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BIOCHEMICAL ANALYSIS OF HIPPURIC ACID AS A MARKER OF SOLVENT EXPOSURE AMONG SUBSTANCE USERS

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ABSTRACT

Background: Inhalant sniffing offers fast and intense sensory experience to abusers which also contribute to its excessive use. Users can be an active user or have frequent exposure to solvents. **Objective:** A biochemical procedure for detection and quantification of hippuric acid as marker of solvent exposure (toluene, paint, thinner, eraser, inhalers) in urine is described and its effectiveness in clinical samples was also demonstrated. **Methods:** Simple chemical reaction for hippuric acid detection and quantification was improvised for clinical application, visual detection as well as quantification by wavelength reader was viable. Solvent exposures (intentional /occupational) among alcohol and drugs users were tested. **Results:** Hippuric acid levels were screened for 194 subjects, among them 46 had history of alcohol use, 33 were opiate users, 74 were cannabis user, 7 had history of poly substance use and 17 were benzodiazepine users. Fourteen subject with solvent use history and ten healthy volunteers also participated. Association between solvent contact and hippuric acid excretion was observed. Validation parameters for recovery ≥100%, accuracy 2.50, coefficient variance (r^2), 0.998 were in acceptable range. **Conclusion:** Hippuric acid, a valid biomarker for solvent exposure has treatment implications also. Current study demonstrates its screening and quantification potential without being affected by diverse abuse patterns of user. This screening procedure for solvent exposure is cost and time effective and hold good sensitivity for further detection of other metabolites of solvent exposure in positive samples can be clubbed with other chromatographic techniques.

KEYWORDS: Hippuric acid, Colorimeter, Enzyme Linked Immuno Sorbent Essay (ELISA), plate reader.

INTRODUCTION

Solvent use among teenagers and adults has been on increase around the world in late nineteenth century. Inhalants are non-medically used ingredient in household or industrial chemical products. These are not intended to be concentrated or inhaled. Inhalants can be abused by sniffing (direct inhalation from a piece of clothing sprayed with the substance), huffing (holding soaked cloth over nose or mouth) and bagging (breathing from a plastic bag containing volatile substance to increase concentration).[1] The substances abused include paint thinner, nail polish remover, petrol, gasoline, correction fluid and glue that contain toluene.

Inhalant abuse is the intentional gasp of volatile substances because of their fast and pleasurable sensory experience.[2] More than 90% of male teenagers and over 60 % of female teenagers have at least sniffed glue once in their lifetime. These substances give an intoxicating high to users and this euphoria is the cause for addiction high to users and this euphoria is the cause for addiction.[3] Toluene is most documented substance inhalant has emerged as growing concern among young adults and teenagers in developing countries.[4] In the absence of definitive diagnostic tests it is difficult to identify their use and that makes management non-existent equally difficult.

Toluene is an organic solvent used in printing, painting, automotive shoe making and other pharmaceutical industries. Toluene has low boiling point, is flammable in nature and evaporates easily. Young people start solvent abuse by inhaling vapours many times (15-20). Inhaled toluene reaches brain within minutes. In the brain NMDA and GABA receptors produce similar effects to that of alcohol.[5] Toluene, increases opiate receptors in the Nucleus Accumbens and gives the feeling of pleasure. Low concentration (0.4-5.0 mg/ml)

produces transient euphoria and confusion that make the user prone to accidents. At high concentrations (6.0-15.0 mg/ml) the user can experience dizziness, sleepiness blurred vision and headaches. User may feel confused unbalanced and experience hallucinations. Higher doses result in seizures, coma and cardiopulmonary arrest.

Exposure to toluene (paint, thinner, glue and nail polish remover, printing and leather tanning industry workers) can lead to serious health consequences. Its chronic effects on central nervous system include neuropsychosis, cerebral and cerebreller degeneration, seizures, choreoathesis, optic and peripheral neuropathies, decreased cognitive ability, optic atropy, blindness and ototoxicity.[5] It affects heart through cardiac automaticity and conduction. Prolonged toluene exposure results in hepatotoxicity.[6] Renal toxicity from toluene exposure includes renal tubular acidosis, hypokalemia, hypophosphatemia, hyperchloremia, azotemia, sterile pyuria, hematuria and proteinuria. Hematologic consequences may include lymphocytosis, macrocytosis, eosinophilia, hypochromia, basophilic stippling and aplastic anaemia.[7]

Many products including paints, thinner and adhesive contain toluene in them and primarily excreted as hippuric acid within 24 hours of exposure. Thus detection of urinary HA would be a good screening test to detect recent and heavy solvent exposure. Various sophisticated instruments (GC-MS, LC-MS)[8] can be employed for confirmation of HA in biological matrices, but for screening simple and sensitive method is need of time. High end instruments are expensive, time consuming and trained technical staff is required for handling. Colorimetric method would be very useful for rapid screening of HA in urine.

The first colour reaction to test Hippuric acid using pyridine and benzene sulphonyl chloride was developed by Umberger and Fiorese.[9] It was further modified and by adding distilled water colour change could be observed with naked eye.[10] But had limitations in terms of its semi-quantitative nature and clinical utility.

The current study presents improved colorimetric method for detecting toluene exposure. Quantification of urinary hippuric acid was done using wavelength detector (ELISA reader), visual detection as well as semi-quantification by colour card would be possible.

MATERIAL AND METHODS

All chemicals were of analytical grade. Bulk solvents and routine chemicals were procured from SISCO research laboratory (Mumbai, India). Hippuric Acid (HA) 98%, Benzenesulphonyl chloroide (BSC) 98%, Toluene 99.3 % and Pyridine 98% were purchased from Sigma Aldrich, USA. Standard stock solutions of Hippuric acid of 6.0 mg/ml strength were prepared in double distilled water and other reagents were ready to use. Uncoated ELISA (Enzyme Linked Immuno Sorbant Assays) plates were procured from Tarson.

Sample Collection

Laboratory tests including blood sugar, urea, liver function test, breath alcohol and Ethyl Glucuronide and Ethyl sulphate are offered as part of the clinical evaluation at the Centre for Addiction Medicine at the National Institute of Mental Health and Neurosciences (NIMHANS), Bangalore Karnataka, India. Samples were collected from referred subjects showing willingness to undergo toxicological investigation attending Out Patient Department of CAM, it is a secondary data and study design is retrospective. Normal healthy controls were willing for toxicological investigations were selected from NIMHANS and had no drug abuse or solvent exposure history. The study was conducted from Dec 2014 to Sept 2015. Requisite permission including institution ethic was obtained. We screened 194 samples belonging to different user groups (Alcohol, Cannabis, Opiates, Benzodiazepines, Multidrug and solvent users) to rule out false results due to cross reactivity with other drugs (Table-1). Solvent users were (n=14) against which healthy controls (n=10) were recruited.

Standard and sample Preparation

For testing patient sample one ml urine sample was centrifuged at 5000 rpm for five minutes. Fifty micro liter (µl) of sample was transferred to uncoated ELISA well followed by 50 µl Benzenesulphonyl chloride (BSC 98%) and 100 µl distilled water. Finally 100 µl of Pyridine (98%) was added drop by drop color change from yellow to red was immediate in case of solvent exposure, absorbance was taken at 510nm within ten minutes using Tecan ELISA plate reader.

HA stock solution (6.0 mg/ml) was prepared in ultra pure water. Calibration curve was in the range of 1.5, to 6.0 mg/ml of hippuric acid, and absorbance was taken at 510nm. Color card is displayed with Figure-1, could successfully calculate the approximate values without taking absorbance.

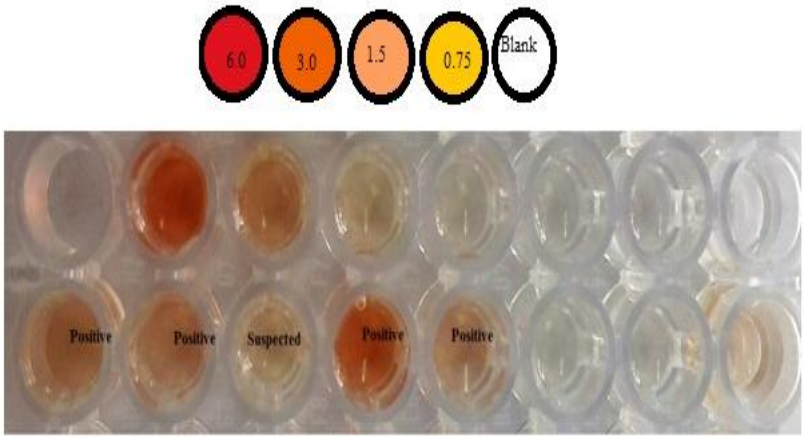

Figure: 1 Colour Chart and ELISA well showing Hippuric acid analysis

RESULTS

Hippuric acid is the major urinary metabolite of toluene, a volatile organic compound (VOC) that accompanies benzene in almost all sources of exposure either on workplace or due to lifestyle. Toluene is metabolized to benzoic acid, which conjugates with glycine and forms hippuric acid. Hippuric acid is the used as indicator for bio monitoring of toluene exposure.

HA can be present in range of 0.1µg/ml to about 1.5 µg/ml in urine samples persons non-exposed to toluene as it is by product of metabolism of common nutrients specially fruits. Analytical techniques and its cut off limits should be sensitive enough to differentiate various exposures.

Screening of solvent abuse was done by simple chemical reaction which takes place between BSC, pyridine and HA. Chloride of BSC reacts with amine/hydroxyl group of HA and forms ester/ether. Pyridine acts as acid indicator and gives deep red colour in the presence of HA, distilled water is added to enhance the colour intensity of reaction. In the absence of HA acid reaction mixture remained colourless, while in the presence of HA reaction mixture becomes red colour intensity was proportional to the concentration of HA in urine sample (Fig 1). For quantification absorbance was taken using Tecan Infinite M 200PRO ELISA reader. Lesser sample and reagent volume made it easy to carry out experiment in uncoated ELISA wells, for quantification absorbance at 510 nm provided good recoveries (99-105%).

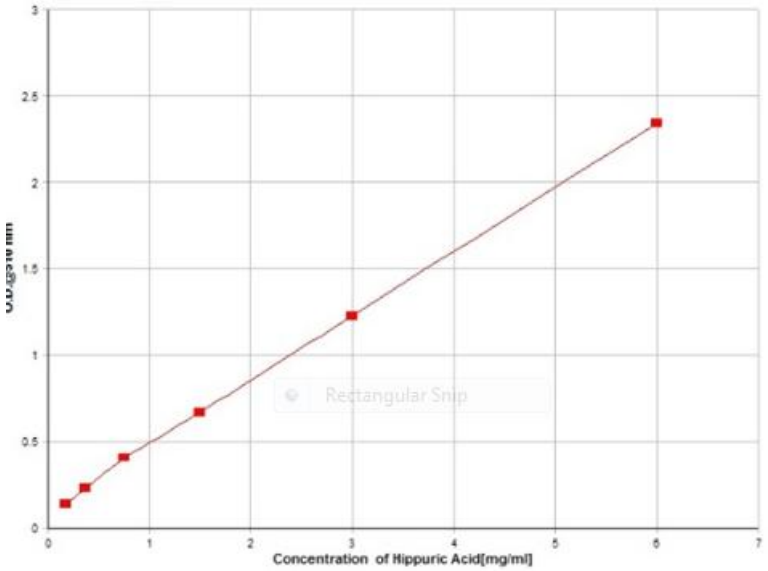

Figure: 2 Confidence limits of analytical method to determine Hippuric Acid

Method Validation

HA measurements were done in triplicate, and the arithmetic means of the three measurements were used in the analysis. Intra and inter day assay precision, expressed as CV, were 3.8% and 5.8%, respectively, at a HA concentration of 1.84 mg/ml. Quantification was

done in the concentration range of 0.75-6.0 mg/ml and r^2 was 0.998. Limit of detection (LOD) and Limit of Quantification (LOQ) for (n=10) was 10.0and 50.0µg/ml respectively.

Among healthy controls (n=10), HA values were less than ≤1.5 mg/ml (mean 0.79±0.29) of hippuric acid, in solvent abuse (n=14) case results varied from 2.0-6.0mg/ml (mean 3.5±1.79). HA values falling out of calibration range were diluted and quantified, in cases where user is unaware of solvent type HA presence indicate toluene exposure.

Analysis was performed using SPSS (Statistical Package for Social Sciences) showed users in different groups (above cut-off only) had statistically significant mean value ≤ 0.001 against control 0.79±0.29 and results were statistically significant p ≤0.001.

DISCUSSION

Benzoic acid is an important source of HA production. Benzoates are used as preservatives for food products (fruit juice, ketchup lemonade) because of its antimicrobial effect against yeast and moulds and bacteria**.** Studies have shown increase in HA after contain benzoic acid.[11] Keeping this in view the HA cut-off was ≤1.5 mg/ml for occupational or accidental exposure while ≥2.0mg/ml considered as intentional exposure. while ≥2.0mg/ml considered as intentional exposure. For more research and bio monitoring of toluene exposure diet restriction (food and drinks containing benzoate) should be taken into consideration.[12]

Following inhalation 60-75% of absorbed toluene is converted to benzoic alcohol by cytochrome P450 enzymes. Benzyl alcohol is oxidized to benzoic acid by alcohol dehydrogenase and aldehyde dehydrogenase. The principle metabolite of it is benzoic acid. Benzoic acid on conjugation with glycine forms hippuric acid[8] its excretion completed in 24 hours.[13,10]

The colorimetric method provide higher HA values compared to other chromatographic techniques, as colorimetric detection covers additional glycine conjugates of benzoic acids such as hydroxyl hippuric acids and methyl hippuric acid, while techniques like GC-MS and LC-MS detects only targeted compound .

Hippuric acid is an indicator for bio-monitoring of toluene exposure. There are reports on interaction of alcohol and other substances with toluene metabolism which could affect its metabolism, like chronic consumption causes stimulating effects while acute consumption leads to inhibiting effect.[6]

Fourteen persons (table-1) with solvent use history were analyzed eight had HA levels above cut-off (1.50mg/ml) mean 4.6±1.37, in rest HA levels were ≤ 1.50mg/ml had solvent exposure 1-2 days back. Among controls HA levels were within range (mean 0.75±0.31).

Table: 1 Distribution of person with solvent exposure

Substance Used	Users with above cut off (1.5mg/ml)	Mean/ S.D.
Alcohol (n=46)	6	2.3±0.48
Cannabis (n=74)	5	3.01±0.67
Opiates (n=33)	3	3.06±0.28
Benzodiazepines (n=17)	0	0
Multidrug use(7)	7	4.01±0.32
Solvent users (n=14)	8	4.5±1.79
Control (n=10)	0	0.79±0.29

Interference due to ethanol and its metabolites with HA was scrutinized among forty six alcohol user (last use 36 hours), Ethyl glucuronide (EtG)[14] levels ranged from 350-11,000ng/ml. Six patients had HA values above cut-off (mean2.3± 0.48), further investigation concluded three had occupational exposure (HA ≥2.0 mg/ml), two had history of solvent inhalation and in one patient HA values were ≥ 2.0 had not reported any occupational or intentional exposure.

Among the 74 subjects cannabis use history, five had HA levels above cut-off 3.01±0.67. Three were positive for cannabis use and had solvent exposure also. Combination of alcohol and cigarette reduces HA excretion is reported[15] but cannabis influence on HA metabolism and excretion is still needs to be investigated.

Among thirty three persons with opiate use three had HA level above cut off (mean 2.1±0.67) and were suspected for occupational exposure (painter). Among 17 persons with benzodiazepine use none had history of solvent exposure and HA values were ≤ 1.0mg/ml.

Use of beverages and its effect on HA excretion is unknown Munaka etal mentioned green tea and coffee can leads to overestimation of urinary HA concentrations and cause false-positive results during the biological monitoring of workers exposed to low doses of toluene.[16]

Overall results indicate viability of biochemical testing for inhalant exposure. Sample collection was easy and non invasive visual detection predicts results within few minutes and use of plate reader produced numeric value

in few minutes. Further research could be planned for establishing benzyl Glucuronide, O and para cresol for their potential role as a confirmatory marker of inhalant abuse using GC-MS or LC-MS. In conclusion we report this method is suitable for screening of solvent exposure and can handle number of samples effectively.

The study had certain limitations in terms of sample size which could be large especially in control group. Though morning sample was collected yet dietary details were not recorded. It would be worthwhile to conduct a similar study on a larger sample size and include other confirmatory metabolites with sensitive technique.

Conflict of interest
None.

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