Simultaneous Separation and Detection of Clobazam, Clonazepam, Flurazepam and Midazolam in Pharmaceutical Dosage Form by Reversed Phase High-Performance Liquid Chromatography

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ABSTRACT: A simple, rapid, and accurate reversed phase high-performance liquid chromatography (RP-HPLC) method was developed for simultaneous separation and detection of benzodiazepines (BZDs) *viz.* clobazam, clonazepam, flurazepam and midazolam in pharmaceutical dosage form. Due to increasing number of pharmaceutical preparations of BZD available, these drugs are frequently encountered in clinical and forensic casework samples involving road traffic offenses and/or drug overdoses. Isocratic elution at a flow rate of 1.0 mL min^{-1} was employed on a symmetry Flexar Quartenary column C18 (250 mm x 4.6 mm, 5µm) at ambient temperature. Four different mobile phase systems were studied. The UV detection wavelength was set at 254 nm and 10 µL sample was injected. The retention time for clobazam, clonazepam, flurazepam, and midazolam were 16.36, 17.91, 28.74 and 21.23 min, respectively. The excipients present in tablets and capsules did not interfere with the developed method. The developed method is simple, rapid, accurate and suitable for routine estimation of pharmaceutical dosage forms and therefore, could be applied to forensic samples.

Keywords: forensic science, benzodiazepine, separation, pharmaceutical dosage forms

Introduction

Benzodiazepines (BZDs) are widely abused class of drugs that used as minor tranquilizers, hypnotics, and muscle relaxant [1]. BZDs are used to aid patients with anxiety and sleeping disorders and, hence, have potential for abuse. Due to the frequency of BZDs 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, which requires faster sample preparation and analysis.

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. Therefore, a rapid and sensitive analytical method became desirable to analyze the trace residual BZDs and/or metabolites in biological fluids, such as urine, and provide valuable information for clinical diagnosis as well as forensic application [3].

In this study, we have chosen four types of BZDs *viz*. clobazam, clonazepam, flurazepam, and midazolam drugs, each having different structural characteristics, 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 [4].

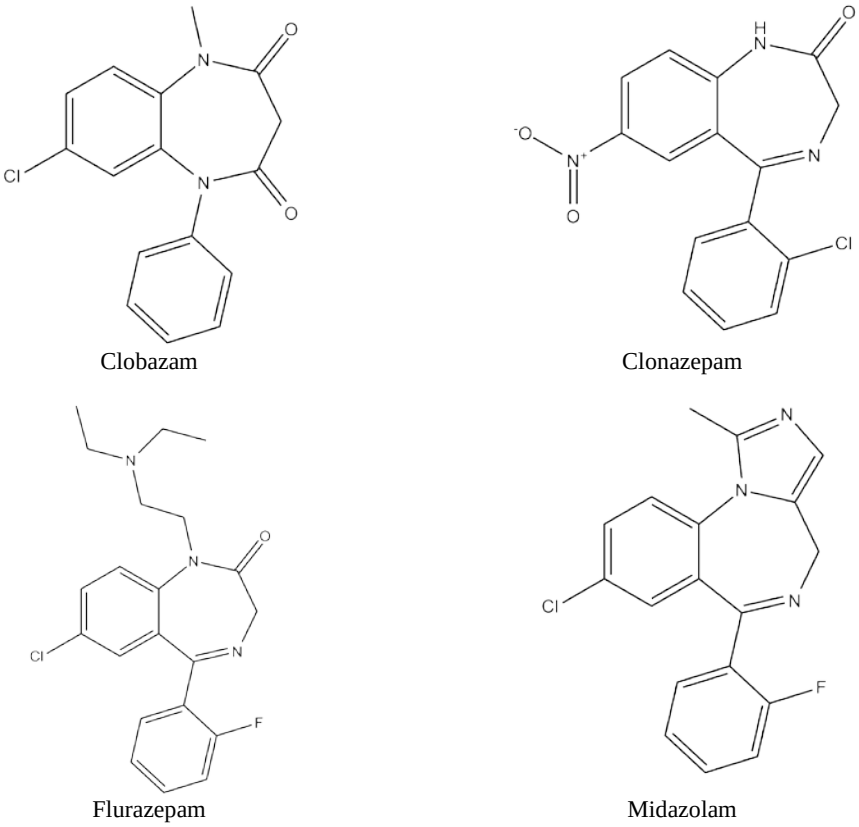

Fig. 1: Structures of studied benzodiazepines

Clobazam (Castillium®, Urbanil®) has been used as an anxiolytic and in the treatment of epilepsy, and it is considered as a relatively safe drug. Paula proenca *et al.* [5] has presented a fatal case with a 49-year-old female, found dead at home. She had been undergoing psychiatric treatment and was a chronic alcoholic. The autopsy findings were unremarkable, except for multi visceral congestion, steatosis and a small piece of a plastic blister pack in the stomach. The clobazam was responsible for the death [6], probably by respiratory depression. Several methods [7,8] for the determination of clobazam have been published.

Gowri Bala Kumari *et al.* [9] have developed and validated a simple, precise and accurate Reversed Phase High-Performance Liquid Chromatography (RP-HPLC) method for rapid assay of clobazam in tablet dosage form. Gazdzik *et al.* [10] has described a method for simultaneous determination of Clobazam and its active metabolite N-desmethylclobazam in various biological samples by RP-HPLC method with UV detection. The determination of both clobazam and N-desmethylclobazam was performed without derivatization.

Clonazepam, a nitrobenzodiazepine and a scheduled drug, is one of the highly abused drugs in recent times. Idris *et al.* [11] has developed an analytical methodology for the detection and quantitation of clonazepam in chocolate sample. Several methods [12-14] have been described for analysing clonazepam. Aboul-Enein and Thiffault [15] have described an accurate and reproducible method for the analysis of flurazepam hydrochloride in pharmaceutical preparations. It was a simple and rapid isocratic HPLC elution method was employed, which requires about 15 minutes to be performed. Dammalapati and Rudra Raju [16] have reported a new, sensitive, fast, precise, RP-HPLC method for the determination of flurazepam in (capsule) dosage form.

Dadgar *et al.* [17] have developed a method for the determination of flurazepam and its metabolites in human blood plasma. Selinger *et al.* [18] described a sensitive isocratic HPLC method, which allows the precise and accurate quantification of flurazepam and four metabolites with a single determination. Mehta [19] had reviewed the methods available to date for the determination of chlordiazepoxide, clonazepam, diazepam,

flurazepam, lorazepam, nitrazepam, oxazepam and their metabolites in biological fluids.

The pharmacokinetics and bioavailability of these compounds and their concentrations in serum in cases of abuse, forensic cases, drug poisoning or suicidal excessive doses have been reported [20]. Dordevic *et al.* [21] reported the use of photodiode array (HPLC-PDA) and mass spectrometric (LC-MS) detection for determination and confirmation of midazolam in biological samples in therapeutic or toxic concentration.

Nishiyama and Hanaoka [22] have reported two cases of overdoses of intramuscular midazolam used as a premedication. Both cases had no resedation or complications, but the accidents happened because of a resident and nurse's lack of experience with midazolam. The postmortem tissue midazolam concentration reported in a death caused by self-injection of midazolam and sulfentanil. Michalodimitrakis *et al.* [23] have reported midazolam related death of a 63-year-old man that occurred during endoscopic retrograde cholangiopancreatography, after receiving 10 mg of midazolam. The acute intoxication due to midazolam overdose was confirmed by HPLC analysis.

HPLC provides a quick and reliable method for rapid separation and detection of pharmaceuticals and forensic samples. For decades, HPLC has been the cornerstone of comprehensive drugs screening in forensic toxicology [3]. Despite the versatility of today's analytical arsenal HPLC continues to maintain a prominent position in the field. In recent years, several important innovations have enabled better utilization of the HPLC technique in screening analysis.

In this study, four different types of mobile phases were studied on clobazam, clonazepam, flurazepam and midazolam drugs. There was lacking in published HPLC methods that are applicable to simultaneous separation and detection of BZD. 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. For these reasons, the analysis of BZDs is of great interest to forensic and clinical toxicologists.

The objective of this research is to develop a universal, rapid, precise and sensitive RP-HPLC method for the separation and detection of clonazepam, clobazam, flurazepam and midazolam in bulk powder and in pharmaceutical dosage form as well as in confiscated materials.

Materials and methods

Materials

Benzodiazepine drugs *viz.* clonazepam, clobazam, flurazepam and midazolam drugs were of pharmaceutical grade (India). All of the solvents (methanol, acetonitrile and water) used were of HPLC grade. The solvents were purchased from Qualikem Fine Chem Pvt. Ltd., New Delhi, India.

Preparation of solutions

Preparation of standard solutions
Standard solutions of Clonazepam, Clobazam, flurazepam and midazolam were prepared by taking 5 mL stock solution of each drug with a calibrated pipette and placing them in a 25 mL volumetric flask. The flask was filled with methanol to get the desired concentrations.

Preparation of stock solutions
Stock solutions of 1 mg/mL of clonazepam, clobazam, flurazepam, and midazolam were prepared. It was dissolved in an appropriate amount of the methanol solution, and stirred using a magnetic stirrer for a period between 15–30 min until it was completely dissolved.

Preparation of working solutions
Working solutions of 150, 100 and 50 µg/mL clonazepam, clobazam, flurazepam and midazolam were prepared separately. These were freshly prepared for every experiment.

Preparation of sample solution
Clonazepam, clobazam and flurazepam, both tablet and capsule samples were prepared separately by weighing 20 tablets individually and the average weight per tablet was calculated. The tablets were ground to get a fine powder. The powder equivalent to 30 mg of clonazepam, clobazam, and flurazepam, were weighed and placed in a 100 mL volumetric flask. The powder was dissolved with methanol and mixed thoroughly on a magnetic stirrer for half an hour. The flask was filled with methanol and mixed. The solution was then filtered through Buchner's funnel with 0.45 mm filters. 50 mL of filtrate

was placed into a 100 mL volumetric flask and was filled with methanol to obtain 1 mg/mL solution of clonazepam, clobazam and flurazepam.

Midazolam ampoule samples were prepared from (50 mg/10 mL) ampoule solution. The ampoule solution was mixed with methanol for 5 min and 3 mL of solution was placed into a 100 mL volumetric flask. The volume was filled with methanol and mixed well to obtain 1 mg/mL solution of midazolam.

Chromatographic Conditions

The analysis of clonazepam, clobazam, flurazepam, and midazolam drugs were carried out on Shimadzu HPLC model (VP series) containing LC-10AT (VP series) pump, variable wavelength programmable UV/visible detector SPD-10AVP and Rheodyne injector (7725i) with 20 µl fixed loop. To develop an HPLC method for quantitative estimation, an isocratic HPLC instrument with Phenomenex C18 column (150 mm x 4.6 mm, 5µm) was used. A 20 µL Rheodyne injector port was used for injecting the samples. The mobile phases were pumped from the solvent reservoir to column at a flow rate of 1 mL/min and the injection volume was 20 µL. The column and the HPLC system were kept at ambient temperature. The mobile phase was prepared fresh and it was degassed by sonicating for 5 min before use. The eluents were monitored at 254 nm with a run time of 10 min.

Results and Discussion

In this study, mobile phases and with its composition are shown in Table 1 to separate and to detect the BZD drugs *viz.* clonazepam, clobazam, flurazepam and midazolam. The relative retention times recorded for the studied BZDs are illustrated in Table 2.

Table 1: 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)

Table 2: Relative retention time for the studied BZD drugs

Mobile phases	Retention times (min)			
	Clobazam	Clonazepam	Flurazepam	Miazolam
A	2.21	3.20	5.01	3.41
B	16.36	17.91	28.74	21.23
C	9.32	15.34	13.07	15.79
D	1.37	2.02	4.99	2.95

Respective standard chromatograms were given to the individual BZD drugs in Fig. 2-5. Simultaneous separation and detection are shown in Fig. 6-9, which were recorded in different concentrations. This HPLC procedure was developed for simultaneous estimation of four BZDs. Clobazam, clonazepam, flurazepam and midazolam. It appeared that the technique is rapid, simple and suitable for routine analysis.

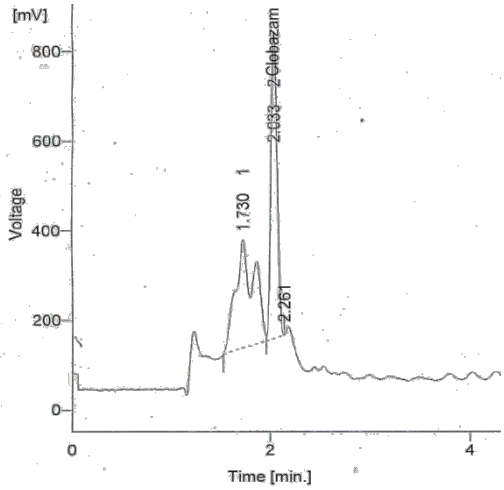

Fig.2: Chromatogram of clobazam in mobile phase A with a flow rate of 1 mL/min at 25°C column temperature

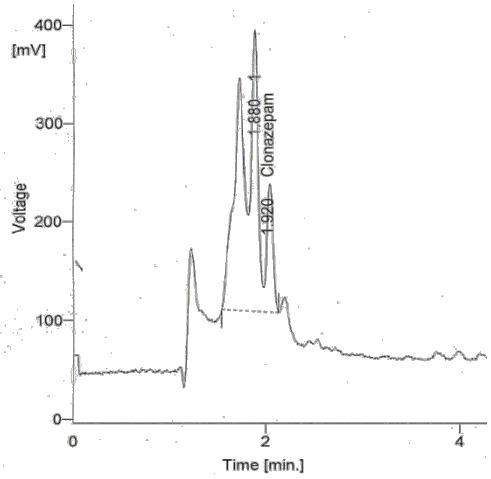

Fig. 3: Chromatogram of clonazepam in mobile phase A with a flow rate of 1 mL/min at 25°C column temperature

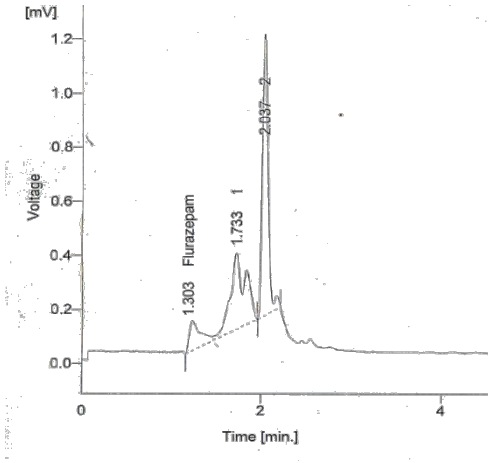

Fig. 4: Chromatogram of flurazepam in mobile phase A with a flow rate of 1 mL/min at 25°C column temperature

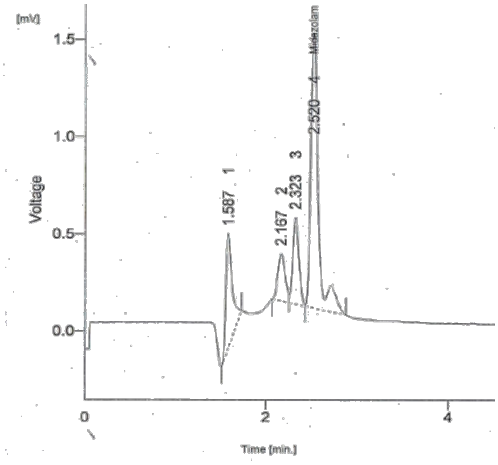

Fig. 5: Chromatogram of midazolam in mobile phase A with a flow rate of 1mL/min at 25°C column temperature

In this pilot study, we could determine four types of BZDs, *viz.* clobazam, clonazepam, flurazepam and midazolam at the retention times of 2.03, 1.92, 1.30 and 2.52 min, respectively. However, we could not quantify these in this study because of the lack of pure standards. It can also be used to quantitate known BZDs. This method could be used in routine forensic applications.

The BZD drug mixture were analysed with various concentration like 50, 100 and 150 µg/mL with the aforementioned chromatographic conditions, Fig. 6-9 for concentration 150 µg/mL. The above-mentioned concentrations for determination of Limit of detection (LOD) was carried out with reliable separation and detection at the concentration of 150 µg/mL.

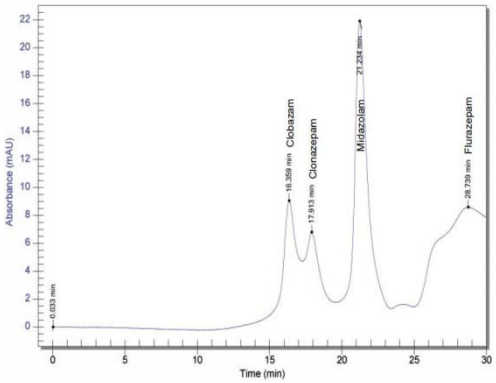

Fig. 6: Chromatograms of clobazam, clonazepam, flurazepam and midazolam in mobile phase B with a flow rate of 1mL/min at 25°C column temperature at drug concentration 150 µg/mL

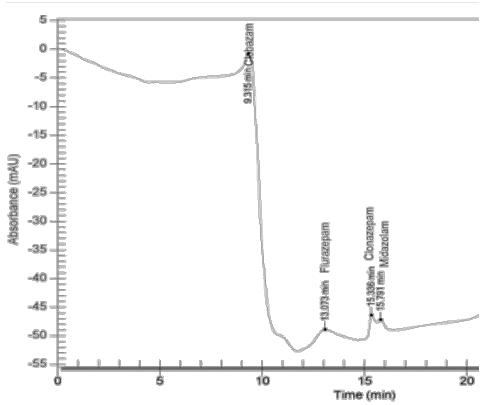

Fig. 7: Chromatograms of clobazam, clonazepam, flurazepam and midazolam in mobile phase C with a flow rate of 1mL/min at 25°C column temperature at drug concentration 150 µg/mL

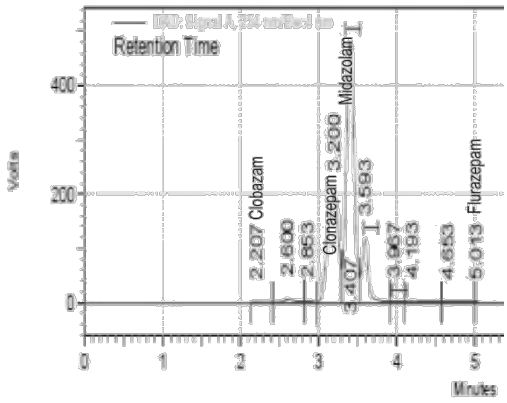

Fig. 8: Chromatograms of clobazam, clonazepam, flurazepam and midazolam in mobile phase A with a flow rate of 1mL/min at 25°C column temperature at drug concentration 150 µg/mL

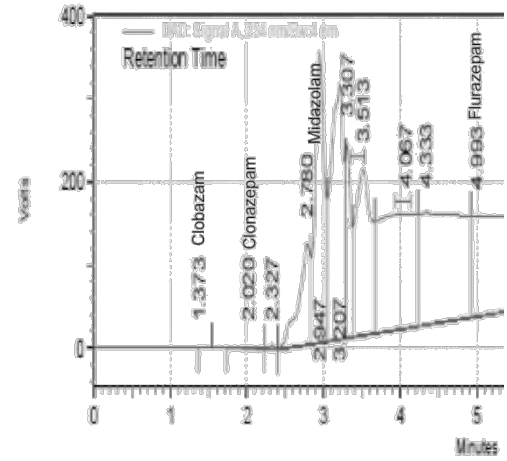

Fig. 9: Chromatograms of clobazam, clonazepam, flurazepam and midazolam in mobile phase D with a flow rate of 1mL/min at 25°C column temperature at drug concentration 150 µg/mL

The nature of the sample, its molecular weight and solubility decide the proper selection of the stationary phase. Clobazam, clonazepam, flurazepam and midazolam are being non-polar is preferably analysed by reverse phase columns and accordingly C18 column was selected. So the elution of the compound from the column was influenced by polar mobile phase. The concentration of the methanol and acetonitrile were optimised to give symmetric peak with short run time based on asymmetric factor and peak area obtained. The obtained chromatogram for the solvent system methanol:acetonitrile:water 45:40:15 (v/v/v) is shown in Fig. 6. The retention time of clobazam, clonazepam, flurazepam and midazolam was found to be 16.36, 17.91, 28.74 and 21.23 min, respectively.

The obtained chromatogram for the system methanol:acetonitrile:water 70:25:5 (v/v/v) which is shown in Fig. 7. The retention time of clobazam, clonazepam, flurazepam and midazolam was found to be 9.32, 15.34, 13.07 and 15.79 min, respectively.

HPLC retention time data for each of four compounds examined are listed in Table 2. The absorbance maxima or points of inflexion were also noted to allow comparison of these values with published data. 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 is made. While not all compounds listed in Table 2 were completely resolved chromatographically, in most cases tentative peak identities were assigned based on retention time and confirmed by examining the UV spectrum.

The reproducibility [24] of retention times in HPLC was poorer than in GC, and reproducibility of methods between laboratories is even less reliable, in spite of the use of alkyl aryl ketones and other markers to calculate retention indices for RP-HPLC, which has been shown to have some merit [25].

In order to evaluate an efficient and universal HPLC method, preliminary tests were performed with 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 mobile phase and the selection of proper column to obtain satisfactory results eliminating tailing problems.

In this study, we chose UV-Vis detection. A UV scans of BZD solution 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 mL min^{-1}.

The chromatogram of excipients used in BZD dosage forms (tablets, capsules and ampoules) shows the absence of interferences for pharmaceutical preparation (Fig. 10).

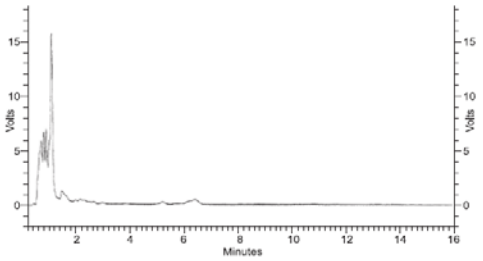

Fig. 10: Representative HPLC chromatogram obtained for a mixture of excipients used in BZD formulation.

Once optimal chromatographic conditions have been established, the method was carried out for simultaneous detection and separation of these compounds. The representative chromatogram of a BZD mixture obtained under the optimal conditions chosen is shown in Fig. 6-9. The chromatogram indicated the appropriate resolution between the compounds investigated. No interfering of peaks was observed in the samples studied (Fig. 6 and 7). This can influence the selectivity of simultaneous separation and detection of these four drugs. It has also been emphasised that the advantage of this developed method is better than existing works.

The proposed method is simple, rapid, accurate, precise and specific. Its chromatographic run time was 10 min, 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 clobazam, clonazepam, flurazepam and midazolam in pharmaceutical dosage form. In most countries, BZDs are classified as controlled drugs, and yet they are frequently encountered in clinical and forensic toxicology analyses involving intoxication, over dosage, and traffic accidents and are sometimes implicated in the commitment of crimes. Therefore, the availability of reliable, sensitive, specific and fast analytical methods for their determination is deemed important.

Conclusion

BZDs are now among the most commonly prescribed drugs, which increases their potential for addiction, abuse and often they are found in combination with other drugs in drug-related fatalities or DFSC. 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.

In this study, HPLC procedure has been established for the simultaneous separation and detection of four BZDs, namely clobazam, clonazepam, flurazepam and midazolam. We could emphasise that the advantage of this developed method is better than existing works. It appears that the developed method is simple, rapid and accurate and is suitable for routine analysis of pharmaceutical dosage forms and it could be applied to the forensic samples.

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