



# Hippuric Acid Quantification by HPLC Among Inhalant Users

Priyamvada Sharma<sup>1\*</sup>; Vijaya Shree Rao<sup>2</sup>; Sandhya Satyan<sup>2</sup>; Vivek Benegal<sup>2</sup>

<sup>1</sup>Department of Clinical Pharmacology and Neurotoxicology, NIMHANS, Bangalore-560029, India.

<sup>2</sup>Department of Psychiatry, National Institute of Mental Health and Neurosciences (NIMHANS), Bangalore-560029, India.

\*Corresponding Author(s): Priyamvada Sharma

Department of Clinical Pharmacology and Neurotoxicology, NIMHANS, Bangalore-560029, India.

Email: ps842010@gmail.com

Received: Nov 18, 2020

Accepted: Dec 17, 2020

Published Online: Dec 21, 2020

Journal: Journal of Addiction and Recovery

Publisher: MedDocs Publishers LLC

Online edition: <http://meddocsonline.org/>

Copyright: © Sharma P (2020). This Article is distributed under the terms of Creative Commons Attribution 4.0 International License

**Keywords:** Hippuric acid; Toluene; Solvents abuse; Color screening; Liquid Chromatography.

**Abbreviations:** HA: Hippuric Acid; HPCL: Liquid Chromatography Mass Spectrometer.

## Introduction

Inhalant abuse is an intentional gasp of volatile substances for its fast and pleasurable sensory experience. The incidence of intentional inhalation of volatile substances (inhalant use, volatile substance abuse, solvent abuse, glue sniffing) is increasing among adolescent [1]. A three-year survey on self-report of inhalant use among middle and high school students [2] concluded that incidence of intentional inhalation of volatile substances is increasing in young adolescents in USA. Studies on general

## Abstract

**Objectives:** Inhalant sniffing is a fast and sensory experience to abusers. Low price, easy access, fast and enjoyable sensory experience makes it popular among youngsters. Despite the substantial prevalence and serious toxicities of inhalant use, most of the diagnosis is made on self-report. This work aimed to detect the direct markers of solvent abuse in biological matrix.

**Material and methods:** Initial screening for solvent exposure was done with color reaction, positive and suspected samples were further analyzed for Hippuric Acid (HA) quantification using Clin Tox complete Kit from Recipe.

**Results:** Solvent users (n= 23) who reported use in last few hours (2-3) had HA levels were  $6690 \pm 3.4 \mu\text{g/ml}$  mg/ml. It was  $87 \pm 0.17 \mu\text{g/ml}$  among abstainers (n= 18). HA levels and  $74 \pm 0.29 \mu\text{g/ml}$  among healthy controls (n= 17). This work presented two-point procedure having screening and quantification for solvent use.

**Conclusion:** Inhalant abuse remains dangerous behavior among young adults, due to euphoria effect it is addictive in nature. Its acute effects include sudden sniffing death syndrome asphyxia and serious injuries. Diagnosis of solvent inhalant use entirely relies on thorough history. This work presents specific laboratory measure in terms of screening and quantification to detect solvent use.

population also yielded prevalence rates of 5-15% in United States, 3-6% in Canada and 6-7% in United Kingdom [3,4]. A nationwide survey conducted in Korea reported that more than 90% of male teenagers and over 60% of female teenagers have at least sniffed glue once in their lifetime [5]. Glue sniffing is a problem in South-East Asian countries specially among school going adolescent. Malaysian media has mentioned glue sniffing as "time bomb" disasters for future generation, when few



**Cite this article:** Sharma P, Rao VS, Sathyan S, Benegal V. Hippuric Acid Quantification by HPLC Among Inhalant Users. J Addict Recovery. 2020; 3(1): 1023.

teenagers were found dead, in Kuala Lumpur school and police recovered three empty glue cans beside them [6]. In India, inhalant abuse emerged only a few decades back, but has shown a rapid upsurge, especially among the urban youth. National

Mental Health survey (2016) [7] also revealed that 0.6% of the 18 plus population were recognized with illicit substance use disorder which included cannabinoids, opiates inhalant and prescription drugs [8].

**Table 1:** Commonly Abused Inhalant Products and Their Constituents (Sharp, Rosenberg 2005).

<b>GLUES AND ADHESIVES</b>	
Airplane glue	Toluene, ethyl acetate
Other glues and cements	Hexane, toluene, methyl chloride, acetone, methyl ethyl ketone, methyl butyl ketone, benzene, xylene, trichloroethylene, tetrachloroethylene, chloroform
<b>AEROSOLS</b>	
Spray paint	Butane, propane (U.S.), Fluorocarbons, toluene, hydrocarbons, xylene
Hair spray	Butane, propane (U.S.), chlorofluorocarbons
Deodorant; air freshener	Butane, propane (U.S.), chlorofluorocarbons
Analgesic spray	Chlorofluorocarbons
Asthma spray	Chlorofluorocarbons
Fabric spray	Butane, trichloroethane
PC cleaner	Dimethyl ether, hydrofluorocarbon
Video head cleaner	Ethyl chloride
<b>ANESTHETICS</b>	
Gaseous	Nitrous oxide
Liquid	Halothane, enflurane, desflurane, isoflurane
Local	Ethyl chloride
<b>CLEANING AGENTS</b>	
Dry cleaning	Tetrachloroethylene, trichloroethane
Spot remover	Xylene, petroleum distillates, chlorohydrocarbons
Degreaser	Tetrachloroethylene, trichloroethane, trichloroethylene
Lacquer; thinners	Acetone, methanol, ethyl acetate, methyl chloride, toluene
<b>SOLVENTS AND GASES</b>	
Nail Polish remover	Acetone, ethyl acetate, toluene (rarely)

The components used for sniffing includes typewriter correction fluid, diluents, glues, nail polish and ink remover. These are freely available at low cost have an easy accessibility to youngsters. Correction fluid has been reported to be the common inhalant abused in India [9]. Another survey from Indian context reported 77 lakhs solvent users in India out of which 22 lakhs are dependent users and 8 lakhs were problematic users [4,10]. Inhalants can be abused by sniffing, huffing and bagging [11,12]. NMDA and GABA receptors react to presence of toluene similarly to those of alcohol [13]. Toluene increases opiate receptors in the Nucleus Accumbens and gives the feeling of pleasure. The low concentration (400-5000 µg/ml) of toluene produces transient euphoria that makes the user prone to accidents. High concentration (6000-15000µg/ml) of solvents having toluene leads to dizziness, sleepiness blurred vision and headaches. User also reported feeling of confusion, unsteady gait and hallucinations. Higher doses result in seizures, coma and cardiopulmonary arrest [14,15]. Toluene absorbed through the respiratory track get oxidized by liver microsomes mainly (68%) to hippuric acid. The use of solvents had adverse health effects, risk of developing other addiction as well as psychiatric co-morbidity associated with the practice of inhalant [12].

It is difficult to carry out diagnosis as well as management of solvent abuse without any testing. The physician carries out a blood count and acid base assessment to assess complications of solvent use. Globally there are no clearly effective diagnos-

tic procedure for the harm reduction and treatment of solvent abuse. A few attempts have been made internationally to develop paper-and-pencil screening assessments of inhalant use, but these instruments are of limited utility. Howard et al prepared Volatile Solvent Screening Inventory (VSSI) [16] and Comprehensive Solvent Assessment Interview (CSAI) [17]. These tests are easily available and requires 20 (approx.) minutes to complete, and assesses past-year and lifetime frequency of use of 55 inhalant chemicals and products. The application of these tests in Indian context is limited [18].

In the absence of definitive solvent abuse measurement technique clinical judgment is made on self-report and patient history. Efforts are under way to improve laboratory diagnosis of inhalant use and abuse [19], but such tests are not yet widely available, nor have they been implemented in routine clinical practice. Findings from the occupational toxicology and inhalant abuse literature suggest that bioassays for hippuric acid, o-cresol levels, and benzyl mercapturic acid may eventually be useful urinary markers of toluene abuse [20]. Thus, there is a need to detect the direct markers of solvent abuse in biological matrix. Research done in past has shown that Hippuric Acid (HA) in urine is one of the most used and easiest way to monitor exposure to solvents.

The novel color screening procedure developed by Gaffney et al [21] and modified by Yoshita [22] could be applied for pre-

liminary screening of solvent exposure. The aim of this work was to describe a procedure having ability to screen recent inhalant use, and facilitate biochemical confirmation.

## Material and methods

Samples were received from the subjects attending the inpatient and outpatient tertiary specialty service from Jan 2018 to Feb 2019. Subjects with solvent abuse history above 18 years were included in the study. Subjects with alcohol abuse and with other psychiatric comorbidities were excluded. Ethics permission from Institute Ethics committee along with patient consent were obtained.

Sampling was convenient stratified while study design was cross sectional, participants were in the age group of 18-45 years. The sample included twenty-three (21 Male & 2 Female) active users with history of solvent use in past 2-6 hours. The seventeen abstainers had no solvents exposure in 24-72 hours. Healthy controls were eighteen (12 Male & 6 Female) and had no exposure to solvents.

## Sample collection and transportation

Urine was the preferred biological specimen for toluene-based inhalants testing because of its availability in adequate quantity and noninvasive collection [13]. Sample collection (2-5 ml) was done in a hygienic, dry and leak proof container and each sample was accompanied with a requisition form enclosing patient details. Sample integrity for creatine, nitrite, glutaraldehyde, oxidant, pH and specific gravity was tested by adulteration strips (ABON, Inc.), qualified samples were considered for HA analysis [9] and were stored at -20°C till analysis.

## Standards and reagents

All chemicals were of analytical grade. Hippuric Acid (HA) 98%, Benzene Sulphonyl Chloride (BSC) 98%, Toluene 99.3% and Pyridine 98% were procured from Sigma Aldrich, USA. For Milli-Q-Plus, ultra-pure Water System (Millipore USA) was used. Control urine lyophilized samples were purchased from Medi-chem Germany. Hippuric Acid quantification was done using Clin Tox complete kit (52000) for HPLC quantification of Hippuric acid and its metabolites.

## Statistical analysis

For analysis of the data one-way ANOVA and Bonferroni multiple-comparison test was applied.

## Sample preparation

To separate positive samples from the negative one screening was done by the procedure described earlier [14,15]. Positive sample and sample with suspected use were further processed for quantification using Clin Tox HPLC complete kit from Recipe as per the manufacturer instructions.

Before HPLC analysis sample preparation was necessary, for that 100 µl of urine equal was mixed with equal volume of internal standard (52012) followed by 2 ml of reagent B (52022). After pretreatment urine samples were subjected to Solid Phase Extraction (SPE) using sample preparation columns (523320). SPE procedure included conditioning, sample loading, washing and elution with two ml of reagent A, followed by HPLC analysis. Agilent 1100 series HPLC with autosampler (DE 91611662) and UV detector was used for HA analysis. Twenty µl of sample was injected (induplicate) and chromatographic separation was obtained on analytical column (52330) using mobile phase

(52010) in isocratic mode. The flow rate was maintained at 1.0 ml/min and absorbance was measured at 230 nm and run time was eight minutes.

## Results

The standard solution (52011) with analyte concentration, was used for qualitative check-up of the HPLC system (integration parameters, retention times, peak separation and shape. Urine calibrator (9922) having weighed analyte amounts served as a test-calibration. (Figure 1).

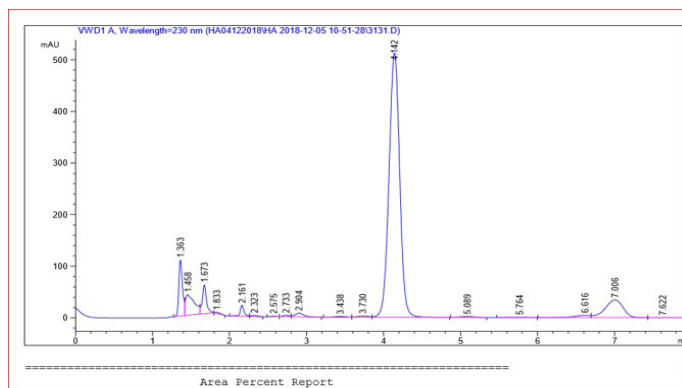


Figure 1: Chromatogram showing peaks of Hippuric Acid.

Sample chromatogram was compared with the urine calibrator chromatogram to check the peak representing analytes corresponds to the retention time. The concentration of unknown samples was calculated using internal standard method via peak areas with the help of equation given below-

$$\text{Concentration (sample } \mu\text{g/ml)} = \frac{\text{area (sample)} \times \text{conc (standard)} \mu\text{g/ml}}{\text{Area (calibrator)} \times \text{recovery}} \times 20$$

## Sample stability

Ten individual samples of urine were used to assess analyte stability and we reported 8-12 hour sample stability at 4-8°C. All urine samples were screened for creatinine, specific gravity, Nitrite, Glutaraldehyde, pH and oxidant/pyridinium chlorochromate. Low creatinine and specific gravity may indicate dilution of urine while pH outside the range of 4-9 indicate the altered samples. Samples having creatinine values between 0.8 to 1.2 mg/dl were only considered for the study.

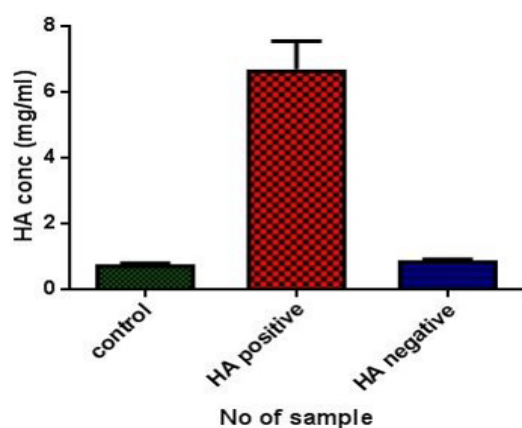
Color test screening by Color test [22] and quantification by ready to use Clin Tox HPLC complete kit from Recipe made current application useful in clinical setting. In chromatographic determination an internal standard was used to calculate correction of losses through different steps of sample treatment. It is widely accepted that greater similarity of physical and chemical properties of the internal standard, results in greater reliability of the results. For the assessment of exposure to the solvents containing toluene the quantitative determination of hippuric acid along its other analytes o-, m-, p-methyl hippuric acids in urine by HPLC proved useful. Since hippuric acid is a physiological component of urine its excretion amount underlies interindividual differences due to the ingestion of benzoic acids used as a preservative in several foods and drugs [9,12]. Thus, the mean normal value for the urinary excretion of hippuric acid in non-exposed person is 1.0 mg/ml. For this reason, screening by color reaction and quantification by HPLC results in determination HA and its metabolites is a way to estimate the toluene exposure.

Among fifty-eight samples analyzed, twenty-three solvent users had mean HA conc.  $6690 \pm 3.4 \mu\text{g/ml}$ . For seventeen healthy and non-exposed controls mean HA was  $74 \pm 0.29 \mu\text{g/ml}$ , abstainers had a mean HA conc. of  $87 \pm 0.17 \mu\text{g/ml}$ , and did not differ much from healthy controls (graph-1, 2). Creatinine ranged  $0.8$  to  $1.2 \pm 0.99 \text{ mg/dl}$  among all the groups.

## Discussion

The current work demonstrates simple on spot screening procedure followed by quantification HA in urine. Researcher has used Gas Chromatography (GC) for determination of hippuric acid, GC analysis require a derivatization procedure in the sample pretreatment step. Some researchers used diazomethane which is an explosive, carcinogenic and highly toxic reagent, other researchers used methylsilyl derivatives that are expensive [23]. The study also discussed Liquid-Liquid Extraction (LLE) for estimation of Hippuric Acid (HA) in urine as a biomarker of the toluene exposure by high-performance liquid chromatography equipped with photodiode array detector (HPLC-PDAD). But with LLE reproducibility of the method remains dubious and HPLC-PDAD again increases the running cost [24].

Usefulness of current application was confirmed on fifty-eight samples. Solvent users were ( $n=23$ ) who have reported use in the last few hours (2-3), while abstainers were ( $n=18$ ), had past history of use but currently were maintaining abstinence, and healthy controls ( $n=17$ ) were also included in study. Solvent users positive by color screening were confirmed by HPLC analysis HA levels  $3000.0$  to  $15,000.0 \mu\text{g/ml}$  (mean  $6690 \pm 3.4 \mu\text{g/ml}$ ). Healthy controls and abstainers showed no color conversion and there was not much variation in HA levels. For healthy controls HA values were  $74 \pm 0.29 \mu\text{g/ml}$  while for abstainers it was  $87 \pm 0.17 \mu\text{g/ml}$  (graph 1). We obtained an excellent relationship between the peak area and the concentrations of HA standards prepared. The same procedure was performed in the urine and excellent recovery was obtained.



**Graph 1:** Hippuric Acid concentration among Subject, Abstainers and Control group.

On comparing users urinary HA levels, the difference between the solvent user and healthy control group were statistically significant ( $\leq 0.05$ ), while among healthy controls and abstainers' results showed no statistical significance.

Research reported variations in urinary HA levels in addition to the toluene abuse can be caused by dietary factors, medical treatment as well as alcohol consumption [23]. For specificity of current method for toluene intoxication calibration range of HA was kept on higher side as mentioned by Poggi et al [25].

HA values should be creatine adjusted or unadjusted scientist always had disagreement on this. Research has reported that creatinine unadjusted and adjusted values of HA were closely correlated and well linked to the unadjusted one because HA levels were partially independent of urinary volume and flow rate [24]. Nicolli A [26] also reported that creatinine unadjusted and adjusted values of HA were closely correlated but HA was well linked to the unadjusted one, because HA levels were independent of volume and flow. In the current work also urine samples were screened for creatinine, specific gravity, Nitrite, Glutaraldehyde, pH and oxidant/pyridinium chlorochromate. The sample having any abnormality were excluded from the study. Since, adjustment of crude hippuric acid values remains almost same, either unadjusted or adjusted to specific gravity and creatinine we reported HA with creatine unadjusted values.

G.S. Kit [24] was developed for on-spot inhalant abuse screening in Malaysia where sniffing was a major problem among youngsters. G.S. kit showed color conversion from yellow to red after adding small quantity of urine sample. He quantified positive samples using UV-Vis at 417 wavelength and values were above  $2.0 \text{ mg/ml}$ . We also report color screening was simple, effective and was able to distinguish between the high values HA from the normal/low values [12,24] with in few minutes. But we observed color of reaction mixtures fades gradually, so results of semi quantification should be read within ten minutes. Suspected samples were quantified by HPLC. Screening by color reaction followed by quantification by HPLC is complementary to each other and applicable in clinical setting for patient care. The limitations observed in the form of assessment of other metabolite of solvent and monitoring of ethyl benzene were not carried out.

**Financial Support and Sponsorship:** This work was supported by the Centre for Addiction Medicine NIMHANS, Bengaluru, Karnataka, India.

**Data Sharing Statement:** De identified individual participant data will not be made available.

## Conclusion

We report screening of solvent abuse by color test followed by confirmation by HPLC provided a laboratory diagnosis solution for solvent abuse detection.

## References

1. Flanagan RJ, Fisher D S. Volatile Substance Abuse and Crime: Data from UK Press Cuttings 1996-2007. *Med. Sci. Law* 2008; 48: 295–306.
2. Broussard L A. The Role of the Laboratory in Detecting Inhalant Abuse. *Clin Lab Sci Bethesda* 2000; 13: 205–209.
3. Lubman DI, Yücel M, Lawrence A J. Inhalant Abuse among Adolescents: Neurobiological Considerations. *Br. J. Pharmacol.* 2008; 154: 316–326.
4. Kurtzman T L, Otsuka K N, Wahl R A. Inhalant Abuse by Adolescents. *J. Adolesc. Health* 2001; 28: 170–180.
5. Park S, Kim Y. Prevalence, Correlates, and Associated Psychological Problems of Substance Use in Korean Adolescents. *BMC Public Health.* 2016; 16: 79.
6. Chan YF, Sidhu GK, Lim PC, Wee EH. Students' Perceptions of Substance Abuse Among Secondary School Students in Malaysia. *Pertanika J. Soc. Sci. Humanit.* 2016; 24: 2-8.



7. Murthy RS. National Mental Health Survey of India 2015–2016. *Indian J. Psychiatry*. 2017; 59: 21.
8. Pradeep BS, Gururaj G, Varghese M, Benegal V, Rao G N, Sukumar GM, Amudhan S, Arvind B, Girimaji S, Vijayasagar KJ. National Mental Health Survey of India, 2016-Rationale, Design and Methods. *PLoS One*. 2018; 13: 10 e0205096.
9. Jain, R, Verma A. Laboratory Approach for Diagnosis of Toluene-Based Inhalant Abuse in a Clinical Setting. *J. Pharm. Bio allied Sci*. 2016; 8: 18-22.
10. Sah SK, Neupane N, Pradhan A, Shah S, Sharma A. Prevalence of Glue-Sniffing among Street Children. *Nurs. Open*. 2020; 7: 206–211.
11. Bowen S, Cruz S L Inhalants: *Addict Sci Clin Pract*; 2011: 6: 18–31.
12. Sharma P, Hegde S, Murthy P, Benegal V. Biochemical analysis of hippuric acid as a marker of solvent exposure among substance users. *EPJMR*: 2017: 04; 359-363.
13. Dick ALW, Pooters T, Gibbs S, Giles E, Qama A, Lawrence AJ, Duncan, J. R. NMDA Receptor Binding Is Reduced within Mesocorticolimbic Regions Following Chronic Inhalation of Toluene in Adolescent Rats. *Brain Res*. 2015; 1624: 239–252.
14. Williams JM, Stafford D, Steketee JD. Effects of Repeated Inhalation of Toluene on Ionotropic GABAA and Glutamate Receptor Subunit Levels in Rat Brain. *Neurochem. Int*. 2005; 46: 1–10.
15. Kozel N, Sloboda Z. Epidemiology of Inhalant Abuse: An International Perspective, *NIDA Res Monogr*. 1995; 148: 1-306.
16. Howard MO, Bowen S E, Garland E L, Perron B E, Vaughn M G. Inhalant Use and Inhalant Use Disorders in the United States. *Addict. Sci. Clin. Pract*. 2011; 6: 18-31.
17. Perron BE, Howard MO. Adolescent Inhalant Use, Abuse and Dependence. *Addiction* 2009; 104: 1185–1192.
18. Menon P G, Rani A, Jaisooriya T S. Solvent Use and Its Management-An Overview. *Kerala J. Psychiatry*. 2015; 28: 42–51.
19. Chakroun R, Faidi F, Hedhili A, Charbaji K, Nouaigui H, Laiba MB. Inhalant Abuse Detection and Evaluation in Young Tunisians. *J. Forensic Sci*. 2008; 53: 232–237.
20. Fustinoni S, Mercadante R, Campo L, Scibetta L, Valla C, Consonni D, Foà V. Comparison between Urinary O-Cresol and Toluene as Biomarkers of Toluene Exposure. *J. Occup. Environ. Hyg*. 2007; 4: 1–9.
21. Gaffney GW, Schreier K, Di Ferrante N, Altman K I. The Quantitative Determination of Hippuric Acid. *J. Biol. Chem*. 1954; 206: 695–698.
22. Yoshida M, Akane A, Mitani T, Watabiki T. Simple Colorimetric Semiquantitation Method of Hippuric Acid in Urine for Demonstration of Toluene Abuse. *Leg. Med*. 2005; 7: 198–200.
23. Yadav A, Basu A, Chakarbarti A. Method for Estimation of Hippuric Acid as a Biomarker of Toluene Exposure in Urine by High-Performance Liquid Chromatography after Extraction with Ethyl Acetate. *Environ. Dis.* 2019; 4: 17-22.
24. Zinalibdin MR, Yacob A. R. Detection of Hippuric Acid: A Glue Solvent Metabolite, Using a Mobile Test Kit. *Arab. J. Chem*. 2013; 6: 115–120.
25. Poggi G, Giusiani M, Palagi U, Paggiaro P L, Loi A M, Dazzi F, Siclari C, Baschieri L. High-Performance Liquid Chromatography for the Quantitative Determination of the Urinary Metabolites of Toluene, Xylene, and Styrene. *Int. Arch. Occup. Environ. Health* 1982; 50: 25–31.
26. Nicolli A, Chiara F, Gambalunga A, Carrieri M, Bartolucci GB, Trevisan A. Reliability of Urinary Excretion Rate Adjustment in Measurements of Hippuric Acid in Urine. *Int. J. Environ. Res. Public. Health*. 2014; 11: 7036–7044.