

UNUSUAL FINDING OF NITRAZEPAM A HYPNOTIC DRUG IN ALCOHOLIC BEVERAGE (TODDY) BY MASTER GAS CHROMATOGRAPHY

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ABSTRACT

Under Narcotic Drug and Psychotropic substances Act 1985, Forensic Science laboratories in Maharashtra state are receiving number of cases. Drugs like Analgesics (paracetamol, aspirin), Sedative drugs (phenobarbital, caffeine); Tranquilizers (Diazepam, Lorazepam), Anxiolytic drug (alprazolam), Hypnotic drug (Nitrazepam) etc. were also used as additives in alcoholic beverages like toddy to increase its effect and hence for large gain. In one of the cases, toddy samples were referred to forensic science laboratory for chemical analysis. Laboratory examination proves that the sample was adulterated with Nitrazepam, a tranquilizer drug which is harmful to human beings. Nitrazepam was detected by HPTLC method and was quantitatively determined using gas chromatography. For separation of Nitrazepam by HPTLC method, Glacial acetic acid: water: methanol and dichloromethane (1:6:30:70) was used as a solvent system which is specific, cost effective and reproducible. Although benzodiazepines are generally considered safe but are dangerous with alcohol. The lowest limit of detection for HPTLC was 0.1 ppm.

Keywords: Additive, Nitrazepam, Toddy, Forensic and Master GC.

INTRODUCTION

Toddy is a sweetish, heavy, milky white, vigorously effervescent alcoholic beverage consumed as mildly alcoholic beverage similar to wine which is collected from palm tree. During ancient period, people drink palm wine in early morning in empty stomach before breakfast for health benefits. Palm wine has a special place in traditional celebrations and ceremonies such as marriages, burials and settling disputes. Generally, it is a popular drink among lower income people and it is believed to be good for the health, eyesight and also serves as a sedative¹ It is made from the fermented sap of tropical plants of Palmate family, such as the coconut palm (*Cocos nucifera*), oil palm (*Elaeis guineensis*) date palm, nipa palm, kitul palm and raffia palm (*Raphia hookeri*)²⁻⁴. The palm wine undergoes lactic, alcoholic and acetic fermentation involving lactic acid bacteria, yeast and acetic acid bacteria as well as *Zymomonas* and *Leuconostoc*. The types and numbers of organisms encountered vary widely, even from tree to tree⁵. The main ingredient of the fresh palm sap is sucrose, which is about 12-15% by weight. There is very little reducing sugar, although glucose, fructose, maltose and raffinose are present. Most consumers prefer palm wine with whitish, pleasant sugary taste and also exhibit vigorous effervescence. Thus, if the collected palm wine is not consumed within a few hours, it begins to develop an unacceptable flavor which is not usually liked by the consumers. Hariharan B. et. al. reported the physicochemical changes occurred in stored coconut toddy was compared with fresh toddy properties⁶.

Liquor is the alcoholic distillate obtained by the fermented mash of grain molasses, jaggery, or mahua flowers (*Madhuca latifolia L*) and contains varying percentage of ethyl alcohol with specific ingredients. Liquors are analyzed and detected as per specifications⁷⁻⁹. Beverages believed to contain ethanol are mentioned in ancient Indian literature dating back to Vedic period around 2000 B. C. 10. Two varieties of drinks are described Soma and sura. Soma, the drink of social elite, was credited with positive qualities. On the other hand, Sura a fermented beverage made from rice and sugarcane was consumed by warriors to enhance their valour and courage among other thing.

HPTLC is important analytical separation technique. The main advantage of HPTLC is simultaneous separation of several samples on a single plate needs small amounts of

developing solvents can separate milligram amount of sample. Use of automatic sample application device, UV/fluorescent detectors, densitometer scanner (which converts the spot on a layer in to a chromatogram with series of peak) coupled with software enable HPTLC tool and other instruments to enhance sensitivity of analyte detection ¹¹⁻¹³.

High performance thin layer chromatography (HPTLC) is a versatile technique for the analysis of large number of chemical substances, drugs and dyes. The technique is easily and conveniently used for routine quality control analysis¹⁴. The term Narcotic is derived from the Greek word Narkotikos, which implies as state of lethargy or sluggishness ¹⁵⁻¹⁶. Narcotic drug are those substances which gives relief from pain and induce sleep but not acceptable socially. Narcotics and psychotropic substances are of natural, semi synthetic and synthetic. On ingestion in body they act on central nervous system and produce an altered mind state and also produce physical and psychological dependencies on the drug ¹⁷⁻¹⁸. Generally samples received in forensic science laboratories are not in pure form and many times are in trace quantities, make analysis very critical. Also many diluents are present in these samples and become difficult to identify all the drugs present in the samples. A toddy sample contains carbohydrate Fresh palm sap i.e. sucrose 2 to 4 % Ethyl Alcohol. Fake toddy is prepared from citric acid, sugar, chloral hydrate, copper sulphate and added alcohol. Although benzodiazepines are generally considered as safe but are dangerous with alcohol preparation reported by Koski et. Al. In one of the case received in forensic science laboratory Nasik Nitrazepam was detected in toddy samples.

Varshney, et al ¹⁹ Described the stability of heroin in methanol. Klemenc, et al ²⁰ found Noscaoine as an adulterant in heroine. Krishnamurthy et al ²¹⁻²³ described simultaneous detection of adulterants and coextractants in illicit heroin, profiling of street narcotic by high performance liquid chromatography and caffeine and paracetamol as the main adulterants in heroin and in brown sugar respectively. Koski, A. ²⁴ reported that Postmortem toxicology frequently finds alcohol both alone and in combination with drugs. Although benzodiazepines are generally considered as safe but are dangerous with alcohol.

MATERIALS AND METHODS

The chemicals Dichloromethane, Glacial acetic acid and Methanol, used was of analytical grade. Water where ever used was of milli Q ultra-pure water. Control samples of Nitrazepam, Oxazepam, Alprazolam, Midazolam and Diazepam received from food and drug laboratory Mumbai.

Preparation of Standard Solution:

Nitrazepam standard 1mg/ml: 10.1mg of Nitrazepam standard dissolved and diluted to 10ml with methanol.

Oxazepam standard 1mg/ml: 10.2mg Oxazepam of standard dissolved and diluted to 10ml with methanol.

Alprazolam standard 1mg/ml: 10.0mg of Alprazolam standard dissolved and diluted to 10ml with methanol.

Midazolam standard 1mg/ml: 10.1mg of Midazolam standard dissolved and diluted to 10ml with methanol.

Diazepam standard 1mg/ml: 10.0mg of Diazepam standard dissolved and diluted to 10ml with methanol.

Preparation of suspected sample Solution: 25 ml of suspected samples were taken and made slightly alkaline by adding ammonium hydroxide solution. Extracted Twice with 25 ml of chloroform: methanol (9:1). Organic layer was filtered and dried with the help of anhydrous sodium sulphate. The extract was evaporated to dryness and again dissolved in 1ml methanol filtered and used for further analysis.

Dragendorffs reagent:

Solution 1:- 2.0032 grams of bismuth sub nitrate dissolved in 25 ml of glacial acetic acid and 100 ml of water added.

Solution 2:- 40.0021 g of potassium iodide dissolved in 100 ml of water.

50 ml of solution 1, 50 ml of solution 2, 100 ml of glacial acetic acid and 500ml of water mixed together to produce Dragendorffs reagent .

Experimental: Precoated HPTLC plates were made of silica gel ⁶⁰F₂₅₄ of size 20cm x 20cm MERCK make ware used. All solvents used were of analytical grade purchased from MERCK. Germany.

Instrumentation:

HPTLC applicator Model No.: AS-30 Make: DESAGA SARSTEDT GROUP

Plate reader: CD 60 The HPTLC system (DESEGA) consist of AS30 sample applicator cm equipped with 10 µl syringe, zero grade nitrogen gas cylinder and TLC scanner (Densitometer CD 60 with Deuterium lamp) was operated using software ‘ProQuint’ located on a personal computer.

Gas chromatography

(A) Gas liquid chromatography (DANI MASTER Gas Chromatograph) coupled with FID

(B) Operating Conditions: The following operating conditions were used

Column (Capillary): Dani–DN-5 MS capillary column (5% phenyl) - 95%

Methylpolysiloxane. Film thickness 0.25µm, max temp. 350°C Non polar bonded and cross-linked, inertness low bleeding and Good thermal stability

Column Material :	Capillary Length- 30mm, O.D -0.25mm, I.D. – 0.25mm.
Carrier Gas :	Nitrogen, flow 30ml/min.
Fuel :	Hydrogen, flow 35ml/ min.
Air :	Flow 350ml/min.
Split flow :	1:50

(C) Programming	A. Oven initial Temp- 240°C
	B. hold- 2 min
	C. Heating Rate- 20°C

D. Oven final Temp- 280⁰C

E. Injector Temp- 280⁰C

F. Detector Temp- 285⁰C

RESULTS AND DISCUSSION

Suspected sample solution prepared in methanol, Standard solutions of Oxazepam (2 μ l of 1mg/ml) in methanol, Alprazolam (2 μ l of 1mg/ml) in methanol, Midazolam (2 μ l of 1mg/ml) in methanol, Diazepam (2 μ l of 1mg/ml) in methanol and Nitrazepam (2 μ l of 1mg/ml) in methanol was applied on precoated silica gel ⁶⁰F₂₅₄ (10x20 cm) glass plate with the help of applicator (AS-30) with spot length 3mm. The developed solvent system Glacial acetic acid: water: methanol and dichloromethane (1:6:30:70) was used for development of plate up to 09 cm distance. The developed plate was dried in air and then sprayed with alcoholic iodine followed by alcoholic HCl and Dragondroff's reagent (Fig.1).

It was very critical to identify narcotic drug in toddy samples. The developed solvent system was Glacial acetic acid: water: methanol and dichloromethane (1:6:30:70) the solvent systems gives very good separation of nitrazepam from other narcotic drugs like diazepam, oxazepam, midazolam and alprazolam. Even the separation spots can be visualization instead of UV; we have done it by spraying Dragondroff's reagent for specificity. The sample gives retardation factor 0.75 matches with nitrazepam standard as shown in Fig 1.

1 ml of Nitrazepam standard 1 mg/ml and suspected sample solutions were injected on gas chromatogram under identical chromatographic conditions and chromatogram were recorded. It was very critical to elute and separate nitrazepam in alcoholic beverages on gas chromatography as the sample matrix was not pure. In alcoholic beverage like toddy it contains higher amounts of higher alcohols, sugars and esters as this is undistilled liquor product. Here we have done it on DANI master gas chromatograph. The modular design of components allows easily changing any GC configurations. Up to three injections units and three detectors units can be mounted simultaneously. DANI selective detectors specifically eliminate matrix interferences while providing maximum sensitivity. The detectors data acquisition rate is up to 300 Hz for a better repeatability and accuracy of chromatographic data. Gas sampling valves, auxiliary ovens, switching valves and other optional devices make

DANI master GC the most suitable gas chromatograph for the development of complex analytical systems in a wide range of applications. DANI master GC can be connected to DDS CLARITY work station through a local area network (LAN). Dedicated turnkey systems are the added value of all DANI instruments. DANI master GC coupled with DANI HSS 86.50 Head space sampler and DANI TD Thermal desorber, covers a wide range of applications for environmental, chemical petrochemical pharmaceutical, food and beverages. Figure 2 shows gas chromatogram of standard nitrazepam. The chromatogram shows retention time of 9.6. Figure 3 shows gas chromatogram of suspected nitrazepam the chromatogram gives response at retention time of 9.6 tallies with nitrazepam standard indicates presence of nitrazepam.

CONCLUSION

New solvent system Glacial acetic acid: water: methanol and dichloromethane (1:6:30:70) was found to be useful for the separation and identification of nitrazepam from diacetylmorphine along with other opium alkaloids by HPTLC, and for quantification of nitrazepam from alcoholic beverages we have used DANI master gas chromatograph with programmable injector

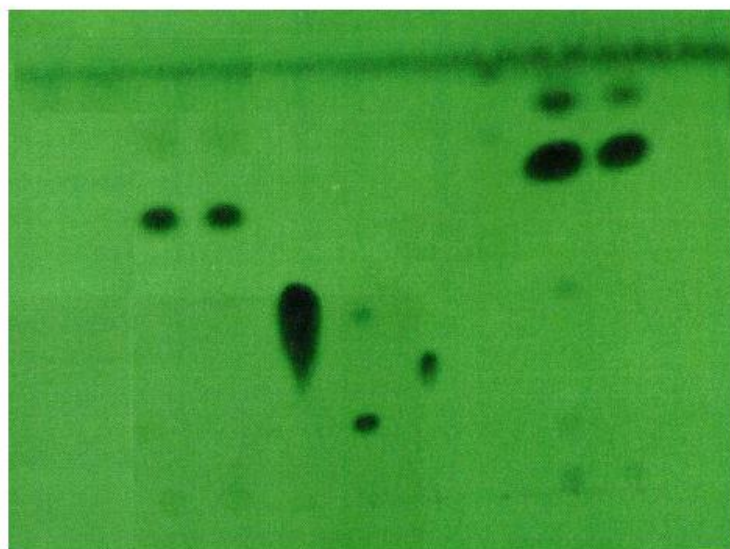
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Solvent system:

**Glacial acetic acid: water:
methanol: dichloromethane
1:6:30:70**

Spray: Dragon droff's reagent

Control:

I - Oxazepam ; II - Alprazolam

III - Midazolam ; IV - Daizepam

V -Nitrazepam ; VI - Nitrazepam

Ex1 Ex-2 I II III IV V VI

Figure 1: HPTLC photograph showing Separation of Nitrazepam

Table 1-Linearity of measurements of Nitrazepam by Gas Chromatography

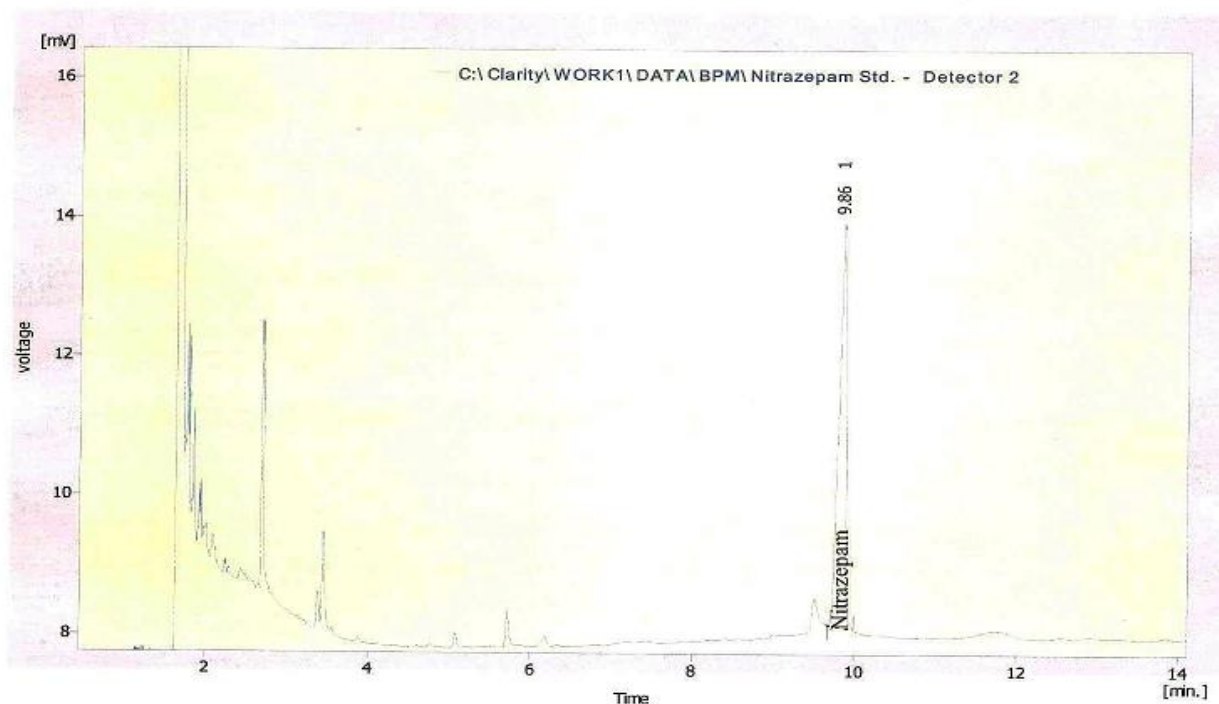
Sr.No.	Nitrazepam Std. Conc.	Area of Heroin standard
1	1 ug/ml	16.26
2	2 ug/ml	33.18
3	5 ug/ml	47.76
4	7 ug/ml	63.25
5	10ug/ml	79.23
6	Sample	36.28



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Chromatogram Info:

File Name	: C:\Clarity\WORK1\DATA\BPM\Nitrazepam Std.,PRM	File Created	: 6/12/2013 12:50:16 PM
Origin	: Acquired	Acquired Date	: 6/12/2013 12:50:16 PM
Project	: c:\Clarity\Projects\Work1.PRJ	By	: Administrator
Base	: Not Used	Calculation	: Uncal
Scale Factor	: Not Used	Units After Scaling	: Not Used
Unretained Time	: 0.00 min	Column Length	: 50.00 mm
Result Table Reports	: All Peaks	Hide ISTD Peak	: Enabled
		Uncal. Response	: 0
		Column Calc.	: From Width at 50% of Height



Result Table (Uncal - C:\Clarity\WORK1\DATA\BPM\Nitrazepam Std. - Detector 2)

Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]	Height [%]	W05 [min]
1	35.401	5.797	100.0	100.0	0.10
Total	35.401	5.797	100.0	100.0	

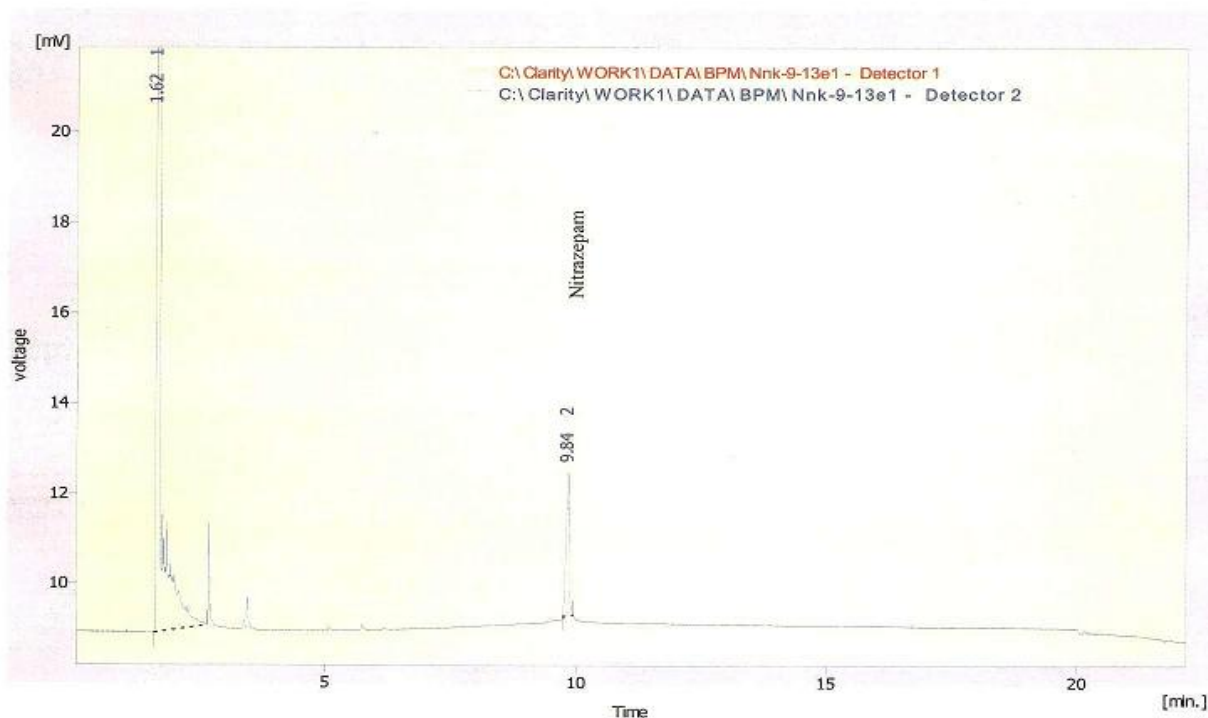
Figure 2: GC chromatogram of Nitrazepam standard



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Chromatogram Info:

File Name	: C:\Clarity\WORK1\DATA\BPM\Nnk-9-13e1.PRM	File Created	: 6/11/2013 12:23:18 PM
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Base	: Not Used	Calculation	: Uncal
Scale Factor	: Not Used	Units After Scaling	: Not Used
Unretained Time	: 0.00 min	Column Length	: 50.00 mm
Result Table Reports	: All Peaks	Hide ISTD Peak	: Enabled
		Calculation	: Uncal
		Uncal. Response	: 0
		Column Calc.	: From Width at 50% of Height



Result Table (Uncal - C:\Clarity\WORK1\DATA\BPM\Nnk-9-13e1 - Detector 1)

	Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]	Height [%]	W05 [min]
1	9.943	0.093	0.005	100.0	100.0	0.02
Total		0.093	0.005	100.0	100.0	

Figure 3: GC chromatogram of Nitrazepam standard