the others gave negative results. At the same time, the National Anti Drug Agency was screening 5 drugs using dip strip kit to the same samples and none of the students are tested positive. The positive hippuric acid samples will be further analysing quantitatively High Performance Liquid Chromatography to confirm that the students are glue abuser.

Under the chromatographic conditions reported at Fig. 7, the sample shows the detection of two components. Before analyzing the samples, the HPLC was calibrated with the mixed standard of 2 mg/ml benzoic acid and hippuric acid. High Performance liquid chromatography spectrum of urine sample 11 shows the presence of benzoic acid and hippuric acid peak. The benzoic acid and hippuric acid is the metabolite of toluene excreted in the urine sample when a person was exposed to toluene or sniffing the glue.

Manabu [10] stated that the amount of hippuric acid level of more than 2.0 mg/ml indicates the high probability level of toluene sniffer and 1.0 to 2.0 mg/ml suggests the possibility of toluene abuse. He also indicates that the normal human body will produce at least 0.10 mg/ml hippuric acid per day. Based on the result in the Table IV, three of the students (11, 15 and 16) have high probability of toluene abuse while one of them is a possible of toluene abuse. Actually, the high amount of benzoic acid is due to the drawback reaction of benzoic acid and glycine to form hippuric acid as shown in Fig. 9.

TABLE IV
RESULT OF AMOUNT OF HIPPURIC ACID USING HPLC SCREENING URINE SAMPLE AT SECONDARY SCHOOL

Sample number	Amount of benzoic acid (mg/ml)	Amount of hippuric acid (mg/ml)
11	3.57	0.05
13	1.40	0.002
15	3.45	0.06
16	5.04	0.14

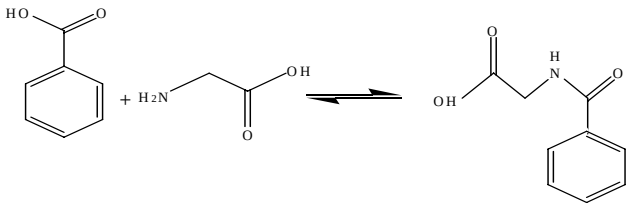

Fig. 9 Benzoic acid and glycine reaction to form Hippuric acid

Other than that, it is because the analysis was not conducted immediately when the urine samples were received. On the other hand, amide group have highly electronegative oxygen atom of the carbonyl group causes a very strong attraction between ion pair of nitrogen electrons and carbonyl group. As a result, the unshared pair of electrons cannot "hold" a proton. Because of the attraction of the carbonyl group for the ion pair of nitrogen electrons, the structure of the C-N bond of an amide is a resonance hybrid. In medium base or acid the amide group will hydrolyze to carboxylic acid and ammonia or an amine [13]. Due to this statement, the drawback reaction will occur in the presence of acidic or bases in urine samples. The amount of hippuric acid can be determined quantitatively based from the amount of benzoic acid. Using simple high performance liquid chromatographic assay for the simultaneous determination of hippuric acid it can be concluded that these 3 students may be a positive glue sniffer.

IV. CONCLUSION

In conclusion, with the rapid and simple High Performance Liquid Chromatography procedure, hippuric acid could be simultaneously determined in a single sample and so could their total or relative concentrations. This method is suitable for measuring the hippuric acid accurately in urine or other biological fluids in clinical chemistry.

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