Separation and Detection of certain Benzodiazepines by Thin-Layer Chromatography

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ABSTRACT: The benzodiazepine drugs are frequently encountered in clinical and forensic casework samples involving road traffic offences and/or drug overdoses. The identification of nine most frequently used benzodiazepine i.e. alprazolam, chlordiazepoxide, clobazam, clonazepam, diazepam, flurazepam, nitrazepam, lorazepam and olanozepam using Thin Layer Chromatographic (TLC) method were performed to assess their degradation products after an "in situ" acid hydrolysis on the chromatoplate. This study aims to develop a simple, rapid and efficient method for the separation of these drugs using pre-coated silica gel G UV254 as stationary phase. We succeeded in the separation of the aforementioned drugs using ten different mobile phase. Each drug can be separated from the others by using an appropriate mobile phase. In this study, we have found that CHCl3: CH3OH (97:3) is the best solvent system among the studied ten systems.

Keywords: Benzodiazepine, thin layer chromatography, separation, detection, acid hydrolysis

Introduction

The Benzodiazepines (BZDs) class of psychoactive drugs is one of the most commonly prescribed medications in the world, and their availability depends on local regulation and, in some instances, illegal distribution. The BZD drugs have hypnotic, tranquilizing and anticonvulsant properties. They are frequently encountered in clinical and forensic casework samples involving road traffic offences and/or drug overdoses. An overdose of BZD can cause respiratory depression, coma and death. The BZD drugs can also amplify the depressant effects of other drugs, including alcohol and opiates, therefore increasing the risk of overdose in polysubstance use [1].

Miscreants to stupefy victims prior to robbery or rape by using these drugs are increasingly reported [1]. Self-induced or accidental overdoses with these drugs are also quite common. The BZDs are generally considered safe for short-term use; however, the risk of overuse, abuse and dependence when use for longer periods has been well documented. The abuse or misuse of BZDs is internationally widespread which means that any forensic laboratory may encounter a range of these compounds

In the United States, three groups of BZDs are available based on their chemical structures [2] viz, 1,4-benzodiazepines & 1,5-benzodiazepines, diazolobenzodiazepines, and triazolobenzodiazepines. In the 1990s a significant increase of BZD misused were identified from forensic and clinical toxicological examinations of biological material. Its identification and determination in biological material was at first associated with various analytical problems [3].

Sioufi and Dubois [4] have reviewed several analytical techniques for the isolation and quantitation of BZDs in biosamples. Both thin-layer chromatography (TLC) and the immunoassay approach remain extremely useful for rapid screening. The TLC of benzophenones was also mentioned in several papers [5,6] as being useful for the identification of pure BZDs and its metabolites. Roets and Hoogmartens [7] have described the separation of the nine benzophenones obtained by acid treatment of nineteen BZDs on the TLC plate.

In most of the TLC techniques for toxicological analysis, the BZDs and its metabolites are first hydrolysed to the corresponding benzophenones, which are then identified by chromatography. These methods are not specific, as different BZDs can give the same benzophenone. The hydrolysis into benzophenones also is not a general method [8] because BZDs such as triazolam, alprazolam and clobazam do not form

benzophenones when treated in the usual way and medazepam is stable towards hydrolysis.

Numerous researchers have extensively used TLC for the separation and identification of BZD drugs. Rajvinder singh et al. [9] introduced a single TLC solvent system, which can be of immense use in the preliminary screening of some commonly encountered BZDs. They believed that it is an improved, alternative TLC solvent system for the separation of BZDs that can be utilized by forensic laboratories. Tewari and Shukla_[10] studied TLC technique for the separation and identification of 1.4-benzodiazepine drugs in biological material.

A study by Inoue and Niwaguchi [11] described a TLC procedure for determining nitrazepam in human urine. Okumura and Nagaoka [12] successfully separated various types of BZDs in methanol-water (2:1 or 3:1), in which the spots were detected by fluorescence quenching. Patil and Shingare [13] also reported on TLC detection of certain BZDs. Rapid separation of mixtures of BZDs drugs was achieved by Stevens and Jenkins [14] using a thin-layer system based on alumina, complemented by a silica gel loaded paper system. Visualisation of the spots was by short-wavelength ultraviolet light and acidified potassium iodoplatinate.

TLC has always been a better choice for the preliminary examination of various drugs of abuse. Literatures have reported some TLC solvent systems for the separation of some BZDs. Detection of intact BZDs, as well as from biological materials of a deceased victim are often necessary in forensic practice. However, the separation of intact BZDs for identification purposes was not extensively reported [5].

In this study, we chose nine BZD drugs viz. (alprazolam, chlordiazepoxide, clobazam, clonazepam, diazepam, flurazepam, nitrazepam, lorazepam, olanozepam) drugs, for the separation and identification as each of them have different structural characteristics (Figure 1).

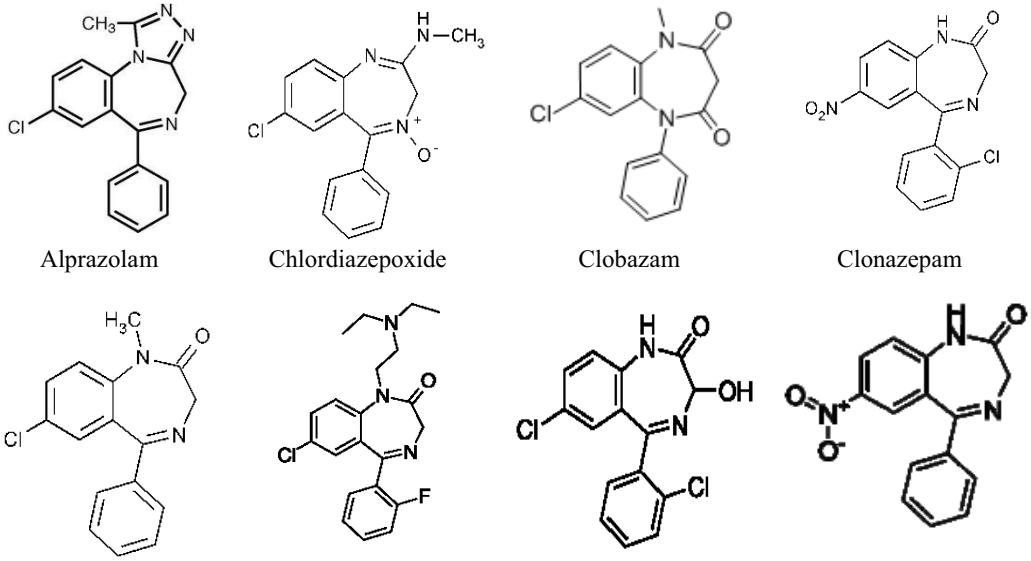

Figure 1:Structure of the Benzodiazepines

The drugs were analyzed to assess their degradation products after an “in situ” acid hydrolysis on the chromatoplate. The present work was undertaken in order to use unimpergnated, thin layers of Silica gel G UV254 as an adsorbent and to develop economical and convenient methods for the separation and detection of the aforementioned BZD drugs.

Experimental

Materials

Silica gel G UV254 precoated commercial plates (20×20 cm, 0.25 mm thickness) were supplied by Merck (Darmstadt, Germany). A 10 µL Hamilton syringe calibrated at 0.1 µL intervals was used. Substances were applied to the plates with the aid of 10 µL Hamilton

syringe and the spots were visualized using a spray gun.

Reagents and chemicals

Benzodiazepine drugs viz. (alprazolam, chlordiazepoxide, clobazam, clonazepam, diazepam, flurazepam, nitrazepam, lorazepam, olanozepam) drugs were of pharmaceutical grade (India).

All of the solvents i.e. methanol (Qualigens Fine chemicals, India), ethyl acetate, ammonium hydroxide (NICE Chemicals, India), ammonia (Merck, India), chloroform (S.D.Fine Chemicals, India), toluene (Rankem, India), acetone (Glaxo Laboratories, India), hexane (Loba Chemie, India) and reagents used were of analytical reagent grade. Deionized water was used to prepare all solutions. Freshly prepared solutions were always employed.

Preparation of reagents

Dragendorff's reagent (Stock solution)

Solution A: Basic bismuth nitrate (Merck, India) of 1.7 gram and 20 gram of tartaric acid (Rankem, India) were dissolved in 80 mL of deionized water.

Solution B: Potassium iodide (Merck, India), of 16 g was dissolved in 40 mL of deionized water. Then equal volumes of solution A and solution B were mixed to get the stock solution. This solution is stable for several months when stored in a refrigerator.

Spray solution: One part of the stock solution was mixed with 10 parts by volume of 1.33 mol/l aqueous tartaric acid solution (Rankem, India).

Iodine methanol solution: prepared by dissolving one gram of iodine (NICE Chemicals, India) in 125 mL of methanol (Qualigens Fine chemicals, Mumbai, India).

Sulphuric acid (10% V/v): prepared by diluting 10 mL of concentrated sulphuric acid (Qualigens Fine Chemicals, India) in 90 mL of distilled water.

Sample preparation

Each type of BZD tablet was accurately weighed and powdered in a mortar. Ten milligrams were taken and dissolved in 10 ml of methanol and sonicated for five minutes. Another 20 mL of methanol was added and sonicated for further 5 minutes. The mixture was mixed thoroughly for 2 minutes and transferred to a 100 mL volumetric flask through a Whatmann No. 40 filter paper. The residue was washed thrice with methanol and the combined filtrate was made up to the mark with methanol.

The prepared sample solution was diluted with methanol to obtain serial sample solutions of 10, 25, 50, 100 and 250 µg/mL concentrations. These solutions were stored in well closed vessels and direct contact with light was avoided.

Procedure

The plates were divided into 1.5 cm wide strips and solute spots were applied using a calibrated Hamilton syringe. Then, 2-10 µL each of the drug solutions were spotted on the TLC plates as separate spots. The plates were developed in a closed glass sintered chamber containing developing solvents with 30 minutes saturation time (listed in Table 1), at 25-30º C temperature. The solvent in the chamber was allowed to reach the lower edge of the adsorbent, though the spot points were not allowed to be immersed. The cover was put in place, and the system was maintained until the solvent ascended to a point 10 to 15 cm above the initial spots; this usually required about 30 to 90 minutes. The plate was removed from the developing chamber when the solvent reached solvent front and later was dried in air.

Non-destructive procedure, such as the use of ultraviolet light (both 254 nm and 356 nm) was used for the localization of separated spots. Next, the plates were sprayed with three different chromogenic reagents (visualization reagent) namely; (i) dragendorff's reagent (ii) iodine/methanol reagent and (iii) sulphuric acid (10%) for the identification of BZD drugs. The Rf values and color spots of the separated drugs were recorded.

In situ hydrolysis

An amount of 0.5 µL of each type of BZD solutions were spotted on the chromatoplate. Then 0.5 µL dilute sulphuric acid (10% vol/vol) was placed over each spot. Next, the plate was covered with a glass plate and kept for 15 minutes in an oven at 120º C. Later, the plate was cooled at room temperature and on each spot, 0.5 µL concentrated ammonia solution (25% v/v) was placed. The spots were dried by heating at 120ºC for 5 minutes.

Table 1: Developing solvent system for Benzodiazepines.

No	System	Developing solvent	Composition[a] (v/v)
1.	I[b]	Methanol: ammonium hydroxide	100:2.5
2.	II	Chloroform: methanol	97:3
3.	III	Ethyl acetate	100
4.	IV	Chloroform: toluene: methanol	40:50:10
5.	V	Ethyl acetate: methanol: ammonia	75: 20: 5
6.	VI	Hexane: ethyl acetate: methanol	50:50:10
7.	VII	Chloroform: acetone	90:10
8.	VIII	Chloroform: toluene: methanol	46:50:4
9.	IX	Ethyl acetate: methanol : ammonia	70:20:10
10.	X	Ethyl acetate: methanol	90:10

(Note: [a]The developing solvents are expressed as parts, not percentages; [b]Developer containing volatile materials such as NH_4OH should be prepared just prior to use and not stored for future.NH_4OH has been prepared as 1.5 mL aq.NH3 in 3 mL of distilled water)

Results and Discussion

Silica gel G UV254 was used as an adsorbent. The use of adsorbents containing fluorescent inorganic pigments is advisable for the detection of substances which absorb in the UV-region. The great advantage of this method is that the separated materials can be detected without being chemically modified [15] in any reaction. There is virtually no difference in the performance between fluorescent and non-fluorescent plates, though the former has the advantage of visibility of a spot under ultraviolet light.

Solvent systems I to X (Table 1) were proposed for BZDs drugs and visualizations of the separate drugs were performed by both short and long wavelength UV-light, 254 nm and 356 nm respectively. Visualisation were also performed using sprayed chromogenic reagents i.e Dragendorff's reagent, iodine in methanol and followed by 10% sulphuric acid. The Rf values in various solvents and the colours developed at each stage for BZDs drugs are given in Table 2. All solvent systems recorded separations with BZDs drug samples except for solvent system I and V. The use of visualization reagents to enhance chromatographic detection was observed whereby Dragendorff's spray detected orange colour spots in all samples developed in the solvent system number II, III, IV, VI, VIII, IX and X. Orange, brown and pink colour spots appeared in all BZDs samples developed from solvent system II, III, IV, VI, IX and X, upon contact with iodine in methanol spray. Similar coloured spots were observed when 10% sulphuric acid reagent reacted with BZDs samples developed using solvent system number II, III, IV, VI, VIII and IX.

Hydrolysis in acid medium:

BZDs are relatively instable, easily hydrolyze in acidic solution and also decompose in UV light. Hydrolysis in acidic solution generally leads to 2-aminobenzophenone derivatives, through the split of the N1-C2 bond of the diazepinic ring (Figure 2).

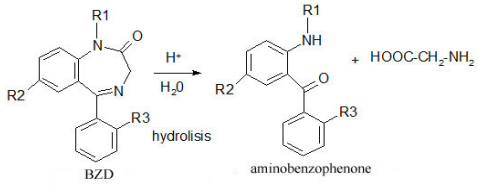

Figure 2: Benzodiazepine degradation in acid medium

TLC of benzophenones obtained by acid hydrolysis of BZD derivatives is widely used for identification purpose however this method is not product specific as different types of BZD can give the same benzophenone derivatives. Alprazolam however do not form benzophenones. The advantage of TLC of the benzophenones is that different metabolites from same benzodiazepine are able to give the same benzophenone on hydrolysis, which makes this method more suitable for identification of these products in biological fluids.

Table 2: The R_f values of certain Benzodiazepine drugs on silica gel G UV_{254}

System			Drug								
			Alprazolam	**Chlordiazepoxide**	**Clobaza**	**Clonazepam**	**Diazepam**	**Flurazepam**	**Lorazepam**	**Nitrazepam**	**Olanozepam**
System II	R_f		0.03	0.18 (0.63)	0.78 (0.86)	0	0.18	0.25	0.88	0.79	0.12
	Colour in	UV	NCD	NCD	NCD	NCD	NCD	NCD	NCD	NCD	NCD
		DDR	Orange	Orange	Orange	NCD	Orange	Orange	Orange	Orange	Orange
		I_2/CH_3OH	Dark Orange	NCD	Orange	NCD	NCD	Dark Orange	NCD	NCD	Orange
		H_2SO_4	NCD	NCD	NCD	NCD	NCD	NCD	NCD	NCD	NCD
System III	R_f		0.02	0.56	0	0	0	0.17	0	0	0.06
	Colour in	UV	NCD	NCD	NCD	NCD	NCD	NCD	NCD	NCD	NCD
		DDR	NCD	Orange	NCD	NCD	NCD	Orange	NCD	NCD	Orange
		I_2/CH_3OH	Orange	NCD	NCD	NCD	NCD	NCD	NCD	NCD	NCD
		H_2SO_4	NCD	Dark orange	NCD	NCD	Purple	NCD	Purple	NCD	Orange
System IV	R_f		0	0.25 (0.34) (0.35)	0	0	0.28	0.43 (0.32) (0.33)	0	0	0.18
	Colour in	UV	NCD	Brown	NCD	NCD	NCD	Yellow	NCD	NCD	Brown
		DDR	NCD	Orange	NCD	NCD	NCD	Orange	NCD	NCD	Brown
		I_2/CH_3OH	NCD	Brown	NCD	NCD	Light brown	Brown	NCD	NCD	Dark brown
		H_2SO_4	NCD	Orange	NCD	NCD	NCD	Orange	NCD	NCD	Orange
System VI	R_f		0	0.68	0	0	0.96	0.18 (0.11)	0	0	0.07
	Colour in	UV	NCD	NCD	NCD	NCD	NCD	Yellow	NCD	NCD	Yellow
		DDR	NCD	Orange	NCD	NCD	Orange	Pale orange	NCD	NCD	Dark brown
		I_2/CH_3OH	NCD	Orange	NCD	NCD	NCD	Orange	NCD	NCD	Brown
		H_2SO_4	NCD	Orange	NCD	NCD	Orange	Orange	NCD	NCD	Orange0
System VII	R_f		0	0.69	0	0	0	0.19	0	0	0.08
	Colour in	UV	NCD	NCD	NCD	NCD	NCD	NCD	NCD	NCD	NCD
		DDR	NCD	NCD	NCD	NCD	NCD	NCD	NCD	NCD	NCD
		I_2/CH_3OH	NCD	Purple pink	NCD	NCD	NCD	Purple pink	NCD	NCD	Purple pink
		H_2SO_4	NCD	Pink	NCD	NCD	NCD	Pink	NCD	NCD	Pink
System VIII	R_f		0	0	0	0.28	0.64	0.18	0	0	0.28
	Colour in	UV	NCD	NCD	NCD	NCD	NCD	NCD	NCD	NCD	NCD
		DDR	NCD	NCD	NCD	Orange	Orange	Orange	NCD	NCD	Orange red
		I_2/CH_3OH	NCD	NCD	NCD	NCD	NCD	NCD	NCD	NCD	NCD
		H_2SO_4	NCD	NCD	NCD	NCD	Orange	Orange	NCD	NCD	Orange
System IX (After acid hydrolysis)	R_f		0	0.97	0	0	0.98	0.99	0	0	0.98
	Colour in	UV	NCD	NCD	NCD	NCD	NCD	NCD	NCD	NCD	NCD
		DDR	NCD	**Orange**	NCD	NCD	NCD	NCD	NCD	NCD	Orange
		I_2/CH_3OH	NCD	Brown	NCD	NCD	NCD	NCD	NCD	NCD	Brown
		H_2SO_4	NCD	NCD	NCD	NCD	Orange	Orange	NCD	NCD	NCD
System X	R_f		0	0.83	0	0	0.05	0.83	0	0	0.84
	Colour in	UV	NCD	NCD	NCD	NCD	NCD	NCD	NCD	NCD	NCD
		DDR	NCD	Pale orange	NCD	NCD	NCD	Pale orange	NCD	NCD	Pale orange
		I_2/CH_3OH	NCD	NCD	NCD	NCD	NCD	NCD	NCD	NCD	NCD
		H_2SO_4	NCD	NCD	NCD	NCD	Orange	NCD	NCD	NCD	NCD

(Note: UV: Ultraviolet light in 254nm & 356nm; DDR: Dragendorff's reagent; NCD: No Colour Development)

TLC of benzophenones is also mentioned in several papers as being useful for the identification of pure benzodiazepines or for the identification of benzodiazepines and metabolites in biological fluids. Technically, a good hydrolysis result can be obtained when the spots were covered with a glass plate after moistening with sulphuric acid; omitting this detail would probably cause rapid evaporation of the sample with partial or no hydrolysis [16]. The use of hydrochloric acid instead of sulphuric acid is unsuitable when UV light is used for detection due to background effects that may prevent normal detection of spots.

It has been observed that out of the ten solvent systems for BZDs, a minimum of three solvent systems is sufficient for separation and identification in TLC. The BZDs in the solvent system number IX i.e. ethylacetate: methanol: ammonia after the acid hydrolysis gave Rf values in the increasing order for chlordiazepoxide, diazepam and flurazepam, the spots were also identified using the aforementioned spray reagents.

The results of in-situ acid hydrolysis obtained were compared to the studied solvent system as given in Table 2. Several solvent systems can be successfully used for separation of the compounds investigated, and it has been observed that the chloroform: methanol (97:3) is the best solvent system for acid hydrolysis. Comparable result were obtained using ordinary TLC methods with three solvents system namely ethylacetate (100), hexane: ethylacetate: methanol (50:50:10) and ethylacetate: methanol (90:10) systems.

Conclusion

It is apparent that TLC is the preliminary method for the identification of BZD drugs. TLC is used to screen and also indicate presence of compounds as confirmation still needs instrumental analysis. Thus, the proposed method is well suited to analyze these BZD drugs. The separation and simultaneous detection of four BZD drugs by TLC was achieved in this work. The purpose of this investigation was to report on the separation and detection of nine types of BZD drugs using ten solvent systems that would rapidly separate these drugs. Hence, this approach could also be applied to detection in drug abuse cases in forensic science laboratories as well as pharmaceutical formulations.

In this paper we describe that, TLC is convenient to check the identity of a BZD or a mixture of BZDs by the use of two or three solvent systems. Separation of BZD using TLC can be achieved using the drug itself or the benzophenone (derivatives) obtained after in situ acid hydrolysis, and is proving to be a very useful method in the preliminary analysis. The chromatographic systems presented in this paper permit an easy and rapid screening of a wide range of BZD currently in use.

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