Tropical Journal of Pharmaceutical Research September 2022; 21 (9): 1951-1957
ISSN: 1596-5996 (print); 1596-9827 (electronic)

Available online at http://www.tjpr.org
http://dx.doi.org/10.4314/tjpr.v21i9.19

Original Research Article

Selective simultaneous ultra-performance liquid chromatographic quantification of some benzodiazepines drug residues in pharmaceutical industrial wastewater

Sherif A Abdel-Gawad[1,2], Mubarak A Alamri[1]
[1]Pharmaceutical Chemistry Department, College of Pharmacy, Prince Sattam bin Abdulaziz University, Al-Kharj-11942, Saudi Arabia, [2]Analytical Chemistry Department, Faculty of Pharmacy, Cairo University, Cairo, ET-11562, Egypt

For correspondence: **Email: sagawad@yahoo.com*

Sent for review: 4 May 2022 *Revised accepted: 25 August 2022*

Abstract

Purpose: *To investigate the sensitivity and selectivity of ultra-performance liquid chromatographic (UPLC) quantification of bromazepam (BRZ) and diazepam (DZP) in pharmaceutical industrial wastewater.*
Methods: *Wastewater samples were collected from the effluents of a pharmaceutical industrial plant producing BRZ and DZP in tablet dosage forms. The quantification of BRZ and DZP was done after their solid-phase extraction. The resolution process was performed on WatersTM column as the stationary phase. The mobile phase was acetonitrile: methanol: 0.05 M phosphate buffer (pH 6.5), at a volume ratio of 5:2:3, with a flow rate of 0.7 mL/min. Detection was carried out at 240 nm in a concentration range of 10 – 250 ng/mL. The method was fully validated in line with ICH-Q2B regulations.*
Results: *The UPLC method was validated for the quantification of BRZ and DZP. The relative percentage recoveries were 99.55 ± 0.48 (n = 5) and 101.34 ± 0.86 (n = 5), for BRZ and DZP, respectively, in spiked distilled water, and 99.16 ± 0.77 (n = 5) and 99.32 ± 0.56 (n = 5), in tap water, respectively. The UPLC revealed effluent content ranging from 20.68 – 44.77 mg/mL for BRZ and 22.77 – 41.83 ng/mL for DZP. These values were not significantly different from their reference standards (p > 0.05).*
Conclusion: *A sensitive and selective UPLC-method has been developed for the reproducible determination of BRZ and DZP in industrial wastewater samples. The effective monitoring of the pharmaceutical industrial pollutant will help to conserve the environment and minimize the hazardous effects of these pollutants.*

Keywords: *Bromazepam, Environmental, Benzodiazepines, Diazepam, Wastewater*

Tropical Journal of Pharmaceutical Research is indexed by Science Citation Index (SciSearch), Scopus, Web of Science, Chemical Abstracts, Embase, Index Copernicus, EBSCO, African Index Medicus, JournalSeek, Journal Citation Reports/Science Edition, Directory of Open Access Journals (DOAJ), African Journal Online, Bioline International, Open-J-Gate and Pharmacy Abstracts

INTRODUCTION

Bromazepam (BRZ) and diazepam (DZP) are considered the most common benzodiazepines that are clinically used for the short-term treatment of anxiety, insomnia, and panic attacks [1]. According to the International Narcotics Control Board (INCB), BRZ and DZP are viewed

as the most commonly prescribed benzodiazepines around the world [2]. This enormous use and the increase in their production may lead to their presence as residues in pharmaceutical industrial wastewater, thereby constituting a great danger to the ecosystem and human health [3].

Long-term intake of BRZ may lead to many side effects, including neurotoxicity, decline in cognitive skills, and common hip fractures [4]. Many studies regarding the determination of pharmaceuticals in wastewater and their negative effects on human health have revealed that there are no permissible limits for their occurrence in natural water or soil [5]. Chemically, BRZ and DZP belong to the benzodiazepine class (Figure 1 a and b) [2].

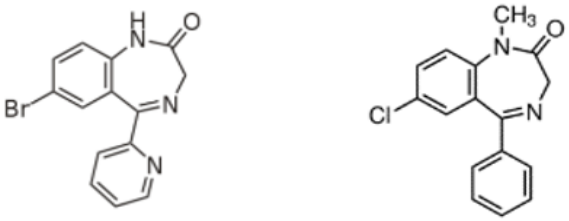

Figure 1: Chemical structures of (a) bromazepam (BRZ) and (b) diazepam (DZP)

Literature survey has revealed that several analytical methods have been used for the detection and quantification of BRZ, including spectroscopy [6], high-performance liquid chromatography (HPLC) [7], and electrochemistry [8]. On the other hand, DZP has been determined using different analytical methods, and in many types of samples, including spectroscopy [9], liquid chromato-graphy (LC) [10], and electrochemistry [11].

An enormous number and amounts of contaminants are discharged into the different water resources. Among these, active pharmaceutical ingredients (APIs) reach the surface water, thereby constituting a great risk to human health and aquatic ecosystems [12]. Despite the common use of BRZ and DZP around the world, it was found that they constitute the least investigated benzodiazepines in surface water [13]. The most frequently applied technique for their determination in the surface water is solid-phase extraction, followed by liquid chromatography coupled to mass spectrometry detection (LC-MS/MS) [14]. This technique suffers from the disadvantages of being multistep, complicated, and non-economical [15]. So, there is a need to develop a simple and economic method that can be adopted for the sensitive and accurate determination of BRZ and DZP in industrial pharmaceutical effluents. The UPLC has many merits over the conventional HPLC including the fact that it is highly sensitive and efficient in resolving mixtures. It also has greater sensitivity and specificity than procedures based on spectrophotometry [16]. A comprehensive survey has revealed the scarcity of articles based on the UPLC quantification of the studied benzodiazepine drug residues in water effluents from the drug manufacturing industry [17]. The main objective of the work was centered on optimization and validation of an accurate UPLC procedure, which can be applied to separate, detect, and quantify BRZ and DZP in water effluents from the drug industry after their pretreatment using solid-phase extraction (SPE).

EXPERIMENTAL

Instruments

Waters™ Acuity system was used for UPLC analysis. It comprised a column 10 cm in length and 2.1 cm in internal diameter. The column was packed with 1.7 µm C18 packing material. The system was supplied with a UV-Vis detector. Solid-phase extraction (SPE) was done with Agilent™ Bondesil cartridges packed with octadecyl silane (ODS).

Chemicals and reagents

Powdered forms of BRZ and DZP bulk were donated by F. Hoffmann-La Roche AG, Grenzacherstrasse, Basel, Switzerland. The percentage purities of the bulk powders as indicated on the labels were 100.17 ± 0.47 and 99.79 ± 0.57 %, for BRZ and DZP, respectively. Methanol, methylene chloride, acetonitrile, and distilled water of HPLC-grade and high purity were products of Sigma-Aldrich (USA). Sodium hydroxide solution (LiChropur™, 49.0 – 51.0 %) was obtained from Merck (Darmstadt, Germany), while KH_2PO_3 was purchased from Fischer Chemicals™ (Zürich).

Standard solutions

Stock standard solutions of BRZ and DZP (100 µg/mL) were made in separate 100-mL volumetric flasks. Dissolution of each drug and making up to the volume (100-mL) were carried out using methanol. Working solutions of BRZ and DZP (1 µg/mL) were prepared *via* dilution of the stock solutions with the same solvent.

Method optimization

Different mobile and stationary phases were

tested to achieve optimum system suitability indices regarding efficiency, selectivity, resolution, and peak symmetry.

Method validation

The full validation scheme was followed in line with the guidelines/protocol of ICH-Q2B [18].

Linearity

Different aliquots (1 – 25 µg) of BRZ and DZP were accurately and separately transferred into a group of 100-mL capacity volumetric flasks. Methanol was used for completing the volume in each flask to get concentrations of 10 - 250 ng/mL. The prepared dilutions were analyzed with a Waters™ column as stationary phase. The developing system was acetonitrile: methanol: 0.05 M phosphate buffer (pH 6.5), in the ratio *(5:2:3, by volume) at a flow rate of 0.7* mL/min. The quantities of the separated analytes were determined at 240 nm. The peak areas were plotted against concentrations, and the resultant plots were used to derive regression relationships.

Accuracy

Accuracy is usually related to the closeness of the measured values to the true ones, and it is presented as % analytes recovery from a stipulated amount [18]. In this study, 9 drug samples, each at strengths of 50, 70, and 90 ng/mL, were chromatographed with the protocol described under linearity.

Precision

It can give an idea about the variability, either intra-day or on different days (between-day). It can be expressed as percent relative standard deviation (% RSD) for a number of experiments which are statistically significant. Three concentrations of BRZ and DZP (50, 70, and 90 ng/mL) were analyzed thrice within the same day (intra-day) and on 3 successive days (inter-day), and the outcomes were expressed as % RSD.

Detection and quantification limits

These parameters present a clear picture about method sensitivity whereby the limit of detection (LOD) is the least concentration that can be detected by the analytical method. On the other hand, the limit of quantification (LOQ) is the least concentration that can be accurately quantified by the proposed method [18]. Both of them were calculated using Eqn 1 and 2:

$$LOD = 3.3 \times \frac{\sigma}{S} \quad \text{..... (1)}$$

$$LOQ = 10 \times \frac{\sigma}{S} \quad \text{..... (2)}$$

where σ represents standard deviation of lowest standard level, and S represents slope of standard curve.

Robustness

Robustness can be evaluated by studying the influence of deliberate variations on the suggested analytical method. It was conducted by measuring the effect of slight variations in the mobile phase composition (changing the percentage of acetonitrile by ± 1 %). In addition, the developing system flow rate was changed by ± 0.1 mL/min.

System suitability

Different parameters which indicate BRZ and DZP migration rates (capacity factors), symmetry of the resolved peaks (tailing factors) and resolution of the separated drugs (resolution factors) were well studied and calculated. Column efficiency, which is measured by the number of theoretical plates (N) and height equivalent to theoretical plates (HETP), were also studied, computed, and presented in a full system suitability sheet.

Method application

Wastewater sample collection and storage

Wastewater samples were collected from the effluents of a pharmaceutical industrial plant 80 Km eastern Cairo international airport. Following filtration through nylon membranes, the samples were stored in a dark and cool place.

Preparation of the wastewater samples

This was done through SPE treatment. First, pre-conditioning of the cartridge packing material was carried out *via* treatment with 5 mL of methylene chloride, 5 mL of methanol and finally 5 mL of distilled water. The wastewater samples were thoroughly homogenized *via* vortex mixing for about ten seconds. Optimization of the type of eluting liquid, its volume, and the flow rate was carefully performed. The volume of sample loaded was 4 mL, followed by passing of 3 mL distilled water while the elution of BRZ and DZP was done with 5 mL of methylene chloride. Centrifugation of the elutes was done for five minutes. Removal of the remaining aqueous layer was done using a pipette. The organic layer

was evaporated to dryness at 40°C under gentile nitrogen stream and reconstitution of the residue was done using 100 µL methanol. Then, careful chromatography was performed under the optimized conditions mentioned under linearity.

Determination of BRZ and DZP in spiked water samples

Evaluation of the extraction procedure was performed through the spiking of distilled and tap water with various levels of BRZ and DZP to reach strengths of 60 ng/mL (BRZ) and 90 ng/mL (DZP), and then subjecting the spiked samples to optimized extraction procedure, and chromate-graphing under the optimum developed conditions.

Determination of BRZ and DZP in industrial wastewater samples

The optimized SPE protocol was used to prepare 5 wastewater samples, followed by chromate-graphing under optimum parameters stipulated under linearity. The concentrations of the five samples were calculated from the plotted standard curves, and comparisons were made between the resultant concentration values and the corresponding values gotten *via* the use of referenced procedures for the measurement of BRZ and DZP following a similar pretreatment protocol [7,10].

RESULTS

This work introduces a sensitive and selective ultra-performance liquid chromatographic method which can be applied for the simultaneous determination of two commonly used benzodiazepines drug residues in industrial wastewater, thereby enhancing the task of monitoring and quantification of these drugs.

Method optimization

Various types of stationary phase and developing systems were tested to get the optimum separation pattern for BRZ and DZP. This was attained using a stationary phase of Waters™ column (100 x 2.1 mm, 1.7 µm). The mobile phase was acetonitrile: methanol: 0.05 M phosphate buffer, pH 6.5 *(5:2:3, by volume).* The optimized mobile phase flow rate was 0.7 mL/min. The absorbance of the eluents was read at a UV wavelength of 240 nm. Figure 2 shows that the values of retention time for BRZ and DZP were 2.102 and 4.091 min, respectively.

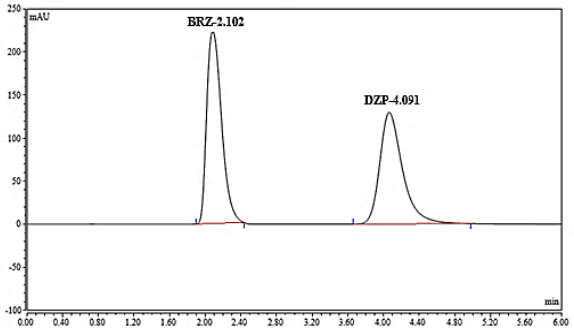

Figure 2: The UPL chromatogram for the separation pattern of a mixture of BRZ and DZP

The calculated suitability indices are presented **in** Table 1. Excellent column efficiency was indicated by the values of N and HETP, while high selectivity and resolution were confirmed through the resolution factor (Rs) value, which ensured baseline-to-baseline separation of the studied drugs. Moreover, excellent peak symmetry was pointed out by the values of the tailing factor.

Table 1: Established system suitability parameters of the optimized ultra-performance liquid chromate-graphic method

Parameter	BRZ	DZP
$t_{R\ (min.)}$†	2.10±0.10	4.09±0.12
Capacity factor (K)	27.89	39.91
Resolution factor (Rs)	-	3.69
Number of theoretical plates (N)	1589	2076
HETP*	$6.29x10^{-3}$	$4.82x10^{-3}$
Tailing factor (T)	1.02	1.02

†Triplicate runs per a sample. * Height equivalent to theoretical plates

Method validation

It was carried out taking into consideration the appropriate protocols [18]. There were linear correlations between peak area and the concentrations of BRZ and DZP within the range of 10 – 250 ng/mL, consistent with the regression relationships in Eq 3 and 4.

Pa (BRZ) = 20.39c – 14.09; r = 0.9998 …… (3)

Pa (DZP) = 10.01c + 2.35; r = 0.9999 …… (4)

where *Pa* is peak area; *c* is concentration (ng/mL), and *r* is correlation coefficient.

The validation sheet presented in Table 2 confirmed excellent accuracy, repeatability, and intermediate precision. Method robustness was

carefully studied by carrying out deliberate variations in the developing system composition, as well as flow rate. These slight variations had no marked influence on the new UPLC procedure, indicating excellent robustness. Moreover, values LOD and LOQ indicated acceptable sensitivity of the method and its suitability for use in detecting and quantifying BRZ and DZP in wastewater samples.

Table 2: Validation results of the ultra-performance liquid chromatographic method

Parameter	BRZ	DZP
Accuracy (mean* ± SD)	101.03±0.94	100.84±0.73
Precision		
Repeatability*	99.56±0.81	101.44±1.15
Intermediate precision*	101.12±1.12	99.61±0.81
Robustness		
Mobile phase composition change	99.14±0.85	100.79±0.92
Flow rate change	99.12±0.74	101.23±1.14
Linearity		
Range (ng/mL)	10-250	10-250
Slope	20.39	10.01
Intercept	– 14.09	2.35
Correlation coefficient (r)	0.9998	0.9999
LOD (ng/mL)	2	2
LOQ (ng/mL)	10	10

*Mean of three readings

Application of the method

The new method was successfully applied for the determination of BRZ and DZP in spiked distilled and tap water samples, as shown in Table 3.

Table 3: Determination of BRZ and DZP in spiked water samples using the optimized ultra-performance liquid chromatographic method

Specimen	BRZ	DZP
Distilled water (Rec±SD)%*	99.55±0.48	101.34±0.86
Tap water (Rec±SD)%*	99.16±0.77	99.32±0.56

*Mean of five measurements

Moreover, the optimized sample pretreatment protocol was carefully applied to the wastewater samples. To validate these results obtained using the proposed method, standard methods for measurement of BRZ and DZP were applied for their quantification in the same wastewater samples after their prior processing with the same sample preparation protocol. The results are presented in Table 4.

DISCUSSION

The problem of environmental pollution has become a major issue and an important challenge for humanity, since it represents a depletion of environmental capabilities which are considered as the most important pillars of human life. Therefore, the issue of environmental analysis has become a crucial task used for monitoring the environmental pollutants to present a clear picture of their levels. This, in turn, plays an important role in protection of health.

Active drugs present in surface water or plants are considered as enormous sources of risk to health because they may bring harmful consequences on people exposed to them.

This study has developed an improved, simple, sensitive, and accurate UPLC procedure for the determination of BRZ and DZP remnants in effluent water samples from drug manufacturing industrial plants. The proposed analytical method can be used for policing the levels of BRZ and DZP in the environment.

First, optimum resolution was obtained through method optimization. This was done *via* application of complete suitability parameters which are presented in Table 1. The values of the capacity factors for the two benzodiazepines indicated a duration which was sufficient for proper interaction between BRZ and DZP with the stationary phase. This optimum interaction had a positive impact during the separation process for BRZ and DZP. The obtained value of resolution factor (R_s) indicated excellent resolution as well as excellent baseline-to-baseline peak separation. The proposed UPLC method had excellent column efficiency, as was

Table 4: Determination of BRZ and DZP in wastewater samples from the industrial pharmaceutical manufacturing facility

Sample number	BRZ*		DZP*	
	UPLC-method	Reference method [7]	UPLC-method	Reference method [10]
Sample 1	20.68	20.59	39.47	39.48
Sample 2	26.23	26.33	31.79	31.69
Sample 3	44.77	44.89	41.01	40.99
Sample 4	33.87	33.58	22.77	22.76
Sample 5	20.97	20.86	41.83	41.11

*Concentrations are calculated in ng/mL

confirmed by the values of N and HETP. In addition, excellent peak symmetry was demonstrated in the values of these factors. These values approached unity, which clear give evidence of an excellent peak symmetry obtained.

Full validation scheme was followed according to the ICH-Q2B guidelines and the results presented in Table 2 indicate that the method was very accurate and very precise. Excellent robustness of the method was also confirmed by the absence of any marked effects due to slight variations in operating conditions. At the same time, the obtained values of LOD and LOQ confirmed acceptable sensitivity which is suitable for the suggested method to be well applied for the effective and successful monitoring and quantification of BRZ and DZP in water effluents.

Application of the optimized sample pretreatment and the quantification procedure were successfully performed for assay of BRZ and DZP in spiked distilled and tap water samples, indicating the effectiveness and accuracy of the pretreatment protocol. The optimized procedure was also used for the determination of BRZ and DZP in waste effluents after their pretreatment with the optimized sample preparation procedure. In order to validate these results, comparison was made between the resultant concentrations and the corresponding levels gotten with reference methodologies for quantifying the studied benzodiazepines [7,10], resulting in acceptable comparability.

CONCLUSION

The proposed method has been successfully validated, thereby ensuring its accuracy and precision as well as sensitivity which guarantees its effective application for the sensitive monitoring and quantification of the studied drugs in actual wastewater effluents. The present work will have a large impact on human life since the effective and successful monitoring of pharmaceutical industrial pollutants in the environment is considered a cornerstone in the conservation of the environment and avoidance of the hazardous effects of these pollutants on human health.

DECLARATIONS

Acknowledgements

The authors extend their appreciation to the deputyship for research & Innovation, Ministry of Education in Saudi Arabia for funding this research work through Project no. IF-PSAU-2021/03/18581.

Funding

None provided.

Ethical approval

None provided.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them.

Open Access

REFERENCES

1. *Honeychurch KC. Review of electroanalytical-based approaches for the determination of benzodiazepines. Biosensors 2019; 9: 130.*
2. *International Narcotics Control Board (INCB) Psychotropic Substances – Statistics, New York, 2015.*
3. *Matsuo H, Sakamoto H, Arizono K, Shinohara R. Behavior of pharmaceuticals in wastewater treatment plant in Japan. Bull Environ Contam Toxicol 2011; 87: 31–35.*
4. *Johnson B, Streltzer J. Risks associated with long-term benzodiazepine use. Am Fam Physician 2013; 88(4): 224-225.*
5. *Seifrtová M, Aufartová J, Vytlačilová J, Pena A, Solich P, Nováková L. Determination of fluoroquinolone antibiotics in wastewater using ultra high-performance liquid*

chromatography with mass spectrometry and fluorescence detection. J Sep Sci 2010; 33: 2094-2108.

6. *Salem AA, Barsoum BN, Izake EL. Spectrophotometric and fluorimetric determination of diazepam, bromazepam and clonazepam in pharmaceutical and urine samples. Spectrochim Acta A 2004; 60(4): 771-780.*
7. *Le Solleu H, Demotes-Mainard F, Vinçon G, Bannwarth B. The determination of bromazepam in plasma by reversed-phase high-performance liquid chromatography. J Pharm Biomed Anal 1993; 11(8): 771-775.*
8. *Al Attas AS. Construction and analytical application of ion selective bromazepam sensor. Int J Electrochem Sci 2009; 4: 20–29.*
9. *Moros J, Garrigues S, de la Guardia M. Quality control Fourier transform infrared determination of diazepam in pharmaceuticals. J Pharm Biomed Anal 2007; 43(4): 1277-1282.*
10. *Sruthi P, Tejaswi N, Thanuja D, Sudheer Kumar P, Sagar V. Simple RP-HPLC method for estimation of diazepam in tablet dosage form. J Pharm Res 2013; 6(1): 140-144,*
11. *Rahimi-Nasrabadi M, Khoshroo A, Mazloum-Ardakani M. Electrochemical determination of diazepam in samples based on fullerene-functionalized carbon nanotubes/ ionic liquid nanocomposite. Sensor Actuat B-Chem 2017; 240: 125-131.*
12. *Luo Y, Guo W, Ngo HH, Nghiem LD, Hai FI, Zhang J, Liang S, Wang XC. A review on the occurrence of micropollutants in the aquatic environment and their fate and removal during wastewater treatment. Sci Total Environ 2014; 473– 474: 619–641.*
13. *Cunha DL, de Araujo FG, Marques M. Psychoactive drugs: occurrence in aquatic environment, analytical methods, and ecotoxicity—a review. Environ Sci Pollut Res 2017; 24: 24076–24091.*
14. *de Araujo FG, Bauerfeldt GF, Marques M, Martins EM. Development and validation of an analytical method for the detection and quantification of bromazepam, clonazepam and diazepam by UPLC MS/MS in surface water. B Environ Contam Tox 2019; 103: 362–366.*
15. *Chen M, Liu K, Zhong D, Chen X. Trimethylsilyldiazomethane derivatization coupled with solid-phase extraction for the determination of alendronate in human plasma by LC-MS/MS. Anal Bioanal Chem 2012; 402: 791-798.*
16. *Nováková L, Solichová D, Solich P. Advantages of ultra-performance liquid chromatography over high-performance liquid chromatography: comparison of different analytical approaches during analysis of diclofenac gel. J Sep Sci. 2006; 29(16): 2433-2443.*
17. *Qriouet Z, Qmichou Z, Bouchoutrouch N, Mahi H, Cherrah Y, Sefrioui H. Analytical Methods Used for the Detection and Quantification of Benzodiazepines. J Anal Methods Chem 2019; 1-11. 10.1155/2019/2035492.*
18. *International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use, ICH harmonized tripartite guideline, validation of analytical procedures: Text and methodology Q2 (B); 2005.*