Reversed-Phase High Performance Liquid Chromatographic Method for the Simultaneous Analysis of Four Benzodiazepines in Pharmaceutical Formulations

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ABSTRACT: Benzodiazepines (BZD) are widely used in pharmacotherapy as antiepileptics, muscle relaxant, hypnotic, and anaesthetic inductors. Because of the increasing number of pharmaceutical preparations of BZD, these drugs are frequently encountered in clinical and forensic case work samples involving road traffic offences and/or drug overdoses. Also, BZDs are now among the most commonly-prescribed drugs, which increase their potential for addiction and abuse, and often they are found in combination with other drugs in drug-related fatalities or drug facilitated sexual assault cases. For these reasons, the analysis of BZDs is of great interest to forensic and clinical toxicologists. A reversed phase high performance liquid chromatographic method for the simultaneous analysis of four frequently prescribed BZDs (alprazolam, chlordiazepoxide, diazepam and nitrazepam) in bulk powder or formulated in tablets. Isocratic elution at a flow rate of 1.0 and 1.5 mL min^{-1} was employed on a symmetry Flexar Quartenary column C_{18} (250 mm x 4.6 mm, 5µ) at ambient temperature. The four different mobile phase systems were studied. The UV detection wavelength was at 254 nm and 10 µL sample was injected. The excipients present in tablets and capsules did not interfere with the developed method. The developed method is rapid and sensitive, and is suitable for routine control of pharmaceutical dosage forms and it could be applied to the forensic samples.

Keywords: forensic science, benzodiazepine, RP-HPLC, pharmaceutical dosage forms

Introduction

The Benzodiazepines (BZDs) are a widely abused class of drugs used as minor tranquilizers, hypnotics, and muscle relaxants [1]. Because BZDs are often seen in forensic and clinical cases, many of these compounds are included in routine drug tests. The increasing number of these samples has created a need for higher sample throughput, requiring faster sample preparation and analysis. BZDs are widely used to aid patients with anxiety and sleeping disorders and, hence, have potential for abuse. For most countries, the BZDs are classified as controlled drugs, and yet they are frequently encountered in clinical and forensic toxicological analyses involving intoxication, over dosage, and traffic accidents and are sometimes implicated in the commitment of crimes.

Although BZDs were considered to have low toxicity, the potential of addiction or dependence has still received much attention from time to time [2]. In addition, abuse of BZDs and some related substances was found to be associated with suicide or drug-facilitated sexual assault (DFSA). In many cases, the DFSA victims might not report the event and receive medical attention until several hours or days after the incident.

Alprazolam, Chlordiazepoxide, Diazepam and Nitrazepam are among the most important BZD derivatives used as anxiolytic, sedative or hypnotic drug, Fig. 1. These compounds are the most commonly prescribed class of drugs in the world for the treatment of anxiety and insomnia, particularly for the elders [3]. Alprazolam is of particular interest, because it is being prescribed for treatment of depression and has been implicated in suicidal ingestions [4]. Alprazolam is also a popular drug of abuse.

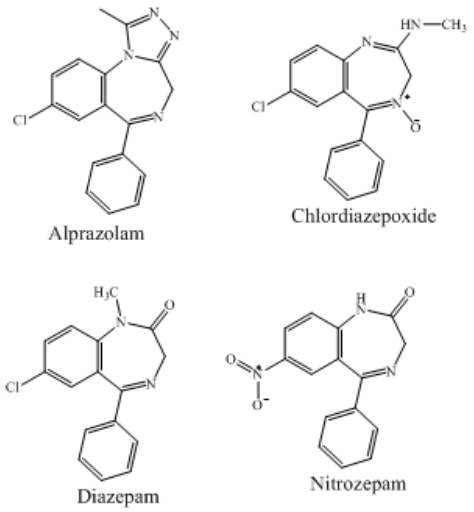

Fig. 1: Structure of the studied benzodiazepines

Alprazolam, and Nitrazepam are used with low doses, and their therapeutic plasma concentrations are rather low. Pharmacotherapy with nitrazepam should not be based solely on dosage, given the relatively low therapeutic index of these drugs.

Chlordiazepoxide is used to treat anxiety and acute alcohol withdrawal. It is also used to relieve fear and anxiety before surgery. Chlordiazepoxide is utilised widely as tranquillisers, hypnotics, sedatives, antidepressants, for both humans and animals, which act by increasing the efficiency of the neurotransmitter γ-aminobutyric acid (GABA) to decrease the communication between neurons, so calming many of the functions of the brain [5]. Diazepam, first approved for use in the early 1960s, is one of the most frequently prescribed drugs of the BZD group. Its uses include; treatment of anxiety and anxiety related insomnia, muscle relaxant, anti-epileptic and preoperative sedative. Therefore, the availability of reliable, sensitive, specific, and fast analytical methods for their determination is deemed important.

Methods based on HPLC with detection of ultraviolet absorbance have been also presented for BZD analysis [6,7]. Several chromatographic methods have been reviewed by Sioufi and Dubois [8]. HPLC combines many of the advantages of other methods by providing adequate separation of components at room temperature with quantification of the drug and metabolites. Since some 1,4-benzodiazepines are thermally labile, operation at room temperature is particularly advantageous [9].

A number of confirmatory techniques are widely used for the BZDs, such as HPLC followed by UV detection. There are many described analytical methods [10-12] for determination of BZDs in biological samples. The most frequently applied method is HPLC with UV detection. Limited HPLC methods are applicable to the simultaneous determination of BZD [13]. None of these methods is universal. In addition, the reported methods could not eliminate important problems like peak tailing in HPLC chromatograms of BZD. The significant peak tailing causes a number of problems including lower resolution, sensitivity, accuracy and precision.

The objective of this research is to develop a universal, rapid, precise, and sensitive HPLC method required for quality control of four BZD in bulk powder and in pharmaceutical dosage form.

Materials and methods

Standards and solvents

Benzodiazepine drugs *viz.* (alprozolam, chlordiazepoxide, diazepam and nitrazepam) drugs were of pharmaceutical grade (India). All of the solvents (methanol, acetonitrile and water) used were of HPLC grade. The solvents were supplied from Qualikem Fine Chem Pvt.Ltd., New Delhi, India. Freshly prepared solutions were always employed.

Instruments and chromatographic conditions

Chromatographic identification was performed at room temperature (25 ± 1º C) with Perkin Elmer NFLR 0400, Flexar Quartenary -10 LC platform delivery system and a programmable UV-visible variable - wavelength was used with Kit-Flexar manual sample injector. The Chromera software was used in the system. The Chromatographic identification was achieved on a (250×4.6 mm) RP C_{18} column (Phenomenex, USA) having a 5 µm packing as a stationary phase. The elution was performed at a flow rate of 1.5 mL/min with UV detection at 254 nm. Samples (10 µL) were injected using Kit-Flexar manual sampler.

Preparation of stock solutions

Stock solutions of 0.75 mg/mL alprozolam, chlordiazepoxide, diazepam and nitrazepam were prepared. 75 mg of alprozolam, chlordiazepoxide, diazepam and nitrazepam were weighed and placed in a 100 mL volumetric flask. It was dissolved in an appropriate amount of the mobile phase solution, and stirred using a magnetic stirrer for a period between 15–30 min until it was completely dissolved. The 100 mL volume of the solution was filled with the mobile phase to obtain the desired concentration.

Preparation of working solutions

Working solutions of 50, 100, 150 µg/mL alprozolam, chlordiazepoxide, diazepam and nitrazepam were prepared. These were freshly prepared for every experiment.

Sample preparation

The tablet contains alprozolam, chlordiazepoxide, diazepam and nitrazepam; twenty tablets were accurately weighed and powdered in a mortar. An amount equivalent to 75 mg of these drugs were taken and dissolved in 100 mL of methanol and sonicated for five minutes. About 20 mL of methanol was added and sonicated for further 5 mins. The mixture was mixed well for 2 mins 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 sample solution thus prepared was diluted with methanol to get the solutions containing different concentrations of BZDs. These solutions were stored in well closed vessels and direct contact with light was avoided.

Results and Discussion

In this study we have chosen the following mobile phases and with its composition, which have shown in Table 1 to separate and to detect the BZD drugs viz. alprozolam, chlordiazepoxide, diazepam and nitrazepam.

Table1: The studied mobile phases and its compositions for the separation

Mobile phases	Solvents
A	Methanol:Water (60:40)
B	Methanol:Acetonitrile:Water (45:40:15)
C	Methanol:Acetonitrile:Water (70:25:5)
D	Methanol:Acetonitrile (60:40)

Respective standard chromatograms were given to the individual BZD drugs in Fig. 1-4. The chromatographic run time selected was 10-30 min. Simultaneous separation and detection has been shown in Fig. 5-8, these were recorded in different concentrations.

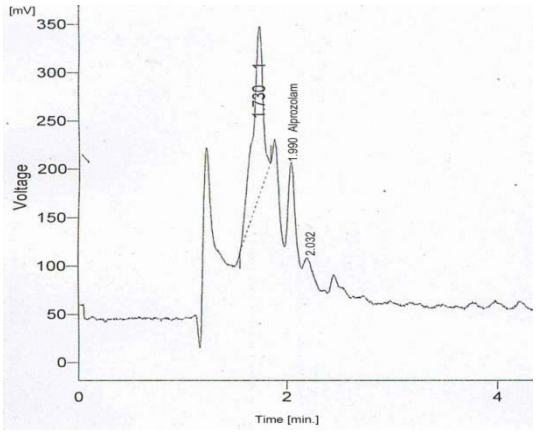

Fig. 1: Chromatogram of Alprozolam in mobile phase A with a flow rate of 1 mL/min at 25°C column temperature

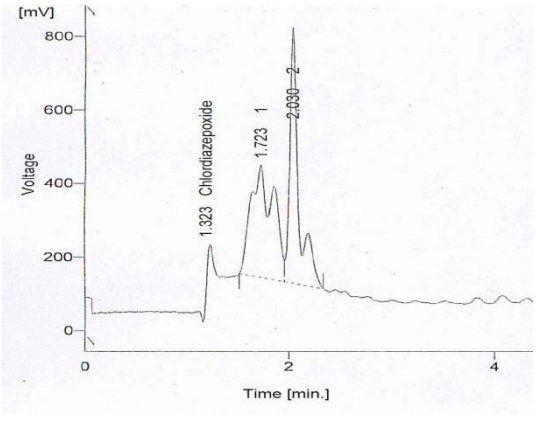

Fig. 2: Chromatogram of Chlordiazepoxide in mobile phase A with a flow rate of 1 mL/min at 25°C column temperature

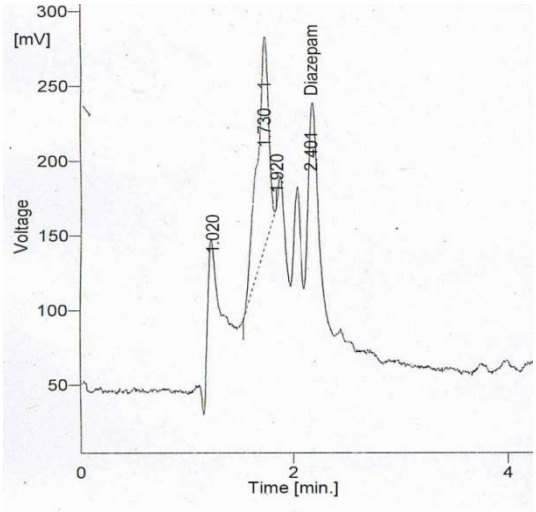

Fig. 3: Chromatogram of Diazepam in mobile phase A with a flow rate of 1 mL/min at 25°C column temperature

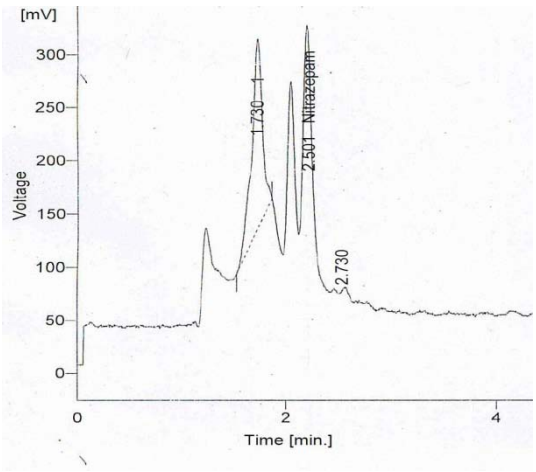

Fig. 4: Chromatogram of Nitrazepam in mobile phase A with a flow rate of 1 mL/min at 25°C column temperature

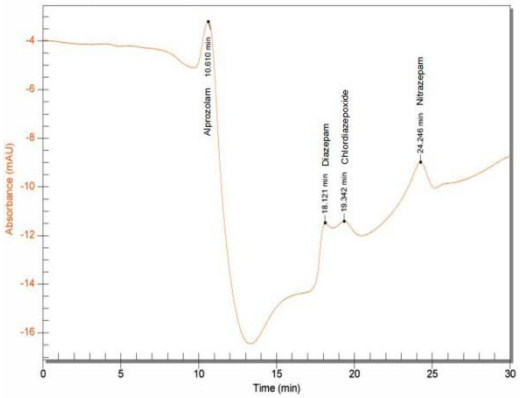

Fig. 5: Chromatograms of alprozolam, chlordiazepoxide, diazepam and nitrazepam in mobile phase B with a flow rate of 1 mL/min at 25°C column temperature at drug concentration 150 µg/mL

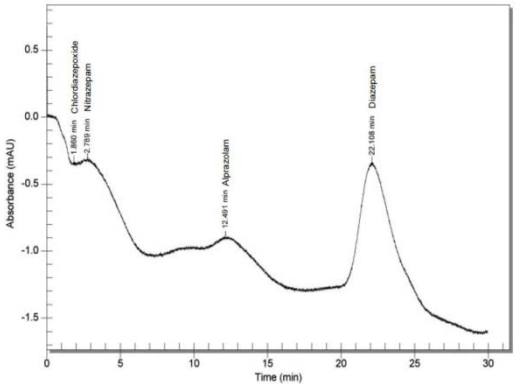

Fig. 6: Chromatograms of alprozolam, chlordaizepoxide, diazepam and nitrazepam in mobile phase C with a flow rate of 1 mL/min at 25°C column temperature at drug concentration 50 µg/mL

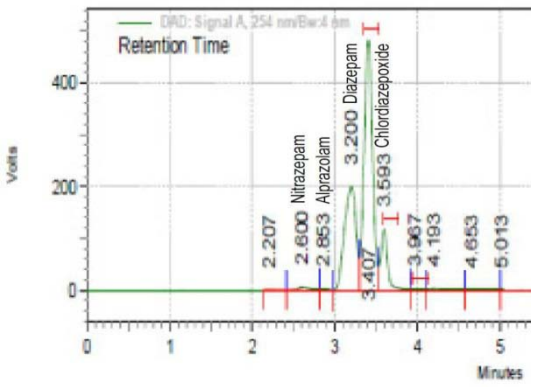

Fig. 7: Chromatograms of alprozolam, chlordaizepoxide, diazepam and nitrazepam in mobile phase A with a flow rate of 1mL/min at 25°C column temperature at drug concentration 100 µg/mL

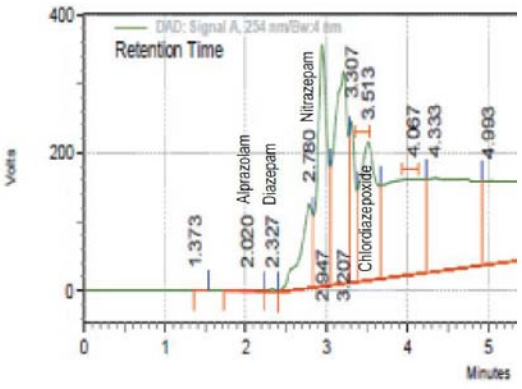

Fig. 8: Chromatograms of alprozolam, chlordiazepoxide, diazepam and nitrazepam in mobile phase D with a flow rate of 1mL/min at 25°C column temperature at drug concentration 100 µg/mL

The mobile phase contains no buffer. Isopropyl amine for the modification of pH can avoid some problems, which often happened in an HPLC assay when the buffer issued. For example, crystal termed in connecting tubing and detector cells, as well as damage to the pump seals [14].

Reversed phase C_{18} column was used and the column temperature was set at 25°C; the details of the mobile phase is given in table1; The flow rate is 1.0 and 1.5 mL/min and UV detector wavelength was set at 254 nm. In this pilot study, this protocol could determine four types of BZDs, such as alprazolam, chlordiazepoxide, diazepam and nitrazepam at the retention times of 1.990, 1.323, 2.401 and 2.501 mins respectively. However, we could not quantify these in this study because of the lack of pure standards. The limit of quantitation was lower than the therapeutic range of BZDs (except for nitrazepam & alprazolam). Therefore, the method can be used in routine forensic applications. The BZD drug mixture at various concentrations (50, 100 and 150 µg/mL) were analysed with the aforementioned chromatographic conditions which has been shown in Fig. 5-8.

The nature of the sample, its molecular weight and solubility determines the proper selection of the stationary phase. The non-polar drug alprozolam, chlordiazepoxide, diazepam and nitrazepam are preferably analysed by reverse phase columns and accordingly C_{18} column was selected. The concentration of methanol and acetonitrile were optimised to give symmetric peak with short run time based on asymmetric factor and peak area obtained.

Different mobile phases were tested but satisfactory separation, well resolved and good symmetrical peaks were obtained with the mobile phase methanol: acetonitrile: water 45:40:15 (v/v/v). The obtained chromatogram for this system shown in Fig. 5. The retention time of alprazolam, chlordiazepoxide, diazepam and nitrazepam was found to be, 10.610, 19.342, 18.121 and 24.246 mins, respectively with a good baseline.

A good reliable separation has also been achieved with the mobile phase methanol: acetonitrile: water 70:25:5 (v/v/v). The obtained chromatogram for this system is shown in Fig. 6. The retention time of alprazolam, chlordiazepoxide, diazepam and nitrazepam was found to be, 12.491, 1.860, 22.108 and 2.789 mins, respectively, indicates a good baseline. The retention times for all of the mixture samples are given in Table 2.

Table 2: The relative retention time for the studied BZD drugs

Mobile phases	**Retention times (min)**			
	Alprazolam	**Chlordiazepoxide**	**Diazepam**	**Nitrazepam**
A	2.853	3.583	3.200	2.800
B	10.610	19.342	18.121	24.246
C	12.491	1.860	22.108	2.789
D	2.020	3.513	2.327	2.780

The absorbance maxima or points of inflexion were also noted to allow comparison of these values with published data [15]. However, this comparison was not always possible as some compounds display a bathochromic or hyperchromic shift depending on the pH and solution conditions under which the measurement [16] is made. While not all compounds in Table 2 were completely resolved chromatographically, in most cases tentative peak identities were assigned on the basis of retention time and confirmed by examining the UV spectrum.

In order to evaluate an efficient and universal HPLC method, preliminary tests were performed with the objective to select adequate and optimal conditions. Parameters, such as optimal mobile phase, optimum pH, stationary phase, detection type, detection wavelength, and flow rate were exhaustively studied. The attention was mainly focused on optimisation of the mobile phase and the selection of proper column to obtain satisfactory results eliminating tailing problems.

In this study, our only choice was the detection in UV-Vis. An UV scan of BZD solution in the used mobile phase showed that the absorption maxima in the spectra of studied BZDs are in the range of 240 to 260 nm. The UV detection at 254 nm was found to be suitable without any interference from tablets or capsules excipients and solvent. Sharper and symmetrical peaks appeared with the flow rate in the range of 1.0-1.5 mL/min. The best flow rate was 1.5 mL/min^{-1}.

The chromatogram of excipients used in BZD dosage forms (tablets) shows the absence of interferences for pharmaceutical preparation (Fig. 9). It was concluded that the developed method is selective in relation to the excipients of the final preparations.

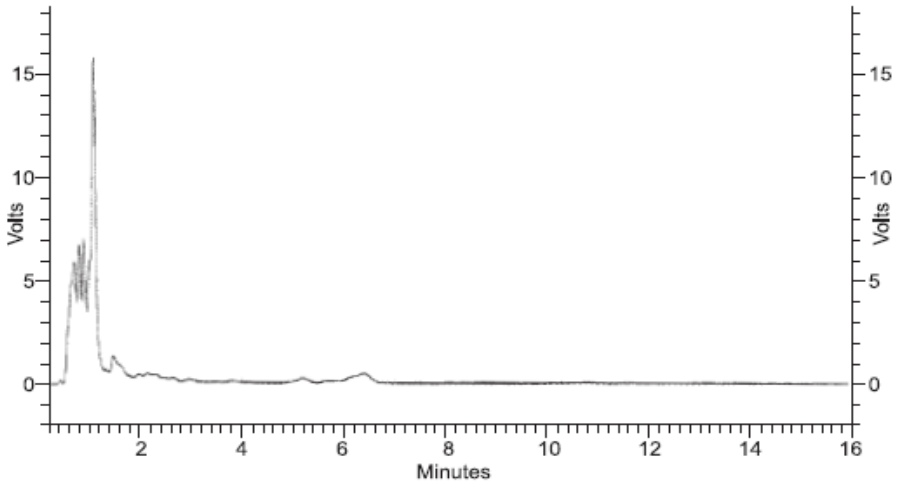

Fig. 9: Representative HPLC chromatogram obtained for a mixture of excipients used in BZD formulation.

Once optimal chromatographic conditions have been established, the method was carried out for the simultaneous detection and separation of these compounds. The representative chromatogram of a BZD mixture obtained under the optimal conditions chosen, Fig. 5-8. This chromatogram indicates the appropriate solution between the compounds investigated. No interfering of peaks was observed in the samples studied (Fig. 5 and 6). This can influence the selectivity of simultaneous determination of these four drugs.

Based on the studied parameters, the developed method is applicable for simultaneous detection and determination of most 1,4-benzodiazepines. The proposed method is simple, rapid, accurate, precise and specific. Its chromatographic run time of 10-30 mins, which allows the analysis of a large number of samples in short period of time. Therefore, it is suitable for the routine analysis of drug alprazolam, chlordiazepoxide, diazepam and nitrazepam in pharmaceutical dosage form.

Conclusions

Numerous aspects of separation science are applicable to forensic science. Because HPLC is so versatile and can be used to determine so many different compounds, the technique is particularly well suited to the demands of a forensic laboratory. Both qualitative and quantitative information can be obtained, often with minimal sample preparation. Because only small volumes are needed for analysis, sample consumption can be minimised. Eluting fractions can be collected for further analysis an important consideration when dealing with trace evidence. HPLC offers a cost effective technique with the ruggedness and reliability necessary for forensic testing and consequently is widely used in forensic laboratories today. This HPLC procedure was developed for the simultaneous determination and detection of four BZDs: alprazolam, chlordiazepoxide, diazepam and nitrazepam. It appears that the technique is rapid, simple and suitable for routine analysis.

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