

## Separation and Detection of Nitrazepam and Clonazepam by Thin-Layer Chromatography

Mali Bhagwat D.\*, Sathe Ravindra A.\*\* , Vaishnav Govind R.\*\*

**Authors affiliation:** \*Assistant Director, Toxicology Division, Regional Forensic Science Laboratory, State of Maharashtra, Cantonment, Aurangabad 431 002. \*\* P.G. Student, Government Institute of Forensic Science, Nipatniranjan Nagar, Caves Road, Aurangabad 431 004.

**Reprints requests:** Mali Bhagwat D., Assistant Director, Toxicology Division, Regional Forensic Science Laboratory, State of Maharashtra, Cantonment, Aurangabad 431 002.  
Email: malibdm@yahoo.co.in

### Abstract

The benzodiazepines are most frequently encountered drugs in emergency toxicology screening, drugs of abuse testing and forensic medicine examinations. The identification of nitrazepam and clonazepam, the most frequently used benzodiazepines is described by simple, rapid and sensitive thin-layer chromatographic method. The structures of these benzodiazepines contain nitro group which is reduced by acidified stannous chloride to give amino group. This couples with diazotized sulphanilic acid to give a yellow brown coloured compound. The detection limit was found to be 3µg and 5µ per spot for nitrazepam and clonazepam respectively. The reaction was not given by other benzodiazepines commonly encountered in toxicological screening.

**Keywords:** Benzodiazepines; Thin-Layer Chromatography; Spray Reagent; Stannous Chloride; Sulphanilic Acid.

### Introduction

Benzodiazepines are the most commonly prescribed medication worldwide. They are known for their hypnotic, tranquilizing and anticonvulsant properties [1]. Due to their wide spread availability they are chronically abused or as seen more commonly in hospital emergency departments, intentionally or accidentally taken in over dose. Such cases cannot be decided by mere medical examination. To have a proper treatment the concerned medical officer collects the stomach wash, gastric lavage, blood, urine etc., of the poisoned patient, and sent to forensic toxicology division. The samples are analysed on top priority and on the basis of report of analysis the line of treatment is decided by the concerned medical officer and thus life of the person can be saved.

Several analytical techniques for the isolation and quantitation of benzodiazepines in biological

samples have been published [2-11]. Though instrumental methods are sensitive they require elaborate instrumental assay. Therefore they are not suited to emergency room determination. Therefore thin-layer chromatography (TLC) is preferred for screening the drugs, due to its simplicity and rapidity. Chromogenic reagents such as Dragendorff [12], UV detection [13-15], Griess reagent [16], Bratton-Marshall reagent [3], chlorine -o-tolidine [8], etc., are reported in literature for detection of benzodiazepines. The objective of this work is to search alternative and sensitive reagent for nitro group containing benzodiazepines. We report stannous chloride-hydrochloric acid followed by diazotised sulfanilic acid reagent for selective detection of nitrazepam and clonazepam.

### Materials and Methods

All chemicals used were of analytical reagent grade

and benzodiazepines were pharmaceutical grade. Distilled water was used throughout. Standard solutions of alprazolam (Cipla Ltd. Solan, H.P.) 1mg/ml in chloroform, clonazepam (Piramal Health Care, Solan, H.P.), 1mg/ml in acetone, diazepam (Ranbaxy, India), lorazepam (Wyeth, India) and Nitrazepam (Anglo French Drugs, Mumbai) mg/ml each were prepared in ethanol. These solutions were diluted appropriately before use.

**Spray reagents:** (i) Stannous chloride-hydrochloric acid - 5.6 gm stannous chloride uniformly dissolved in 10ml of 20% hydrochloric acid. (ii) Diazotized sulphanilic acid reagent was prepared by dissolving 0.5gm sulphanilic acid and 1 gm solid sodium nitrite in 100 ml of 10% hydrochloric acid.

### Thin-layer Chromatography

Standard glass TLC plates (10X15 cm) were coated with slurry of silica gel G (Sisco Research Laboratories, Mumbai) in water (1:2) to produce uniform 0.25 mm layers. These were left to dry at room temperature. Plates were activated by heating in oven at 110°C for ca. 1 hour. Before use the plates were stored in desiccators. Standard solutions of 10µl each of alprazolam, clonazepam, diazepam, lorazepam and nitrazepam were spotted 1.5cm from the bottom of the plate by means of a micropipette and spots were left to dry in air. Plates were developed by ascending technique, in pre-saturated TLC chamber using two solvent systems chloroform: acetic acid (9+1) and chloroform: acetone (8+2) at 25°C temperature. The mobile phase was allowed to migrate to a distance of

about 10 cm. Approximately 20 ml solvent was required for run (development time ca. 20 min). The plate was removed from the chamber, dried in air, and sprayed uniformly with stannous chloride-hydrochloric acid reagent and was air dried. It was kept in oven for about 10 min at 100°C temperature. The plate was removed from oven and cooled to room temperature. It was sprayed uniformly with diazotized sulphanilic acid.

### Results and Discussion

After detection only nitrazepam and clonazepam appeared as yellow brown coloured spots; however other benzodiazepines such as alprazolam, diazepam and lorazepam do not show any colour reaction with the reagent (Fig 1). The structure of these benzodiazepines reveals that only nitro group containing benzodiazepines (nitrazepam and clonazepam) show colour reaction. A representative reaction for nitrazepam is presented in Fig 2. Nitrazepam (I) on reduction with stannous chloride gives amine (II) which couples with diazotized sulphanilic acid (III) reagent to give yellow brown coloured compound (IV). The colour of spots remains stable for couple of days. Both the mobile phases give compact spots. The  $R_f$  values, detection limit and spot concentration/cm<sup>2</sup> for nitrazepam and clonazepam is listed in Table 1. Both the mobile phases give compact spots.

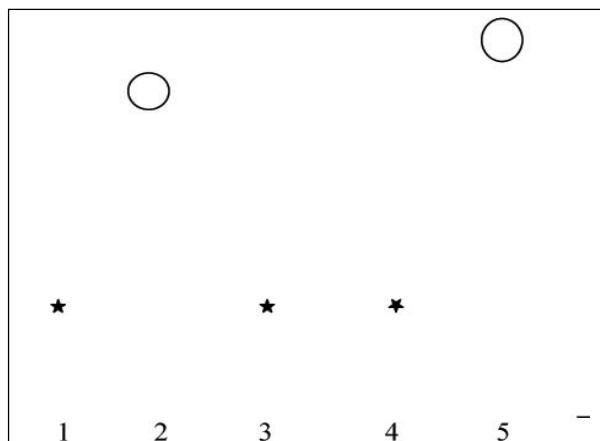
The method described in this paper permit simple and rapid identification of nitrazepam and clonazepam. Other benzodiazepines such as alprazolam, diazepam and lorazepam do not give colour reaction. The reagent described is very sensitive and selectively used for screening two benzodiazepines in single run.

**Table 1:**  $R_f$  values and detection limits of benzodiazepines

Benzodiazepines	$R_f$ in solvent system I	$R_f$ in solvent system II	Detection limit µg	Spot conc./cm <sup>2</sup>
Nitrazepam	0.63	0.40	3	6µg
Clonazepam	0.68	0.30	5	8µg

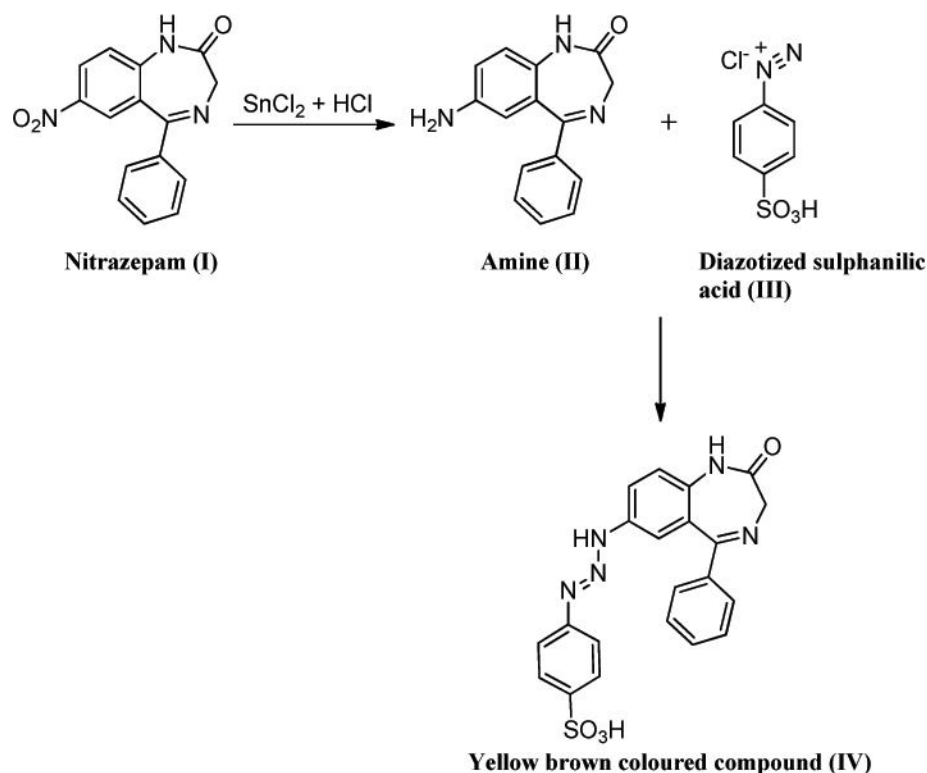
Solvent system I Chloroform: Acetic acid (9+1)

II Chloroform: Acetone (8+2)



**Fig.1:** Thin-layer chromatogram obtained from: 1, alprazolam; 2, clonazepam; 3, diazepam; 4, lorazepam; 5, nitrazepam. Asterisks indicate no spots were visualized.

Fig. 2: Proposed mechanism of Nitrazepam with diazotized sulphanilic acid



## References

- Mandrolini R, Mercolini M, Raggi M. Benzodiazepine Metabolism: Analytical perspective. *Curr. Drug Metab.* 2008; 9: 827-844.
- Meola J. Rapid procedure for routine drug overdose screening GC and Nitrogen-selective and flame ionization detector chromatography. *Clin. Chem. Newsletter.* 1977; 5: 1-3.
- Sioufi A, Dubois JP. Chromatography of benzodiazepines. *J. Chromatogr.* 1990; 531: 459-480.
- Otusubo K, Seto H, Futagami K, Oishi R. Rapid and sensitive detection of benzodiazepines and zopiclone in serum using HPTLC. *J. Chromatogr. B* 1995; 669: 408-412.
- Jain R, Utility of thin-layer chromatography for detection of opioids and benzodiazepines in a clinical setting. *Addictive Behav.* 2000; 25: 451-454.
- Zevzikovas A, Kikiuvience G, Ivanauskas L, Dirse V. Analysis of benzodiazepine derivative mixture by gas-liquid chromatography. *Medicina(Kaunas).* 2002; 38: 316-320.
- Dhavale N, Gandhi S, Sabnis S, Bothra K. Simultaneous HPTLC determination of escitalopram oxalate and clonazepam in combined tablets. *Chromatographia.* 2008; 67: 487-490.
- Marcin J, Kararzyna K, Tomasz B. UV densitometric HPTLC method for analysis of nitrazepam in pharmaceutical formulations. *J. Planar Chromatogr.* 2011; 24: 44-47.
- Hancu G, Eniko F, Aura R, Eleonora, M, Gyeresi A. Thin-layer chromatographic separation of benzodiazepine derivatives. *Analele Univ. Bucuresti-Chemie.* 2011; 20: 181-188.
- Subbiah T, Ahilandam D, Madurai V. Separation and detection of certain benzodiazepines by thin-layer chromatography. *Malaysian J. Forensic Sci.* 2013; 4: 47-53.
- Khedkar TS, Reddy YR, Mali BD. Thin-layer chromatographic detection of some benzodiazepines. *Indian J. Med. Tox. And Legal Med.* 2012; 15: 61-63.
- Drummer OH. Methods for the measurement of benzodiazepines in biological sample. *J. Chromatogr.* 1998; 713: 201-225.
- Clarke ECG. Isolation and Identification of Drugs, First Edn. London: Pharmaceutical Press. 1978; 294.
- Chiorotti M, Giovanni N, Fiori A. Analysis of benzodiazepines. *J. Chromatogr.* 1986; 358: 169-178.

15. Kastner P, Klimes J. Analysis of benzodiazepines by adsorption and ion-pair RPTLC. J. Planar Chromatogr. 1996; 9: 383-387.
16. Patil VB, Shingare MS. Thin-layer-chromatographic detection of certain benzodiazepines. J. Planar Chromatogr. 1993; 6: 497-498.
17. I.L. Finar, Organic Chemistry Vol I, Fourth Edn: Longmans, Green and Co. London, 1964; 596.
- 

## Instructions to Authors

Submission to the journal must comply with the Guidelines for Authors.

Non-compliant submission will be returned to the author for correction.

To access the online submission system and for the most up-to-date version of the Guide for Authors please visit:

<http://www.rfppl.co.in>

Technical problems or general questions on publishing with JFCT are supported by Red Flower Publication Pvt. Ltd's Author Support team (<http://www.rfppl.co.in>)

Alternatively, please contact the Journal's Editorial Office for further assistance.

Publication-in-Charge  
Journal of Forensic Chemistry and Toxicology  
Red Flower Publication Pvt. Ltd.

48/41-42, DSIDC, Pocket-II

Mayur Vihar Phase-I

Delhi – 110 091

India

Phone: 91-11-22754205, 45796900, Fax: 91-11-22754205

E-mail: [redflowerppl@gmail.com](mailto:redflowerppl@gmail.com), [redflowerppl@vsnl.net](mailto:redflowerppl@vsnl.net)

Website: [www.rfppl.co.in](http://www.rfppl.co.in)