

## Profiling of Benzodiazepines using Fluorescence Spectrofluorometry: A Systematic Review

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### Abstract

Benzodiazepines (BZDs) are one of the most widely used psychoactive drugs for the treatment of anxiety and panic disorders, insomnia, muscle relaxation, epilepsy among other purposes. Given its rampant consumption worldwide, BZDs are used in a number of drug facilitated sexual assaults (DFSA), suicides and driving under the influence of drugs. Therefore, BZDs and their metabolites are commonly detected in both clinical and forensic cases. Hence, there's a need to develop a simple and efficient method for the detection and determination of BZDs in different biological specimens. This paper provides a summary of methods for the detection and quantification of BZDs and their metabolites in commonly used biological matrices. The commonly used methods are: GC-MS, HPLC, TLC, spectrophotometry and spectrofluorometry.

**Keywords:** Benzodiazepines, Biological specimen, Central nervous system, spectrofluorometric.

**List of abbreviations:** BDZs: Benzodiazepines; GC-MS: Gas Chromatography–Mass Spectrometry; HPLC: High Performance Liquid Chromatography; TLC: Thin-Layer Chromatography; DFSA: Drug Facilitated Sexual Assaults

## Introduction

Introduced in the 1960s, Benzodiazepines (BZDs) are a class of psychoactive drugs, which are useful for treating insomnia, anxiety, epilepsy, muscle relaxation and relief from spasticity caused by central nervous system pathology [1,2]. BZDs are one of the most widely prescribed drugs in the world [1]. In 2007, more than 112 million people were prescribed benzodiazepines in the United States [1]. Women are prescribed BZDs at a rate two times higher than men and their usage increases with age [1,2]. The annual prevalence of BZDs is around 2% to 17% and varies from country to country [1]. BZDs amplify the effect of Gamma-aminobutyric acid (GABA), the major inhibitory neurotransmitter in the brain [3]. BZDs are classified on the basis of short, intermediate and long elimination half-life. Long-acting BZDs have the highest average elimination half-life of 40-250 hours, followed by intermediate-acting BZDs (12-40 hours) and short-acting BZDs (1-12 hours) [1]. BZDs are also characterized in terms of relative potency. The first lot of BZDs such as chlordiazepoxide, oxazepam and temazepam were of low to medium potency and were useful in the treatment of insomnia and anxiety [1]. Later, high-potency BZDs (alprazolam, lorazepam, and clonazepam) were found effective against panic disorders, obsessive-compulsive disorder and in the treatment of acute mania or agitation [1]. The commonly used biological matrices for detection and quantification of BZDs are blood, urine, and saliva [4]. There are many reliable analytical methods for the determination of Benzodiazepines (BZDs) in biological specimens and pharmaceutical preparations are TLC, HPLC, GC, immunoassays, capillary electrophoresis, capillary electro-chromatography, spectrophotometric, spectrofluorometric and potentiometric, polarographic, voltammetry [3]. However, a quick, cost-effective and sensitive method for the detection of BZDs is yet to be discovered [4]. It will help prevent the increasing misuse of BZDs around the world [4]. This review aims to analyze previous studies on the detection and determination of Benzodiazepines in biological samples by fluorescent spectrofluorometry.

## Methods reviewed so far

A specific spectrofluorometric micro method was developed for the determination of chlordiazepoxide in plasma of humans, rats and dogs. The extraction of chlordiazepoxide was followed by hydrolysis to a lactam derivative. Under the influence of light, the lactam is converted into a compound that gave rise to fluorescence in alkaline solution. The method had a limit of sensitivity of 0.25 $\mu$ g/ml for either compound and was sufficient for measuring plasma levels of chlordiazepoxide in human (10-30 mg) after normal therapeutic doses. The half-lives of chlordiazepoxide were 20-24 hr, 14-20hr, and 4-6hr in humans, dogs, and rats, respectively. Though, lactam showed a half-life similar to that of chlordiazepoxide, when administered orally to dogs [5]. A fluorometric method was developed for detecting chlorazepate, chlordiazepoxide, diazepam, oxazepam and their metabolites in gastric content, blood and urine at low therapeutic concentrations by first hydrolyzing drugs to their respective benzophenones and converting them to highly fluorescent products, 9-acridanones. This procedure failed to detect therapeutic concentrations of flurazepam and its metabolites in blood. The fluorometric response for chlordiazepoxide and diazepam was a linear function of concentration from 0.2 to 10 mg/liter whereas; the linearity of the fluorometric response of other benzodiazepines was not studied [6]. This paper explained a simple procedure for the toxicological detection of BDZs in serum and methods and concentrations associated with high-dose intravenous therapy with diazepam. The five BDZs (oxazepam, clorazepate, chlordiazepoxide, flurazepam and diazepam) and their metabolites were extracted from a buffered serum sample at pH 9.2 by a solvent comprising toluene, hexane, isoamyl alcohol. The extracted organic phase was separated by centrifugation and a small aliquot of the organic layer was injected into a GC-ECD system which resulted in identification and quantitation of drugs. This method takes around 30 minutes. This technique was proved by analyzing samples from a patient suffering from delirium tremens and was administered high doses of diazepam intravenously. It was found that the concentration of serum diazepam, N-desmethyldiazepam, and oxazepam were several times higher than usual therapeutic dose [7]. This simple Thin-Layer Chromatography (TLC) technique was proposed for detection of benzodiazepines and tricyclic antidepressants by fluorescence in serum from emergency-room patients. Post absorption of compounds on charcoal and extraction via an organic solvent mixture composed of Acetone, dichloromethane and concentrated Ammonium hydroxide; the drugs are analyzed by TLC and are easily detected by their fluorescence. Being quick and fast, this method requires 1.0 mL of serum and is ideal for emergency toxicological detection. It also allow for the detection of benzodiazepines and tricyclic antidepressants in serum instead of urine. Serum better detects a patient's toxicity compared to urine. The limit of detection for each of the drugs is around 0.75 mg/L [8]. The micro analytical method

was reported for determination of diazepam and its pharmacologically active metabolites in biofluids by Bond Elut™ Column and Reversed-Phase High-performance liquid chromatography.

The drug and its major metabolites were extracted from 50-100µl biofluid samples using Bond Elut™ C<sub>18</sub> Column and quantitated by high-performance liquid chromatography. The extraction and recovery of drug and its active metabolites from blood were 88% higher than other compounds. The minimum detection range of each compound was around 2.5 ng per 100µl sample [9]. The two fluorimetric and photochemical - fluorimetric methods were described for the determination of lorazepam in serum and tables. Irradiation of lorazepam in 0.2M NaOH solution resulted in fluorescent photoproduct. The fluorimetric method, applied for the detection of lorazepam in dosage form, had a detection limit of 0.3 ng ml<sup>-1</sup> while a photochemical - fluorimetric method, applied for the determination of lorazepam in serum, has a detection limit of 0.3 ng ml<sup>-1</sup> [10]. The molecular rearrangement of benzophenones to 9- acridones was studied. Acridanones were synthesized from sixteen benzodiazepines by using dimethyl sulphoxide (DMSO) and sodium hydroxide as cyclization solvent and were analysed by HPLC coupled with a fluorescence detector. This method is suitable for the detection of these compounds in biological specimens at therapeutic concentrations. The limit of detection was 50 ng/ml of urine for clonazepam, flunitrazepam, flurazepam and lormetazepam, 500 ng/ml for nitrazepam and 2 ng/ml for oxazepam, chlordiazepoxide, clorazepate, nordiazepam, diazepam, lorazepam, pinazepam, prazepam, camazepam, temazepam and 2,5-dichloronordiazepam. Calibration curves for diazepam and oxazepam gave a correlation coefficient of 0.9994 and 0.9915, respectively. It takes about 15 minutes to separate all synthesized acridanones [11]. A spectrofluorimetric method was described for the determination of tofisopam in DMSO (dimethyl sulphoxide) in the presence of NaOH at 90-100 °C. It resulted in 1-ethyl-4-(3,4-dimethoxyphenyl)- 6,7-dimethoxy-2-naphthol with intense fluorescence. The excitation spectra had maxima at 400nm while the emission spectra had maxima at 488nm. The linear calibration graph for tofisopam in the concentration range was  $5 \times 10^{-5}$  to  $5 \times 10^{-5}$  g ml<sup>-1</sup>. Though there was deviation from linearity at higher concentration possibly due to concentration quenching [12]. A smallbore HPLC method was developed for the determination of clonazepam in pharmaceutical dosage forms. This method utilized a 150×3.0 mm i.d. column packed with 3 mm octyldecylsilane particles. While the current USP used 300×3.9 mm i.d. conventional octyldecylsilane column for analysis of clonazepam. The retention times for clonazepam and the internal standard on the 3.0 mm i.d. column were 4.0 and 12.5 min, respectively. The intra- and interday RSDs on the 3.0 mm i.d. column were < 0.55% (n=4) using the internal standard, and < 0.19% (n=4) without the internal standard at the lower limit of the standard curve, 50 mg ml<sup>-1</sup> and had a limit of detection of 24 ng ml<sup>-1</sup> [13].

The author developed a simple, novel and fully automated technique for the fluorimetric detection of 1,4-benzodiazepines in pharmaceutical formulations using Flow-injection technique. The FI technique is based on the formation of fluorescent products post hydrolysis with sulphuric acid in ethanolic or methanolic medium at room temperature. The linearity of oxazepam, diazepam and nitrazepam were found in the range of 0.025–0.150 mg ml<sup>-1</sup>, 0.010-0.125 mg ml<sup>-1</sup> and 0.010-0.150 mg ml<sup>-1</sup>, respectively, while the limit of detection were 0.01, 0.005 and 0.005 mg ml<sup>-1</sup>, respectively. The measurement throughput is approximately 60 h<sup>-1</sup> using a 200µl sample derived by dissolution of formulations in alcohol [14]. A high-throughput analysis of benzodiazepines in human urine by using LC/MS/MS. Using a simple liquid-liquid extraction in 96-well plates, the analytes were extracted from 1152 human urine samples along with their deuterium-labeled internal standards. Using an electronic switching box, the autosamplers were synchronized with the mass spectrometer. This is to ensure that injections were made as soon as the mass spectrometer was ready to collect data. Each run required 30 s to complete. For the duration of the analyses, Chromatographic integrity and ion current response remained relatively constant. The results were satisfactory, accurate and showcased the viability of using fast separations with tandem mass spectrometry for high-throughput analysis of biological samples containing multiple analyses [15].

Two spectrophotometric methods were developed for the determination of clonazepam and bromazepam in pure and pharmaceutical formulations. Based on the formation of C.T complexes between each of bromazepam, clonazepam and chloranil in chloroform or ethanol at alkaline pH, the proposed methods measured the absorbance of the formed C.T. complexes at  $\lambda=330$  nm and  $\lambda=375$ nm for bromazepam and clonazepam, respectively. For bromazepam, linearity was obtained in the range of 31.6–316.0 µg/mL, while for clonazepam it was 63.2–316. µg/mL. The average recovery obtained for bromazepam was 99.00–99.60%, while for clonazepam was

98.57–99.60%. Standard deviations of 0.6–2.00 and 0.75–1.11 were obtained for bromazepam and clonazepam tablets, respectively [16]. Two new photometric and fluorimetric methods were developed for the detection of diazepam, bromazepam and clonazepam in its pure form, pharmaceutical formulations and urine samples. Fluorimetric methods are highly sensitive with low detection limits as opposed to photometric methods. In photometric methods, the linear calibration curves for diazepam, bromazepam and clonazepam were found in the range of 2.85–28.5, 0.316–3.16, and 0.316–3.16 ng ml<sup>-1</sup> with detection limits of 1.27, 0.08 and 0.13 µgml<sup>-1</sup>, respectively. For fluorimetric methods, the linear calibration curves for diazepam, bromazepam and clonazepam were found in the range of 0.03–0.34, 0.03–0.32 and 0.03–0.38 µgml<sup>-1</sup> with detection limits of 7.13, 5.67 and 16.47 ng ml<sup>-1</sup>, respectively [17]. In this paper, author developed high-performance liquid chromatographic method for assay of Clonazepam in plasma by using a non-porous silica column packed with 2mm particles. Through column extraction, clonazepam in plasma was first purified and injected onto a non-porous silica column. The linearity of calibration curve was 5- 200 ng/ml. The recovery of clonazepam was more than 94.0% with a coefficient of variation from 5.1–13.8% [18]. In this paper, author developed and validated the liquid chromatography-tandem mass spectrometry (LC-MS-MS) method for the determination of eight benzodiazepines in human urine by using solid phase extraction technique. The solid phase extraction with the polymer-based mixed mode column was done then the sample measuring 0.5ml was hydrolyzed with β-glucuronidase at 60°C for 2 hrs. Except 7-aminonitrazepam and α-hydroxyalprazolam, deuterated analogues were used as internal standards for all analytes. 7-aminonitrazepam and α-hydroxyalprazolam were quantified using 7-aminoclonazepam-d4 and alprazolam-ds, respectively. The concentration range for 7-aminonitrazepam, 7-aminoclonazepam, 7-aminoflunitrazepam, alprazolam, and α-hydroxyalprazolam was 0.1–8.0 µM and 0.5–40 µM for other compounds (Oxazepam, 3-OH-Diazepam and N-Desmethyldiazepam). The average recovery ranged from 56% to 83% for different analytes. The limits of quantification were found to be between 0.002 and 0.01 µM for other compounds [19].

A sensitive and selective gas chromatography–tandem mass spectrometry method was presented for determination of diazepam and its main metabolites in urine. Using xenon as collision gas, this method involves electron capture ionization and multiple reactions monitoring performing CID. The transfer of energy produced three product ions- two for nordazepam and diazepam and one for decomposed oxazepam. The method used liquid/liquid extraction and produced recovery yields between 68 and 95% with coefficient of variation below 6% for 10 samples. The method used 1mL of urine to yield quantitation limits of 0.15ng/mL, 1.0 ng/mL and 1.5ng/mL for diazepam, nordazepam and oxazepam, respectively [20]. The spectrofluorimetric method was developed for quantification of bromazepam using a highly selective optical probe based on Eu<sup>3+</sup>–bromazepam complex in pharmaceutical preparations and serum samples. The bromazepam increased the luminescence intensity of the Eu<sup>3+</sup> ion in Eu<sup>3+</sup> -bromazepam complex at ex 90nm. The working range, detection limit (LOD) and quantitative detection limit (LOQ) for the determination of bromazepam were 2.3×10<sup>-8</sup> to 6.2×10<sup>-7</sup>, 3×10<sup>-9</sup> and 1.2×10<sup>-8</sup> M, respectively. Whereas, in case of quantum yield the working range, (LOD) and LOQ) were 3.7×10<sup>-8</sup> to 3.4×10<sup>-7</sup> M, 3.4×10<sup>-9</sup> and 9.2×10<sup>-8</sup> M, respectively [21].

Two simple and rapid UV-Derivative Spectrophotometry and HPTLC UV-Densitometric methods were described for the determination of oxazepam in tablet dosage form. The UV-derivative spectrophotometry method was used for quality control of oxazepam because some of its derivatives and wavelengths were linear, precise, and accurate and showed satisfactory recovery. When calibration curve was estimated using nonlinear regression analysis, HPTLC UV-densitometric method showed good results. The HPTLC method was developed with silica F<sub>254</sub> plates, a mobile phase of benzene, ethanol (5:1), and densitometric detection at 204nm receiving R<sub>f</sub>=0.47 [22]. A UV derivative spectrophotometry method was developed for diazepam quantitative assay in human plasma sans separation of the drug from the biological sample using the fourth derivative of the spectra containing small amounts of diazepam (approx. 2-10 µg/mL), by integrated area of the peak ranging from 306 to 333 nm. The method can help determine the therapeutic blood level of diazepam at the preliminary stage of treatment to avoid accidental intoxication [23]. The author developed and validated two simple and accurate spectrophotometric and spectrofluorimetric methods for the detection of tofisopam in pure and pharmaceutical formulations. The spectrophotometric method was based on the reduction of Fe (III) to Fe (II) in 1, 10- phenanthroline to give an orange –red colored ferroin complex exhibits absorbance at 510 nm while, spectrofluorimetric method was based on the oxidative coupling reaction, in which 3-methylbenzothiazolin-2-one hydrazone (MBTH) hydrochloride reacts with tofisopam in presence of cerium (IV) ammonium sulfate in an acidic medium. Ce (III) was obtained upon reaction of

Ce (IV) with MBTH and the fluorescence intensity of the reaction product was measured at  $\lambda_{em}$  = 345 nm with  $\lambda_{ex}$  = 296 nm. The proposed methods obeyed Beer's law in the range of 2-12  $\mu\text{g ml}^{-1}$ . The mean percentage recovery for spectrofluorimetric method was 100.04 whereas, it was  $99.29 \pm 0.563$  for spectrophotometric method [24]. The two spectrophotometric and spectrofluorimetric methods were developed for the detection of alprazolam (ALP) in pharmaceutical dosage forms using As (III)-SDS system. Both these methods were based on complex formation of ALP-As (III) in the presence of SDS. The absorption spectrum of ALP exhibited  $\lambda_{max}$  at 255 nm and a weak band at 325 nm while, the complex spectra exhibited  $\lambda_{max}$  = 265 nm and  $\lambda_{em}$  = 520 nm with respect  $\lambda_{ex}$  = 325 nm. The spectrofluorimetric method was linear over a concentration range of 0.05-9.5  $\mu\text{g ml}^{-1}$  with a limit of detection of  $1.048 \times 10^{-2} \mu\text{g ml}^{-1}$  while spectrophotometric method had 8.0-17.0  $\mu\text{g ml}^{-1}$  range with limit of detection of 13.52025  $\mu\text{g ml}^{-1}$ . Mean recovery was found to be 99.54% with %RSD less than 0.478 and 100.22% with %RSD less than 0.296 for spectrophotometrically and spectrofluorimetrically, respectively [25]. The current available simple, accurate and rapid analytical methods for the detection of 1,4-benzodiazepines (BDZs) in biological matrices and pharmaceutical preparations were studied.

The methods reviewed were capillary electro-chromatography, Photometric (spectrophotometric, spectrofluorometric), chromatography (HPLC, TLC, GC, MLC), capillary electrophoresis and electroanalytical (potentiometric, polarographic, voltammetry) along with modern isolation techniques e.g. ASPEC, SFE, DLLME, MISPE. LC-MS was found to be the most effective technique in sensitivity terms for the detection of ng ml<sup>-1</sup> levels of BDZs in human hair [26].

A simple, validated and accurate spectrofluorimetric method was proposed for the determination of clonazepam (CLZ) in pharmaceutical preparations by reducing the nitro group of clonazepam with zinc/CaCl<sub>2</sub>. This yielded a highly fluorescent product which exhibited strong fluorescence intensity at  $\lambda_{em}$  = 383 nm after excitation at  $\lambda_{ex}$  = 333 nm. The proposed method showed LOD of 0.0057 ng/mL and a LOQ of 0.017 ng/mL and was successfully applied to the determination of CLZ in dosage forms with a mean percentage recovery of  $100.10 \pm 0.75\%$  [27]. This precise, accurate and validated method was developed for the forensic screening of lorazepam, clonazepam, diazepam, midazolam, flurazepam, and chlordiazepoxide in adulterated soft drinks by HPLC-UV. All BDZs showed good linearity ( $r^2 > 0.996$ ) with calibration curves in the concentration range of 0.5- 10  $\mu\text{g/ mL}$ . The limits of detection and quantification were found in the range 0.01- 0.02  $\mu\text{g/mL}$  and 0.03- 0.05  $\mu\text{g/mL}$ , respectively. Recovery rates for BDZs ranged from 93.7- 108.7%. The coefficient of variation for all selected drugs in inter-day and intra-day were found to be in the range 0.45 - 7.69 % [28]. A spectrofluorometric method was developed for the determination of clonazepam (CNP) in pharmaceutical dosage forms and in spiked human plasma. This highly sensitive, simple and cost-effective method was determined and validated by chemical reduction of Zn/HCl followed by enhancement of its fluorescence through utilizing carboxymethylcellulose (CMC). CMC increased the fluorescence intensity of the produced fluorophore by nearly 100%. Calibration curve showed good linear regression ( $r^2 > 0.9998$ ) within test ranges of 20-400 ng ml<sup>-1</sup> with a lower detection limit of 0.67 ng ml<sup>-1</sup> and lower quantification limit of 2.22 ng ml<sup>-1</sup> upon using CMC [29].

A method was developed for detecting Phenazepam in biological samples of patients with acute poisoning. The study found out that TLC gave more accurate results than ICA (Immune Chromatographic Analysis). LC-MS/MS method was proved effective for confirmatory analysis for the native substance, while GC-MS for the products of hydrolysis after derivatization [30].

A switchable solvent based liquid-liquid microextraction combined with differential pulse voltammetry was proposed for electrochemical determination of nitrazepam. With the assistance of HCl and NaOH as pH adjustment reagents, Analyte was extracted using the N,N-dipropylamine. Spiked with the analyte, a switchable solvent was added to the aqueous sample that resulted in the formation of a two phase mixture. Afterwards, a clear and monophasic solution was obtained by adding HCl drop by drop. By the addition of NaOH, the separation of phases was achieved. This resulted in switching back N,Ndipropylamine to its primary hydrophobic form. Lastly, following the evaporation of solvent, the extracted nitrazepam was analyzed by voltammetric methods. Two linear ranges of 0.03-20 ng mL<sup>-1</sup> and 20-450 ng mL<sup>-1</sup> with the correlation coefficients of 0.996 and 0.998 obtained. While the Limits of detection and quantification was obtained 9 ng L<sup>-1</sup> and 0.03 ng mL<sup>-1</sup> respectively [31]. The author developed and validated a precise and accurate UV spectrophotometric method for the quantitative estimation of Diazepam in its pure and pharmaceutical dosage form. Using methanol as solvent, the UV spectroscopic determination method emitted absorption maximum of 266.5 nm.

The linear method obeyed beers law in the concentration range 2-20 µg/ml with a correlation coefficient 0.999 [32]. This research analysed chromatography methods for separation and detection of certain BDZs drugs in pharmaceutical dosage forms and forensic samples (Table 1). The following methods were used for the analysis of selected BZDs: thin layer chromatography (TLC), high-Performance liquid chromatography (HPLC) and gas chromatography-mass spectrometry (GC-MS). It was found that HPLC and

Year	Author	Technique	Sample	Drugs
1963	<i>Spectrofluorometric</i> BERNARD	Spectrofluorometric Micro	Plasma of Humans, Dogs, and Rats	Chlordiazepoxide
1975	Valentour et al.	Fluorometric	gastric content, blood and urine	diazepam, chlordiazepoxide, oxazepam, chlorazepate, and their major metabolites
1979	Kelly et al.	GC-ECD	Serum	Diazepam
1981	Meola et al.	TLC	Serum	Benzodiazepines and Tricyclic Antidepressants
1982	S. N. Rao et al.	Bond Elut™ Column and Reversed-Phase High-performance liquid chromatography	whole blood, serum or plasma	Diazepam and its metabolites
1987	Procopio et al.	spectrofluorimetry and ultraviolet spectrophotometry	Tablets and serum	Lorazepam
1988	Applxations	High-performance liquid chromatography fluorescence	Urine or serum	clonazepam, flunitrazepam, flurazepam, lormetazepam, nitrazepam, oxazepam, chlordiazepoxide, clorazepate, nordiazepam, diazepam, lorazepam, pinazepam, prazepam, camazepam, temazepam and 2,5-dichloronordiazepam
1989	Acta	Spectrofluorimetric method	Grandaxin tablets	Tofisopam
1998	Spell & Stewart	smallbore HPLC	tablet	Clonazepam
1999	Dolejšová et al.	Flow-injection fluorimetric	pharmaceutical formulations	oxazepam, diazepam and nitrazepam
1999	Zweigenbaum et al.	High-Throughput Bioanalytical LC/MS/ MS	Urine	Bromazepam, carbamazepine, estazolam, norfludiazepam, alprazolam, triazolam
2002	Salem et al.	spectrophotometric	Pure and pharmaceutical Dosage form	Bromazepam and clonazepam
2004	Nakamura et al.	HPLC	Human Plasma	Clonazepam
2004	Salem et al.	Spectrophotometric and fluorimetric	pharmaceutical and urine	diazepam, bromazepam and clonazepam
2007	Kinani et al.	gas chromatography- tandem mass spectrometry	Urine	Diazepam and its main metabolites
2009	Attia	Spectrofluorimetric	pharmaceutical and serum	Bromazepam
2009	Koba et al.	HPTLC UV- Densitometric and UV-Derivative Spectrophotometry	pharmaceutical formulations	Oxazepam
2012	El Lakiss et al.	UV Derivative spectrophotometry	Human blood plasma	Diazepam

Year	Author	Technique	Sample	Drugs
1963	<i>Spectrofluorometric</i> BERNARD	Spectrofluorometric Micro	Plasma of Humans, Dogs, and Rats	Chlordiazepoxide
1975	Valentour et al.	Fluorometric	gastric content, blood and urine	diazepam, chlordiazepoxide, oxazepam, chlorazepate, and their major metabolites
1979	Kelly et al.	GC-ECD	Serum	Diazepam
1981	Meola et al.	TLC	Serum	Benzodiazepines and Tricyclic Antidepressants
1982	S. N. Rao et al.	Bond Elut™ Column and Reversed-Phase High-performance liquid chromatography	whole blood, serum or plasma	Diazepam and its metabolites
1987	Procopio et al.	spectrofluorimetry and ultraviolet spectrophotometry	Tablets and serum	Lorazepam
1988	Applxations	High-performance liquid chromatography fluorescence	Urine or serum	clonazepam, flunitrazepam, flurazepam, lormetazepam, nitrazepam, oxazepam, chlordiazepoxide, clorazepate, nordiazepam, diazepam, lorazepam, pinazepam, prazepam, camazepam, temazepam and 2,5-dichloronordiazepam
1989	Acta	Spectrofluorimetric method	Grandaxin tablets	Tofisopam
1998	Spell & Stewart	smallbore HPLC	tablet	Clonazepam
1999	Dolejšová et al.	Flow-injection fluorimetric	pharmaceutical formulations	oxazepam, diazepam and nitrazepam
1999	Zweigenbaum et al.	High-Throughput Bioanalytical LC/MS/ MS	Urine	Bromazepam, carbamazepine, estazolam, norfludaizepam, alprazolam, triazolam
2002	Salem et al.	spectrophotometric	Pure and pharmaceutical Dosage form	Bromazepam and clonazepam
2004	Nakamura et al.	HPLC	Human Plasma	Clonazepam
2004	Salem et al.	Spectrophotometric and fluorimetric	pharmaceutical and urine	diazepam, bromazepam and clonazepam
2007	Kinani et al.	gas chromatography- tandem mass spectrometry	Urine	Diazepam and its main metabolites
2009	Attia	Spectrofluorimetric	pharmaceutical and serum	Bromazepam
2009	Koba et al.	HPTLC UV- Densitometric and UV-Derivative Spectrophotometry	pharmaceutical formulations	Oxazepam
2012	El Lakiss et al.	UV Derivative spectrophotometry	Human blood plasma	Diazepam
2012	Ramadan et al.	spectrophotometric and spectrofluorimetric	Pure and pharmaceutical Dosage form	Tofisopam
2013	Mohd et al.	spectrophotometric and spectrofluorimetric method	pharmaceutical formulation	alprazolam

Year	Author	Technique	Sample	Drugs
2014	Nasir Uddin	HPLC, TLC, GC, MLC, capillary electrochromatography, capillary electrophoresis, immunoassays, spectrophotometric, spectrofluorometric and potentiometric polarographic, voltammetry	biological materials and pharmaceutical formulations	1,4-Benzodiazepines
2016	Ibrahim et al.	Spectrofluorimetric	pharmaceutical formulations	clonazepam
2016	Soltaninejad et al.	HPLC-UV	Spiked Soft Drinks	chlordiazepoxide, diazepam, clonazepam, midazolam, flurazepam, and lorazepam
2017	Belal et al.	Spectrofluorimetric	Dosage form	clonazepam
2018	Belova et al.	TLC,GC-MS,LC-MS/MS,ICA	Urine	Phenazepam
2018	P. V. Rao et al.	UV spectrophotometric	Pure and pharmaceutical Dosage form	Diazepam
2018	Shahraki et al.	Switchable solvent based liquid-liquid microextraction combined with Differential pulse voltammetry	Urine	Nitrazepam
2019	Qriouet et al.	Nuclear magnetic resonance (NMR), chromatography (GC-MS,HPLC, TLC), immunoassay (ELISA, RIA, LFA, CEDEA, FPIA, and KIMS),and electroanalytical methods (voltammetry and potentiometry).	saliva, urine, and blood matrices	Benzodiazepines
2019	Gurusamy & Thangadurai	TLC,HPLC,GC-MS	Pharmaceutical, Forensic sample (viscera)	Alprazolam, Chlordiazepoxide, Clobazam, Clonazepam, Diazepam, Flurazepam, Midazolam, Nitrazepam.

**Table 1:** Shows tabulated summary of profiling and detection of benzodiazepines

GC-MS are the most widely used method for forensic analysis [33]. Common studied techniques for the detection and quantification of Benzodiazepines were: GC-MS, HPLC, TLC, nuclear magnetic resonance (NMR), immunological tests and electro analytical methods. The frequently used biological specimens for the toxicological screening of psychotropic drugs include blood, urine and saliva, whereas HPLC and GC-MS remain the most commonly used analysis methods [34].



## Commonly Used Biological Specimens for the Analysis of BZDs:

**Saliva:**-Due to its noninvasive nature and ease of sampling saliva is ideal for psychotropic drug screening. Some of the common detected drugs are amphetamines, 3,4-methylenedioxy-methamphetamine (MDMA), cocaine, opiates (heroin and codeine), cannabis, and BZDs [4].

**Urine:** Urine is widely used as a biological specimen for the detection of a wide range of substances including BZDs qualitatively [4].

**Blood:** Though blood sample is restrictive to collect compared to urine or saliva, it's the only specimen to indicate whether an individual is under the influence of BZDs, or not, at the time of collection [4].

## Analytical Methods Used For Analysis of BZDs

**High-Performance Liquid Chromatography (HPLC):** HPLC is the go to method for BZDs analysis and quantification as compared to GC method, HPLC does not expose molecules to thermal degradation. Hence, it makes possible to overcome derivatization reactions. A simple sample preparation technique either liquid-liquid or solid-phase extraction is usually suffice [4].

**Gas Chromatography Coupled to Mass Spectrometry:** The GC-MS method is the most powerful tool for identifying BZDs in biological specimen because of its high sensitivity and specificity. It offers dual advantage of quantification and identification of BZDs with often low detection limits. Even though, GC is not perfect given issues related to the thermolability of most BZDs, it's still a reliable method for confirming ambiguous diagnosis [4].

**Thin-layer chromatography (TLC):** The thin-layer chromatography (TLC) is the simplest of the chromatographic methods, but is rarely used for toxicology analysis. Though TLC provided faster results, it lacks specificity and sensitivity and especially the interpretation is delicate [4].

**Potentiometry:** In Potentiometry technique the potential between two electrodes is measured using a high impedance voltmeter. It makes it possible to evaluate the composition of the sample with BZD [4].

**Fluorimetry:** Fluorometry is better than spectrophotometry in terms of sensitivity and specificity. But, Fluorimetric methods are selective with low detection limits compared to photometric methods which showed relatively high detection limits [3].

## Results & Discussion

The most commonly used biological samples for the detection and determination of BZDs are saliva, blood, and urine whereas HPLC and GCMS are the most quantitative and qualitative analysis methods [7]. But, spectrofluorometric method was found to be the most efficient, sensitive and cost-effective for the determination and detection of BZDs in commonly used biological specimens. Various biological specimens and analysis methods have been conjointly used for the detection and quantification of psychotropic drugs, as described in our systematic review is in accordance with the other papers published in this regard. However, the most of the work is carried out on saliva, blood, and urine, whereas the commonly used analysis methods remain the quantitative and qualitative ones including HPLC and GC-MS as of now.

Thin-layer chromatography (TLC) is a handy technique to constrict the possible identities of unknown drugs in biological matrices, but this technique still lacks sensitivity and specificity. Gas chromatographic (GC) methods offer great sensitivity, but compared to HPLC they have lengthy clean-up procedures and in some cases the method lead to formation of more volatile derivatives or hydrolysis prior to analysis. Though for certain benzodiazepines, HPLC still lacks the sensitivity of GC, particularly if the sensitivity

range of the ultraviolet detector is restricted to 0.01 absorbance unit's full scale (AUFS). Of late, liquid chromatography tandem mass spectrometry (LC-MS/MS) has been regularly used for drug analysis in biological samples. Electrokinetic micellar chromatography has also proved to be effective in the analysis of benzodiazepines in urine. Highperformance liquid chromatography (HPLC) is generally used for the separation of benzodiazepines. LC-MS has come across as the best method in sensitivity terms for identification and detection of ng mg<sup>-1</sup> levels of BZDs in human hair. HPLC and GC are widely used to identify specific BZDs in a specimen

## Conclusion

The widespread use of this class of drugs has raised concern about benzodiazepine abuse and has led to the erroneous impression that benzodiazepines have a relatively high abuse liability among recreational drug users. Therefore, the separation and identification of these compounds is of great interest. Methods reported in our paper allowed for a simple, accurate, rapid and reproducible quantification of BDZs as biological samples. The described approaches might be very useful for forensic science laboratories in which extensive benzodiazepines analyses are performed as a part of toxicological analysis. The scope of using spectrofluorometric techniques is increasing gradually.

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