IJPSR (2023), Volume 14, Issue 3 (Research Article)

INTERNATIONAL JOURNAL OF PHARMACEUTICAL SCIENCES AND RESEARCH

Received on 23 July 2022; received in revised form, 03 November 2022; accepted 20 November 2022; published 01 March 2023

THIN LAYER CHROMATOGRAPHIC SEPARATION OF BENZODIAZEPINES BY SEVEN DIFFERENT SOLVENT SYSTEMS

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Keywords:

Benzodiazepines, TLC, Solvent system, Rf value, Separation

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ABSTRACT: Benzodiazepines are psychoactive drugs used to treat anxiety, epilepsy, convulsions, and psychiatric and sleeping disorders. These medicinal compounds possess considerable importance in forensic toxicology due to their tranquilizing, hypnotic, and anticonvulsant properties. Thus, identifying and separating these compounds found significance in pharmaceuticals. The high-performance liquid chromatography (HPLC) technique is employed to separate benzodiazepines. The present research article aims to provide a more straightforward and efficient method for separating benzodiazepines. The thin layer chromatography (TLC) technique follows to separate benzodiazepines containing silica gel 60 F_{254} as a stationary phase. The seven different mobile phases for the separation of benzodiazepines are employed. The combination of Acetone: Toluene: Ethanol: Ammonia (45:45:7:3) is the best solvent system for separating benzodiazepines among the seven chosen solvent systems.

INTRODUCTION: Benzodiazepines are bicyclic heterocyclic compounds with benzene and diazepine rings containing two nitrogen atoms in their constitution. Benzodiazepines depend on chemical structures divided into five different groups. Benzodiazepines are essential in forensic toxicology, pharmaceutical analysis, and therapeutic drug monitoring [1-4]. Benzodiazepines for insomnia, anxiety, epilepsy, anesthesia, *etc*., are used.

QUICK RESPONSE CODE

DOI: 10.13040/IJPSR.0975-8232.14(3).1442-51

This article can be accessed online on www.ijpsr.com

DOI link: http://dx.doi.org/10.13040/IJPSR.0975-8232.14(3).1442-51

They increase the effect of neurotransmitter gamma-aminobutyric acid at their receptors, leading to anticonvulsant, sedative, anxiolytic, hypnotic and muscle relaxant properties [5-9]. Benzodiazepines are synthetic depressant drugs with variable chemical structures and impart several chemical properties and pharmacological effects. Flumazenil is a safe and effective antidote recommended for benzodiazepines.

1, 4-benzodiazepine derivatives are the largest group of benzodiazepines, especially 1,4-benzodiazepine-2-one derivatives, used to detect the relationship between the structure and activity of these compounds [10-12]. Substituent groups like Br, Cl and F improve the action of benzodiazepines. This group generally used drugs that possess a nitro group or a halogen atom in this

particular ring position. The chlorine-substituted atom provides anxiolytic and sedative properties to the compound. Thus, forensic samples use several analytical techniques to separate and characterize benzodiazepines. Gas chromatography and high-pressure liquid chromatography are popular due to their selectivity [13-16].

Forensic toxicology and a routine drug screening consist of immunoassay testing for a broad panel of drugs. Immunoassays involve an antibody-antigen complex that can be detected using fluorescence. Immunoassays are preliminary tests followed by the confirmatory mass spectrometry method. Mass spectrometry is one of the essential methods used in forensic seized-drug analysis and forensic toxicology. The mass spectrometric analysis required separation, obtained through gas chromatography and high-pressure liquid chromatography. Mass spectrometric analysis with a chromatographic method is applied to identify substances. Liquid chromatographic methods follow the quantitative and qualitative analysis of drug mixtures. Gas chromatography-mass spectrometry is not suitable for detecting and quantifying benzodiazepines due to their thermally unstable nature. Another method, Ultra-Performance Liquid Chromatography (UPLC), can also be used to determine benzodiazepines [17-18].

This method is treating for screening and identifying tranquilizers. This technique is also helpful in the determination of antidepressant drugs in pharmaceuticals. This technique is vital in determining the therapeutic concentration and toxic dose of benzodiazepines in blood samples. The benzodiazepine derivatives were analyzed in biological fluids like blood serum or plasma by the HPTLC method. Thin-layer chromatography (TLC) is the oldest chromatographic technique employed for drug analysis [19-22]. In forensic science, it TLC predominantly screens and separates drugs in solid dosage form and drugs found in biological fluids. TLC provides a simple, affordable analytical method and fast screening tool for drug separation in a complementary manner. The TLC method is helpful in the detection of benzodiazepines at nanogram concentrations [23-25]. TLC solvent systems reported in the literature for separating some benzodiazepines. Thus, in the present article, we provide a different solvent system for separating some benzodiazepines by the TLC method. It depends on the polarity of the sample and mobile phase that the sample either adsorbs by the solid phase or elutes by the mobile phase. The position of a sample zone in a thin-layer chromatogram characterize by the fundamental parameter retardation factor or R_f value. Retardation factors will be measured using the different mobile phase systems. The instrument's detection limit will be determined using each mobile phase without chemical visualization reagents.

MATERIALS:

Apparatus: The TLC system consisted of pre-coated silica gel 60 F_{254}10x20 cm of aluminum sheets as stationary phase, a microcapillary, and a Desaga Sarstedt- Gruppe HP-UVIS chamber.

Benzodiazepines: All of the benzodiazepines include alprazolam (triazolobenzodiazepines), clonazepam (1,4-benzodiazepine), chlordia-zepoxide (1,4-benzodiazepine), diazepam (1,4-benzodiazepine), lorazepam (1,4-benzodiazepine), nitrazepam andoxazepam (1,4-benzodiazepine) were pharmaceutical-grade and widely accessible. We picked seven of the most often abused benzodiazepines, each with a unique structural characteristic provided in **Fig. 1.**

(Reagents: Conc. ammonia, acetone, chloroform, diethylamine, ethanol, ethylacetate, hexane, isopropanol, methanol, toluene, and acidified potassium iodoplatinate reagent spray.

Sampling: The control samples of benzodiazepine were taken in new fresh test tubes and dissolved in HPLC methanol. The samples in alphabetical order, were loaded on a pre-coated silica gel chromatographic plate.

METHOD: The chromatographic chamber with mobile phase saturate for 40 minutes. The solvent front at 15 cm from the bottom of the plate. The baseline was 1.5 cm from the bottom of the plate. The samples loaded at the baseline are equidistant from each other. The plate examines under UV radiation at 254 nm. The acidified potassium iodoplatinate reagent was sprayed over the plate to visualize spots and measure the R_f value of the spots. One plus seven cameras with a 48MP + 5MP rear camera employ to capture the photographs of

the developed plate and the visualized plate under UV radiation.

Solvent System: Seven solvent systems / mobile phases employ for the separation of benzodiazepines [26-27]. The critical distinction between the present research and other research articles is the use of alternate solvent systems. **Table 1** mentions all seven solvent systems.

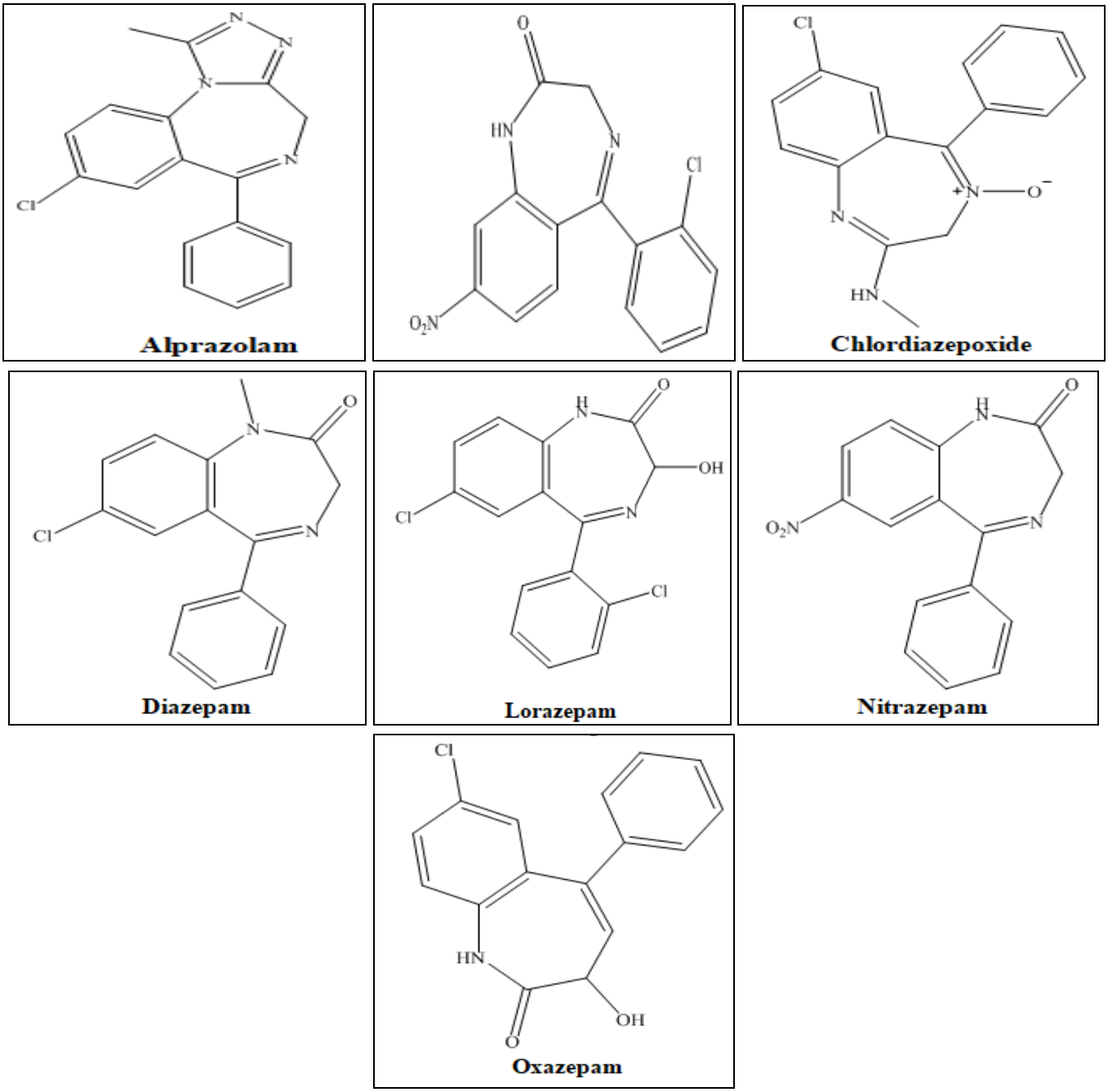

FIG. 1: CHEMICAL STRUCTURES OF THE DIFFERENT TYPES OF BENZODIAZEPINES USED IN THE STUDY

TABLE 1: SOLVENT SYSTEMS USED FOR THE SEPARATION OF BENZODIAZEPINES

S. no.	SolventSystem	Ratio
1	Hexane: Acetone	65:35
2	Ethyl Acetate	100
3	Hexane: Chloroform: Methanol	45:45:9
4	Acetone: Toluene: Ethanol: Ammonia	45:45:7:3
5	Acetone: Chloroform: Isopropanol	8:1:1
6	Chloroform: Toluene: Methanol	40:50:10
7	Toluene: Diethylamine	85:15

RESULTS & DISCUSSION: All the benzodiazepines are well-differentiated in the solvent systems I (Hexane: Acetone), III (hexane: chloroform: methanol), and IV (acetone: toluene: ethanol: ammonia) means these solvents provide different R_f values except lorazepam and oxazepam which possess almost similar R_f values other solvent systems. The similar R_f values are due to the structural similarity in these two compounds. The only difference in the chemical structure of lorazepam and oxazepam is an extra chlorine atom on the benzene ring in lorazepam. In separating benzodiazepines, the II solvent system (ethylacetate) is the least suitable than the other solvent systems as four samples of benzodiazepines (clonazepam, lorazepam, diazepam and nitrazepam) provide almost similar R_f values **Table 2**. This solvent system is unsuitable for the separation of benzodiazepines, but this solvent system employs for separating lorazepam and oxazepam due to different R_f values. This solvent system is most suitable for differentiating alprazolam and chlordiazepoxide from the other benzodiazepines effectively. **Table 2** contains the R_f values studied from the spots obtained from each solvent system.

TABLE 2: RF VALUES OF BENZODIAZEPINES IN DIFFERENT SOLVENT SYSTEMS

S. no.	Benzodiazepines	I	II	III	IV	V	VI	VII
1	Alprazolam	0.03	0.07	0.41	0.48	0.28	0.32	0.16
2	Clonazepam	0.33	0.70	0.58	0.70	0.76	0.49	0.12
3	Chlordiazepoxide	0.17	0.27	0.60	0.56	0.58	0.48	0.22
4	Diazepam	0.56	0.72	0.76	0.81	0.77	0.72	0.77
5	Lorazepam	0.28	0.70	0.45	0.46	0.74	0.39	0.20
6	Nitrazepam	0.35	0.71	0.58	0.68	0.77	0.52	0.12
7	Oxazepam	0.25	0.66	0.46	0.44			0.15

The R_f values of all benzodiazepines in the V (acetone: chloroform: isopropanol) solvent system reflect that this solvent system separated alprazolam and chlordiazepoxide superlatively from the other benzodiazepines. In contrast clonazepam, diazepam, lorazepam and nitrazepam gave almost similar R_f values in this solvent system. Alprazolam, Diazepam, Lorazepam, and Nitrazepam have distinct R_f values insolvent system VI (chloroform: toluene: methanol), but this solvent system possesses indistinguishable R_f values for clonazepam and chlordiazepoxide. In the VII solvent system (toluene: diethylamine), alprazolam and oxazepam were not well differentiated due to similar R_f values. Clonazepam and nitrazepam were also indistinguishable due to identical R_f values in this solvent system. Chlordiazepoxide and lorazepam also possess the same R_f values and cannot be distinguished in this solvent system. The only benzodiazepine determined in this solvent system is Diazepam.

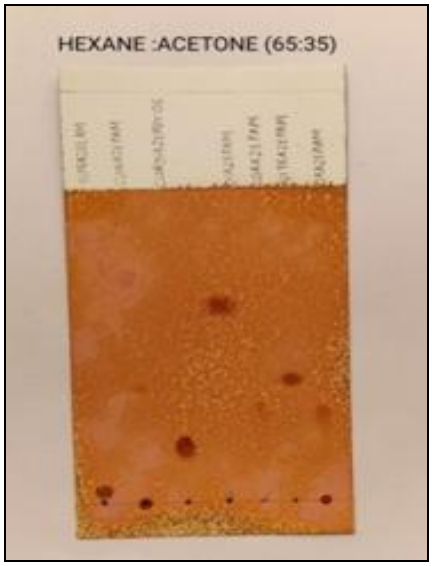

FIG. 2: SHOWING TLC PLATE AFTER SPRAYING WITH ACIDIFIED POTASSIUM IODOPLATINATE REAGENT SPRAY; HEXANE: ACETONE (65:35)

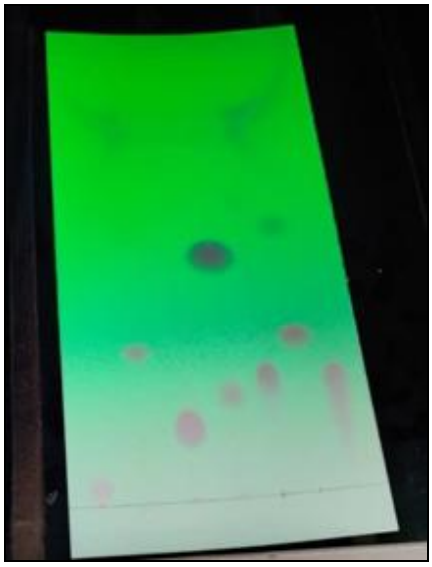

FIG. 3: SHOWING TLC PLATE UNDER UV RADIATION AT 254nm WAVELENGTH; HEXANE: ACETONE (65:35)

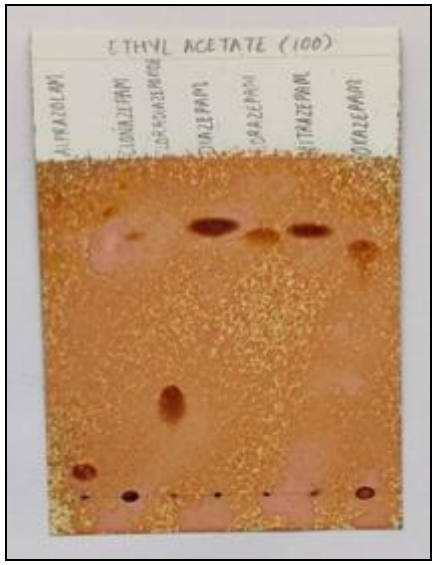

FIG. 4: SHOWING TLC AFTER SPRAYING WITH ACIDIFIED POTASSIUM IODOPLATINATE REAGENT SPRAY; ETHYL ACETATE (100)

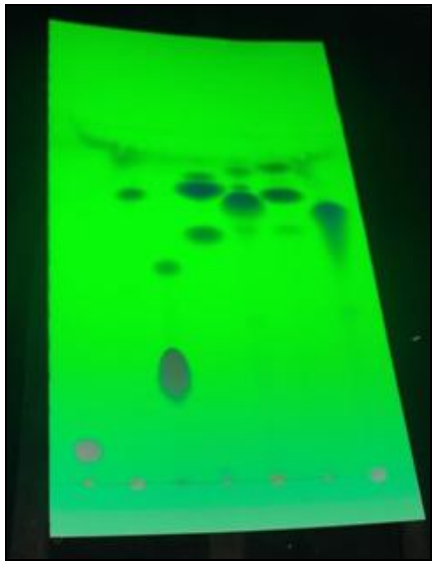

FIG. 5: SHOWING TLC UNDER UV RADIATION AT 254 NM WAVELENGTHS; ETHYLACETATE (100)

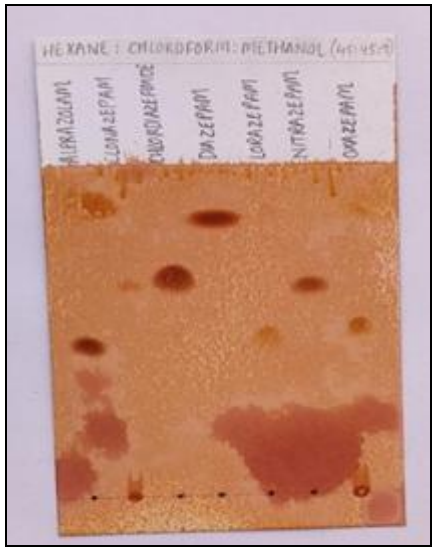

FIG. 6: SHOWING TLC AFTER SPRAYING WITH ACIDIFIED POTASSIUM IODOPLATINATE REAGENT SPRAY; HEXANE: CHLOROFORM: METHANOL (45:45:9)

FIG. 7: SHOWING TLC UNDER UV RADIATION AT 254nm WAVELENGTHS; HEXANE: CHLOROFORM: METHANOL (45:45:9)

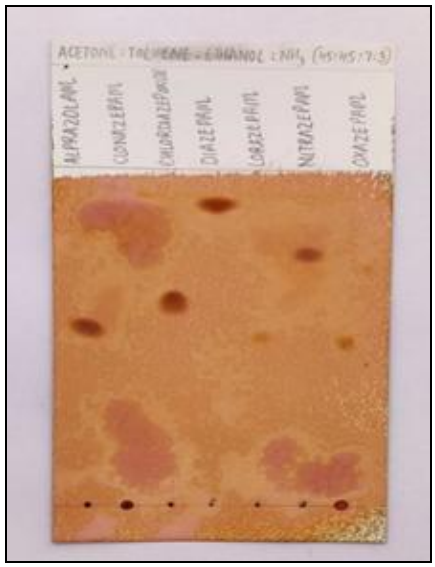

Fig. 8: SHOWING TLC AFTER SPRAYING WITH ACIDIFIED POTASSIUM IODOPLATINATE REAGENT SPRAY; ACETONE: TOLUENE: ETHANOL: AMMONIA (45:45:7:3)

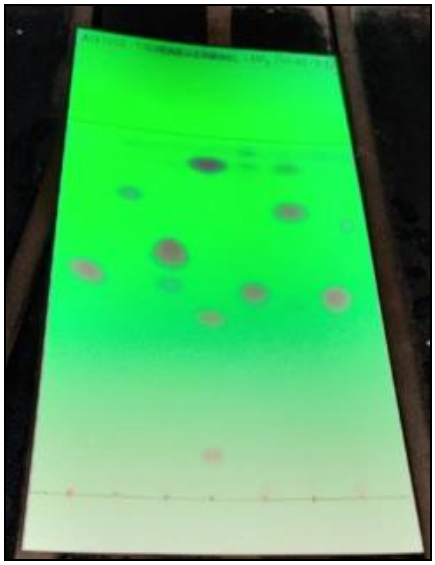

FIG. 9: SHOWING TLC UNDER UV RADIATION AT 254nm WAVELENGTH ACETONE: TOLUENE: ETHANOL: AMMONIA (45:45:7:3)

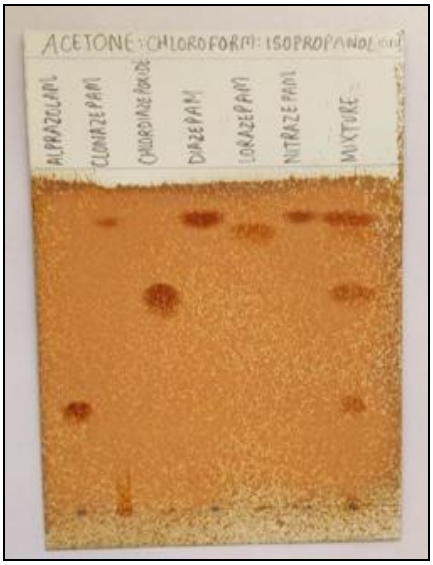

FIG. 10: SHOWING TLC AFTER SPRAYING WITH ACIDIFIED POTASSIUM IODOPLATINATE REAGENT SPRAY; ACETONE: CHLOROFORM: ISOPROPANOL (8:1:1)

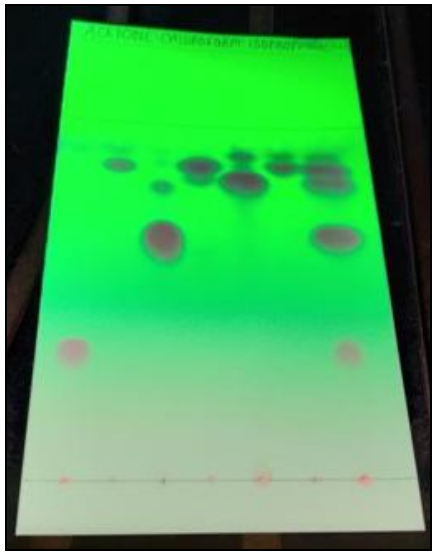

FIG. 11: SHOWING TLC UNDER UV RADIATION AT 254nm WAVELENGTH; ACETONE: CHLOROFORM: ISOPROPANOL (8:1:1)

FIG. 12: SHOWING TLC AFTER SPRAYING WITH ACIDIFIED POTASSIUM IODOPLATINATE REAGENT SPRAY; CHLOROFORM: TOLUENE: METHANOL (40:50:10)

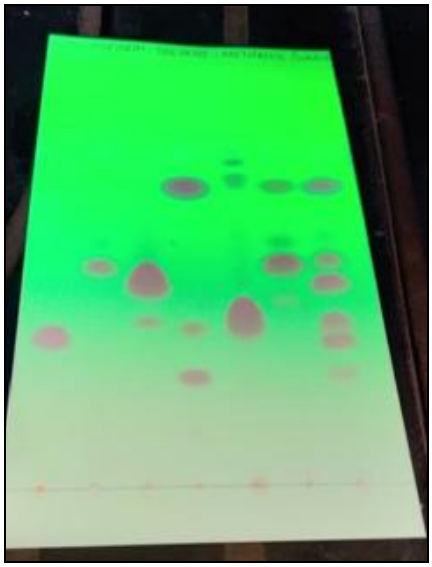

FIG. 13: SHOWING TLC UNDER UV RADIATION AT 254nm WAVELENGTH; CHLOROFORM:TOLUENE: METHANOL (40:50:10)

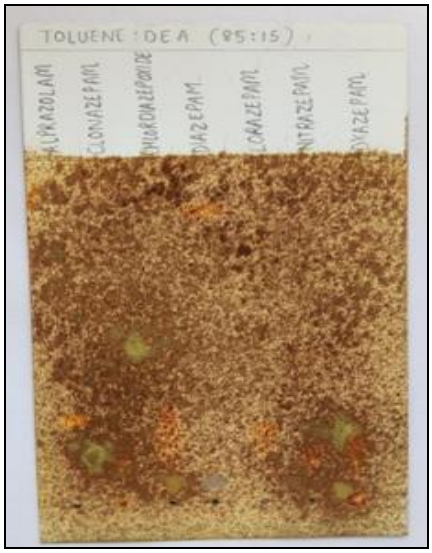

FIG. 14: SHOWING TLC AFTER SPRAYING WITH ACIDIFIED POTASSIUM IODOPLATINATE REAGENT SPRAY; TOLUENE: DIETHYLAMINE (85:15)

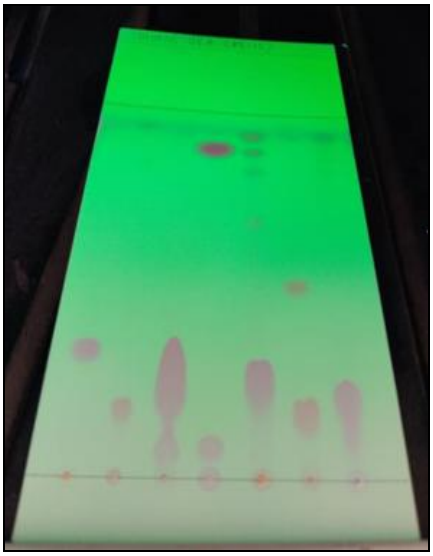

FIG. 15: SHOWING TLC UNDER UV RADIATION AT 254nm WAVELENGTH; TOLUENE: DIETHYLAMINE (85:15)

CONCLUSION: The research of this article concludes that the chromatographic separation of benzodiazepines requires at least two different solvent systems for proper verification **Fig. 2-15**. As per the findings of experiments, the best solvent system for separating benzodiazepines is solvent system IV (acetone: toluene: ethanol: ammonia), which gives the best differentiation among the seven chosen mobile phases. The solvent system II (ethylacetate) is applied to treat lorazepam and oxazepam well differentiated. These are indistinguishable from other solvent systems. The solvent system (chloroform: toluene: methanol) follows to distinguish all the benzodiazepines except for clonazepam and chlordiazepoxide. The benzodiazepine mixture remains unseparated by the solvent system (toluene: diethylamine), so it is not an excellent mobile phase.

ACKNOWLEDGMENTS: The authors are most grateful for providing laboratory support to Jaipur's Forensic Department.

Funding: This research possesses no external funding.

CONFLICTS OF INTEREST: There is no conflict between the authors.

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How to cite this article:
Sharma A, Wadhvani G, Sharma SK and Sharma S: Thin layer chromatographic separation of benzodiazepines by seven different solvent systems. Int J Pharm Sci & Res 2023; 14(3): 1442-51. doi: 10.13040/IJPSR.0975-8232.14(3).1442-51.