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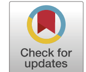

Development of a sample preparation and analysis method for therapeutic monitoring of diazepam and major metabolite in alcohol withdrawal syndrome treatment

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ABSTRACT

Diazepam, a widely prescribed benzodiazepine, is frequently used for the management of alcohol withdrawal syndrome, anxiety, seizures, and muscle spasms. Its monitoring is critical due to its potential for abuse and the therapeutic importance of its metabolite nordiazepam. A sustainable and environmentally friendly high-performance liquid chromatography method was developed and validated for the quantification of diazepam and its active metabolite nordiazepam in human plasma samples. The chromatographic analysis was conducted on an HPLC system equipped with a UV detector. Separation was achieved using a Kinetex F5 column (150 ×4.6 mm, 5 μm). The mobile phase consisted of acetonitrile and phosphate buffer adjusted to pH 4.3 (30:70, v/v), delivered in isocratic mode at a flow rate of 1.0 mL/min, with the pH adjusted to optimize resolution. The method demonstrated excellent sensitivity, reproducibility, and linearity for both compounds, highlighting its applicability for drug monitoring and toxicological studies. Moreover, the environmentally conscious selection of materials and conditions underscores the method's compliance with green analytical chemistry principles. Both analytes were successfully detected and quantified in all fifty-three patients under the treatment of alcohol withdrawal syndrome. The measured concentrations of diazepam (0.23–1.4 μg/mL) and its metabolite nordiazepam (0.22–3.78 μg/mL) displayed considerable variability among individuals.

1. Introduction

The anxiolytic benzodiazepine diazepam (DZP) was initially introduced and patented in the United States in 1963. It is classified as a long-acting, fast-acting benzodiazepine. It is commonly prescribed to manage various conditions such as acute recurring seizures, severe muscle spasms, stiffness associated with neurological disorders, anxiety disorders, and alcohol withdrawal. DZP is particularly effective in alleviating symptoms of acute alcohol withdrawal, including agitation, tremors, alcoholic hallucinosis, and acute delirium tremens [1]. Due to their superior safety profile, reduced side effects, and the ability to counteract oversedation in cases of benzodiazepine overdose through the use of flumazenil, a benzodiazepine receptor antagonist, benzodiazepines have largely replaced barbiturates in the treatment of anxiety and sleep disorders [2].

The Food and Drug Administration (FDA) has authorized the utilization of DZP for various clinical indications, including the treatment of anxiety disorders and the transient mitigation of anxiety symptoms, as well as the management of refractory epilepsy in certain patient subsets. Additionally, it is sanctioned for the management of spasticity stemming from upper motor neuron disorders, adjunctive therapy for muscle spasms, preoperative anxiety attenuation, and the treatment of severe

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recurrent convulsive seizures and status epilepticus. However, DZP is also employed off-label, denoting its use in applications not officially approved by the FDA, such as short-term therapy for spasticity in children afflicted with cerebral palsy and for sedation within the intensive care unit setting [3].

The interaction between DZP and gamma-aminobutyric acid (GABA) A-type receptors ($GABA_A$) is assumed to be the mechanism by which DZP exerts these effects. GABA is the primary inhibitory neurotransmitter in the central nervous system. The opening of the $GABA_A$ receptor that occurs as a result of the binding of GABA to the receptor makes it possible for chloride ions to enter neurons. The ability of neurons to depolarize and create action potentials is diminished because of this, and researchers believe that seizures are associated with excessive action potentials. DZP is hypothesized to boost the effects of GABA by increasing the affinity between GABA and its receptor. This, in turn, causes GABA to bind more tightly to the $GABA_A$ receptor, amplifying the effects of GABA [4].

Following oral ingestion, more than 90 % of DZP is absorbed into the

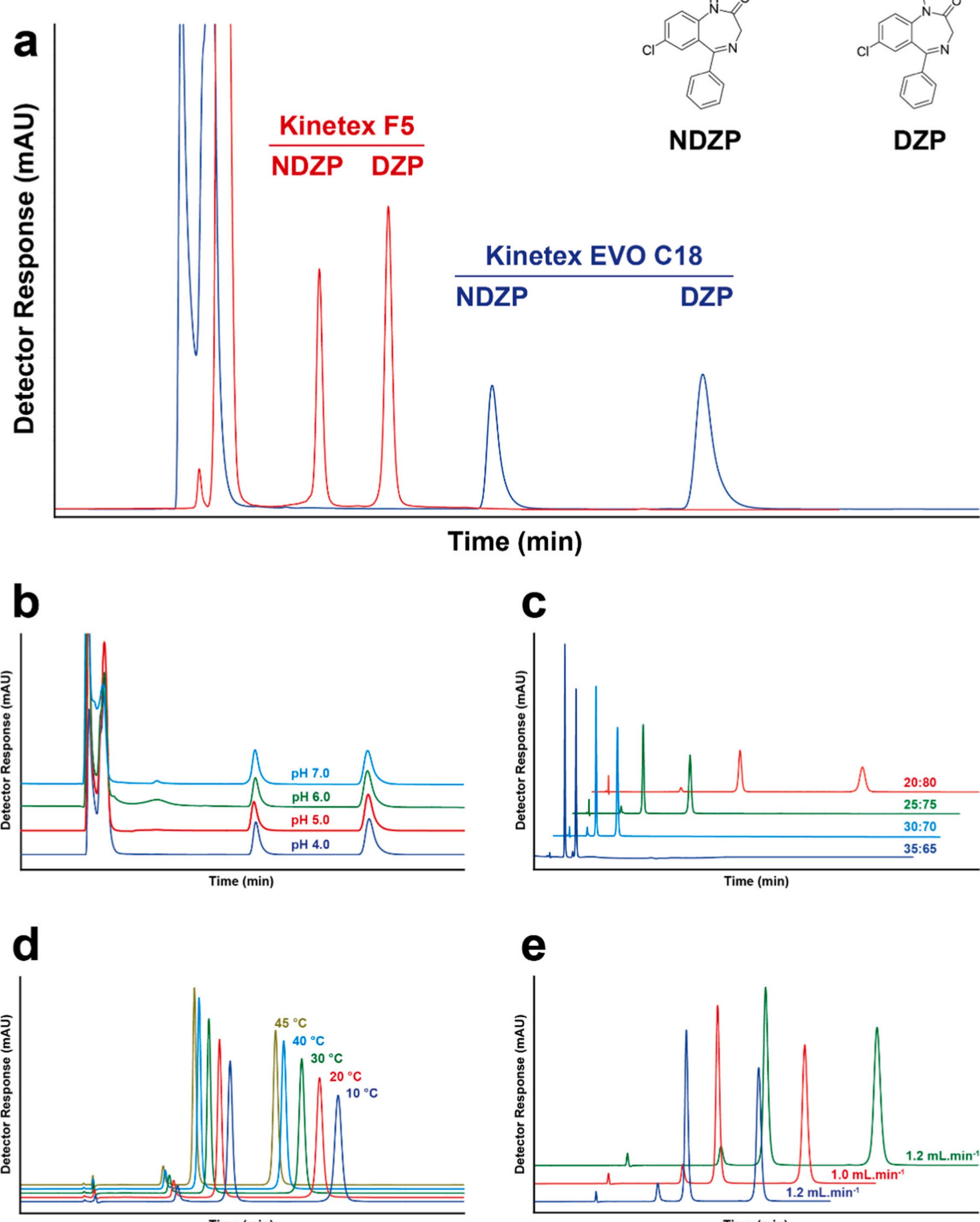

Fig. 1. HPLC Method optimization results of NDZP and DZP (10 μg/mL). (a) Comparison of two different stationary phases. (b) Comparison of different pH of buffer solution. (c) Comparison of different mobile phase compositions. (d) Comparison of the effects of column temperatures. (e) Comparison of the effects of flow rate.

bloodstream. The highest levels of DZP in the blood are achieved within 1–1.5 hours. However, this peak concentration is delayed to around 2.5 hours if taken with food. DZP exhibits a high affinity for lipids and rapidly undergoes redistribution within the body, displaying a substantial binding affinity to plasma proteins (98 %). It crosses the blood-brain and placental barriers and is present in breast milk, with a distribution volume of 0.8–1.0 L/kg. DZP is primarily metabolized by the enzymes CYP2C19 and CYP3A4, forming active metabolites such as nordiazepam (NDZP). The average half-life of DZP is approximately 46 hours, whereas the half-life of NDZP is around 100 hours. The drug and its metabolites are mainly eliminated through urine, and taking numerous doses causes the drug to accumulate in the body, somewhat extending the time it takes for the drug to be eliminated [5,6].

Drug monitoring (DM) of DZP and its metabolite NDZP (the molecular structure is given in Fig. 1**a** as an inset) is essential for various reasons. Firstly, DZP, like all the other benzodiazepines, is classified as a controlled substance that has a prolonged half-life, can result in the development of tolerance and physical dependency, and tends to build up in the body with repeated doses, which can result in a higher likelihood of experiencing side effects and toxicity if not adequately controlled. The extended half-life of the active metabolite NDZP significantly enhances this risk. Further, DZP exhibits a high affinity for lipids and has a strong binding affinity for plasma proteins [7]. This characteristic results in variations in how individuals respond to the drug and requires precise dosage modifications based on plasma concentrations. Furthermore, the existence of food might hinder the process of absorption, affecting the effectiveness of the medication and necessitating close monitoring to ensure that the desired therapeutic levels are sustained. Additionally, the metabolism of DZP through CYP2C19 and CYP3A4 enzymes implies that genetic variants or interactions with other medications can significantly affect its levels in the bloodstream. Also, DM plays a crucial role in the prevention and identification of DZP abuse. Through regular monitoring of plasma levels, healthcare providers might detect trends that suggest abuse, such as abnormally high concentrations or inconsistent dose regimens. This can aid in the implementation of suitable therapies and the prevention of any consequences connected to abuse. Monitoring these levels improves the therapeutic effects while reducing unwanted effects, guaranteeing patients a safe and successful treatment. According to a research study published by Jones and Holmgren [8] in 2013, the average ratio of DZP to NDZP in blood samples from 1000 cases of driving under the influence of drugs (DUIDs) was determined to be 1.29. Among 90 drivers, the ratio of ones with DZP levels above the upper therapeutic limit was 1.8. In his paper, Hiemke et. al.[9] stated that a DZP/NDZP ratio of 0.9 can be regarded as within the normal range. The study conducted by Schoretsanitis and colleagues [10] in 2018 found that plasma levels of DZP plus NDZP within the range of 100–2500 ng/mL are deemed to be within the normal range. However, a laboratory warning threshold is set at 3000 ng/mL.

Several studies in the literature have focused on the analysis of DZP and NDZP. These analyses are performed using various techniques, with studies employing Gas Chromatography-Mass Spectrometry [11], High-Performance Liquid Chromatography with Ultraviolet detection (HPLC-UV) [12,13], Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS) [14–17], and capillary electrophoresis [18]. HPLC-UV methods are often used due to their reliability and accessibility, providing accurate quantification of these compounds in various biological matrices. On the other hand, LC-MS/MS and LC-QTOF offer greater sensitivity and specificity, making them particularly useful for detecting low concentrations of DZP and NDZP, as well as multi-analyte drug detection [14–17,19]. All techniques contribute valuable insights into the pharmacokinetics and therapeutic monitoring of these substances, highlighting the importance of method selection based on the specific requirements of the analysis. Also, there are various sample preparation techniques for the analysis of DZP and NDZP. These techniques include Solid phase extraction (SPE) [20,21], liquid-liquid extraction (LLE) [22–24], dispersive liquid-liquid microextraction (DLLME) [25], and solid phase microextraction (SPME) [26]. SPE is commonly used due to its efficiency in concentrating and purifying the analytes from complex biological matrices. LLE is another widely used technique, valued for its simplicity and effectiveness in isolating DZP and its metabolites. LPME and SPME, more recent techniques, offer the advantages of being cost-effective and requiring smaller sample volumes while still providing high sensitivity and precision. Each of these methods has its advantages and is selected based on the specific requirements and goals of the analysis, contributing to the robustness and accuracy of DZP and NDZP quantification in various studies.

This study aimed to develop a novel sample preparation method prior to HPLC-UV analysis of DZP and NDZP simultaneously. The developed protein precipitation method enables the green, rapid, and miniaturized preparation of human plasma samples for analysis. At the same time, this optimized HPLC-UV technique allows for the fast, accurate, affordable, and green detection and quantification of the target analytes for DM and confirmation purposes.

In recent years, concepts related to sustainability have emerged as a primary emphasis throughout scientific fields, including analytical chemistry. Analytical greenness is essential for assessing the environmental impact of analytical methodologies, highlighting the necessity for sustainable practices while maintaining analytical efficacy. This methodology adheres to the tenets of green chemistry, which promotes the minimization of hazardous materials, energy usage, and waste production during the analytical procedure.

The Analytical GREEnness Metric (AGREE) [27] and Analytical Greenness Metric for Sample Preparation (AGREEPrep) [28], are prominent instruments that quantitatively evaluate the compliance of an analytical approach with green chemistry principles. AGREE and AGREEprep enables the comparison and optimization of analytical techniques by evaluating parameters such as reagent toxicity, energy efficiency, solvent consumption, and waste management. This allows researchers to make educated choices when creating or selecting procedures that reduce environmental effects while ensuring high accuracy, precision, and reliability.

Additionally, incorporating AGREE and AGREEPrep into method creation promotes innovation by facilitating the use of alternative techniques, including downsizing, automation, and renewable resources. These developments not only promote environmental sustainability but also improve the efficiency and cost-effectiveness of analytical processes.

Also, other analytical greenness index calculators demonstrated the greenness evaluation of the analytical process and sample preparation method, including the Complementary Green Analytical Procedure Index (Complex GAPI) [29], Blue Applicability Grade Index (BAGI) [30], and Red Analytical Performance Index (RAPI) [31].

2. Materials and methods

2.1. Reagents and materials

DZP and NDZP were purchased from Lipomed (Cambridge, MA, USA). Clozapine (CLZ) was purchased from Cerilliant (Round Rock, TX, USA). Trichloroacetic acid (TCA), zinc sulfate heptahydrate ($ZnSO_4$), sodium chloride (NaCl), acetone (ACE), and acetonitrile (ACN) were purchased from Merck (Darmstadt, Germany). Drug-free human plasma was obtained from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Instrumentation

Chromatographic analysis of DZP and NDZP was achieved on an Agilent 1100/1200 Series HPLC system with a UV detector (Santa Clara, CA, USA). Separation was performed on a Kinetex F5 column (150 ×4.6 mm i.d., 5 μm) acquired from Phenomenex (Torrance, CA, USA). The analytical column was protected by the Restek Trident Level 3

LC Column Protection System (Bellefonte, PA, USA). A Mettler Toledo pH meter (Columbus, OH, USA) was used for performing pH measurements.

In the process of the analysis, a mobile phase composition consisting of acetonitrile and phosphate buffer in a ratio of 30:70 (v/v) was utilized. The mobile phase was set up in isocratic mode with a flow rate of 1.0 mL/min. The temperature of the column thermostat was adjusted to 40°C. The injection volume was 5 μL, and the detector wavelength was set up at 238 nm. The solutions passed filtration using a 0.45 μm membrane filter and were thereafter degassed using an ultrasonic bath for about 20 minutes. Agilent Technologies Chemstation Rev. B.04.03-SP1 (Santa Clara, CA, USA) was used to process the chromatograms.

2.3. Sample Collection

Fifty-three male volunteers undergoing alcohol withdrawal syndrome treatment at the Alcohol and Substance Addiction Treatment Center (AMATEM) provided real blood samples. The study was conducted with the approval of the Human Research Ethics Committee of Ankara University (110−618−21). Written informed consent was acquired from each volunteer. The level at which the drug concentration continuously stabilizes is referred to as the steady-state concentration. Achieving a steady-state concentration is typically essential for efficient pharmacological treatment of medical conditions [32]. Before the samples were taken, it was ensured that the patient's plasma drug levels reached a steady state concentration, and blood samples were collected right before the next drug dose [33]. A 4 mL heparinized tube with blood samples was centrifuged for 20 minutes at 1000 g. Up until the day of analysis, plasma was split into microcentrifuge tubes and kept in a deep freezer at −80°C.

2.4. Sample preparation

A simple protein precipitation technique was applied to remove proteins from the plasma samples. For real samples, 20 μL of 4 % (w/v) TCA and 80 μL of ACN containing CLZ (20 ng/mL) were added to 100 μL of plasma sample and mixed in a vortex mixer for 30 s. The sample was centrifuged at 4°C, 22000 g, for 10 minutes to precipitate proteins. Then, 100 μL of the supernatant was transferred to inserts and analyzed. The sample preparation procedure is visualized in Fig. 2.

2.5. Validation

According to FDA's Bioanalytical Method Validation Guidance for Industry guidelines' [34] criteria, validation experiments were carried out to ensure selectivity, linearity, recovery, accuracy, and precision, as well as limit of detection (LOD) and lower limit of quantification (LLOQ). Specificity was evaluated during the validation procedure by examining plasma samples from six distinct people. Analyte detection limits were calculated using the calibration curve's standard deviation. Quality control (QC) samples were prepared using four different concentrations while considering the working range. The calibration curve was created utilizing six distinct concentrations. By adding DZP and NDZP standards to each plasma matrix, which corresponded to 100 % of the predicted content, accuracy and precision were evaluated.

3. Results and discussion

3.1. Development and optimization of the chromatographic method

Two different stationary phases from Phenomenex (Torrance, CA, USA) were tested to achieve the best separation conditions. These included core-shell octadecyl silane (C18) and core-shell pentafluorophenyl propyl (PFP) phases. Core-shell silica-based particles offer several advantages in conventional HPLC analysis, such as improved separation efficiency, reduced backpressures, and enhanced performance for separating large molecules [35]. Furthermore, C18 columns are widely used for pharmaceuticals, environmental analysis, and food science and separate primarily based on hydrophobicity, while PFP incorporates additional interactions like π- π, dipole-dipole, and hydrogen bonding due to the electronegative fluorine atoms. This column type is much more successful in separating halogenated compounds, pharmaceuticals, and structurally similar compounds like metabolites. The Kinetex F5 (150 ×4.6 mm i.d., 5 μm) analytical column was used in the following studies, which considered the compounds' peak shapes, separation efficiency, and analysis time. Considering the performance attributes of these column alternatives, PFP was chosen to provide the best possible separation and detection of the target analytes and short analysis time (Fig. 1a).

Subsequently, experiments were conducted to determine the most optimal chromatographic parameters. The parameters involved modifying the buffer pH between pH 4 and 7, adjusting the amount of organic solvent in the mobile phase between 20 % and 35 %, controlling the column temperature between 10 and 45 °C, and regulating the flow rate between 0.8 and 1.2 mL/min.

The pKa of DZP and NDZP is approximately around 3.0. For this reason, both compounds will be predominantly in their neutral form at pH values above their pKa. To optimize the separation of both compounds, it's generally recommended to use a mobile phase pH that is either above or below their pKa values. This ensures that the analytes are predominantly in a single ionization state, leading to sharper and more symmetrical peaks. A pH above 4.0 was selected as the starting point. During the studies conducted on buffer pH, it was observed that pH levels of 4–7 had no significant impact on the retention times or peak

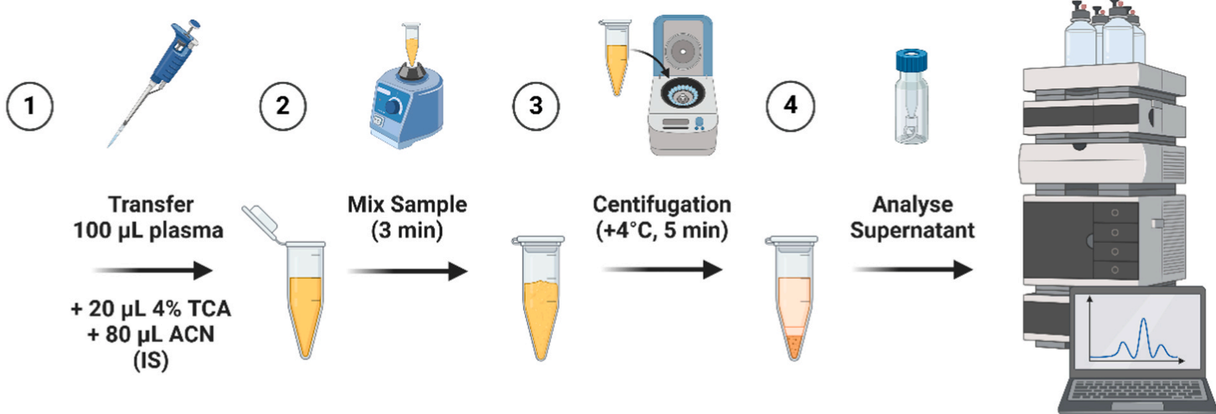

Fig. 2. Optimized sample preparation method.

shapes of DZP and NDZP peaks (Fig. 1b). However, a different observation was made for CLZ, which was used as the internal standard. Consequently, pH 4.3 was determined to be the optimal condition to ensure that the CLZ peak does not overlap with the other two analytes and that its peak shape remains well-defined (Figure S1).

The mobile phase compositions were tested by increasing the organic phase from 20 % to 35 % in 5 % increments. While increasing the organic phase significantly reduced the retention times of the target analytes, it also decreased the resolution between the analytes. Moreover, it increased the likelihood of overlap between signals from plasma interferences and those of the analytes. Based on the experiments, 30 % organic phase was determined to be the optimal percentage, providing high resolution and a short analysis time (Fig. 1c).

The column temperature (10 – 45 °C) was found to have a significant effect on the elution of the compounds of interest and peak shapes. To shorten the analysis time, achieve sharper peak shapes, reduce the backpressure, and avoid pushing the upper-temperature limit of the analytical column, 40 °C was determined to be the most suitable condition (Fig. 1d).

One of the critical parameters optimized during the development of the chromatographic method was the flow rate, as it significantly impacts key performance attributes of the analysis. In this study, the flow rate was systematically varied between 0.8 and 1.2 mL/min to evaluate its effect on analytical performance, including retention time, peak shape, and system backpressure. After a comprehensive analysis, a flow rate of 1.0 mL/min was identified as the optimal condition, providing a balance between efficient separation, acceptable retention times, well-defined peak shapes, and manageable system backpressure. This optimized flow rate ensured the method's reliability and robustness for the subsequent analysis of plasma samples (Fig. 1e).

The optimized HPLC conditions were evaluated using system suitability tests. Parameters such as theoretical plate count, tailing factor, resolution, etc. were assessed. The results of these tests met the predefined acceptance criteria, indicating that the system was operating within acceptable limits and capable of delivering reliable and accurate data for the separation and quantification of the target analytes (Table S1). Under these chromatographic conditions, the retention times of NDZP, CLZ, and DZP were 4.12, 4.99, and 6.02, respectively.

3.2. Development of sample preparation method

The study employed the protein precipitation technique as the chosen method for sample preparation. TCA, $ZnSO_4$, NaCl, ACN, and their combinations were applied in this approach in different proportions. Based on the acquired results, it was observed that NaCl did not yield adequate precipitation. The precipitation induced by ACN and $ZnSO_4$ was found to be sufficient; however, it did not effectively reduce the interferences observed in the chromatogram. In the conducted experiment involving a mixture of TCA and ACN, it was noted that more accurate chromatograms were produced, along with adequate precipitation. The addition of ACN in the procedure showed an improved signal and reduced interference in the chromatogram compared to the findings obtained with TCA alone.

3.3. Method validation

DZP, NDZP, and CLZ were used to spike plasma samples from six individuals, and the resulting samples were analyzed for selectivity. Selectivity was assessed by comparing the chromatograms of six distinct persons who did not use DZP with the chromatogram of the spiked sample. The analysis showed that there was no interference with the results for the target analytes.

The blank plasma samples were enriched with analytes at six distinct concentration levels, and the calibration curves were established by graphing the concentration of each analyte against the ratio of the peak area of the analyte to the peak area of the internal standard. The linearity spanned from 0.2 to 5 μg/mL with a coefficient of determination (R^2) above 0.998. All regression data were tabulated in Table 1.

The detection limit is determined using the following formula: LOD $= 3.3\ \sigma/S$. The formula utilized the standard deviation of y-intercepts of regression lines. The values obtained using the given formula were 0.066 μg/mL for DZP and 0.052 μg/mL for NDZP.

To ensure consistency and precision in the research focused on repeatability and accuracy, quality control (QC) samples were prepared at four distinct concentrations: LLOQ, the low-quality control (LQC), the medium-quality control (MQC), and the high-quality control (HQC). The samples underwent analysis in five duplicates, and the repeatability was determined by calculating the percent relative standard deviation (RSD %). The intraday repeatability results obtained during the validation studies ranged from 0.97 % to 6.24 % for DZP and from 0.64 % to 3.21 % for NDZP. The inter-day repeatability results were between 2.92 % and 8.38 % for DZP and between 0.42 % and 9.11 % for NDZP, respectively. Intraday accuracy findings for DZP ranged from 90.17 % to 104.47 %, while for NDZP, the range was between 93.86 % and 109.75 %. The results have been compiled in Table 2 and are found to be within the indicated limits as per the guidelines [34].

The recovery study was conducted by spiking a patient's plasma sample, in which NDZP and DZP concentrations were initially determined as 0.25 μg/mL and 0.51 μg/mL, respectively. The spiking was performed to achieve final concentration increments of 0.50, 1.00, and 1.50 μg/mL for both analytes. The obtained data were calculated as percent recovery. The results indicated that NDZP recovery ranged from 99.4 % to 105.6 %, while DZP recovery varied between 98.5 % and 106.6 %, demonstrating the accuracy and reliability of the applied method. A comprehensive summary of the entire recovery study is presented in Table 3.

3.4. Real samples analysis

The method we developed was applied using plasma samples obtained from 53 male volunteers. The demographic information of the volunteers is tabulated in Table 4.

The established method successfully determined patient plasma levels of DZP and NDZP, as shown by the sample chromatogram in Fig. 3. DZP and NDZP were successfully detected and quantified in all fifty-three volunteers. Despite the collection of blood samples from volunteers receiving oral administration of 5–25 mg/day, the measured concentrations of DZP (0.23–1.4 μg/mL) and its metabolite NDZP (0.22–3.78 μg/mL) displayed considerable variability among individuals. Metabolic variations among people are believed to contribute to the development of this variance significantly. However, there were six volunteers (11.3 %) who had a plasma NDZP/DZP ratio above the therapeutic range (>1.9). In addition, the plasma DZP+NDZP concentration in one volunteer was above the laboratory alert level (3 μg/mL). The variations in metabolizer enzymes (Table S2), CYP2C19 and CYP3A4, which metabolize DZP, may cause such abnormal plasma concentrations, also abuse of DZP should not be ruled out.

Table 1
Regression data of the calibration curve.

	DZP	NDZP
Linearity range (μg/mL):	0.2–5.0	
Slope:	17.477	15.872
SE of slope:	0.147	0.107
Intercept:	−2.8709	−2.1193
SE of intercept:	0.3464	0.2512
Determination coefficient R^2:	0.9988	0.9993
LOD (μg/mL):	0.066	0.052
LLOQ (μg/mL):	0.2	

Table 2
Repeatability and accuracy results of the validation study.

		QC Level	SD*	RSD% *	Accu% *	Bias%*
NDZP	*Intra-day*	*LLOQ (0.2 µg/mL)*	0.08	**3.21**	**93.86**	**+ 6.14**
		QCL (0.3 µg/mL)	0.05	**1.65**	**109.75**	**−9.75**
		QCM (1.5 µg/mL	0.29	**1.29**	**104.30**	**−4.3**
		QCH (4.0 µg/mL)	0.39	**0.64**	**98.62**	**+ 1.38**
	Inter-day	*LLOQ (0.2 µg/mL)*	0.20	**9.11**	**91.20**	**+ 8.80**
		QCL (0.3 µg/mL)	0.13	**4.06**	**110.34**	**−10.34**
		QCM (1.5 µg/mL	0.66	**2.89**	**104.19**	**−4.19**
		QCH (4.0 µg/mL)	0.26	**0.42**	**99.00**	**+ 1**
DZP	*Intra-day*	*LLOQ (0.2 µg/mL)*	0.12	**6.24**	**90.17**	**+ 9.83**
		QCL (0.3 µg/mL)	0.03	**0.97**	**104.47**	**−4.47**
		QCM (1.5 µg/mL	0.42	**1.93**	**93.68**	**+6.32**
		QCH (4.0 µg/mL)	0.70	**1.12**	**93.28**	**+ 6.62**
	Inter-day	*LLOQ (0.2 µg/mL)*	0.17	**8.38**	**92.58**	**+ 7.42**
		QCL (0.3 µg/mL)	0.07	**2.92**	**102.88**	**−2.88**
		QCM (1.5 µg/mL	1.25	**5.51**	**97.79**	**+2.21**
		QCH (4.0 µg/mL)	2.39	**3.75**	**95.21**	**+ 4.79**

* Five replicated analyses.

3.5. Comprehensive assessment of method performance: greenness, sustainability, practicality, and reliability

To evaluate the environmental sustainability of an analytical procedure, various indices and metrics have been developed, including AGREE, AGREEprep, Complex GAPI, BAGI, and RAPI. When assessed through the lens of these metrics, our developed method demonstrates attributes of a green analytical procedure, underscoring its environmental compatibility and sustainability (Figure S2).

The AGREE provides a comprehensive evaluation based on 12 green chemistry criteria, offering a visual score of up to 1 [27]. AGREEPrep extends this approach by specifically assessing the environmental impact of sample preparation steps, and also using up to 1 scoring system [28]. Complex GAPI enhances the original GAPI method by providing a color-coded categorical evaluation of sustainability across all analytical stages [29]. BAGI evaluates the practicality and reliability of analytical methods, assigning a score up to 100, where higher values indicate greater applicability [30]. In contrast, the RAPI focuses on analytical reliability by assessing accuracy, precision, and detection limits, using a scoring system up to 100, where higher values indicate superior performance [31].

Among these, AGREE and AGREEPrep are most suitable for assessing environmental sustainability, while Complex GAPI provides a more detailed, step-by-step analysis. BAGI complements green metrics by evaluating method applicability, whereas RAPI ensures analytical robustness. A combined approach incorporating these metrics allows for a more holistic assessment, balancing both environmental impact and analytical performance.

The developed analytical method was assessed using multiple green and performance-related metrics to ensure its environmental sustainability, applicability, and analytical reliability. The AGREE score of 0.71 indicates that the method aligns well with the 12 principles of green analytical chemistry, demonstrating a considerable level of environmental friendliness. The AGREEPrep score of 0.78 further supports this finding, highlighting the sustainability of the sample preparation steps, which is a critical aspect in reducing the environmental impact of an analytical procedure.

In addition to green chemistry assessments, the method was evaluated using applicability and performance indices. BAGI score of 80 suggests a high level of practicality, robustness, and ease of implementation in routine analysis. Similarly, the RAPI score of 77.5 indicates strong analytical reliability, with good precision, accuracy, and detection capabilities.

Table S3 provides a summary of sample preparation and analytical methods from various studies, including their AGREEPrep scores. Overall, these results confirm that the developed method achieves a well-balanced compromise between environmental sustainability and analytical performance. The relatively high AGREE and AGREEPrep scores demonstrate its suitability as a green analytical procedure, while the BAGI and RAPI scores confirm its practical applicability and reliability for real-world analytical applications.

4. Conclusions

The HPLC-UV method described in this paper is fast, precise, and sensitive. The results of the precision and accuracy studies fall within the limits specified by the guidelines, demonstrating that our method is both precise and accurate. Indicate excellent performance; hence, the accuracy of the established method is highly reliable. The recovery rates, ranging from 98.5 % to 106.6 %, achieved will encourage the use of the approach. The simplicity and greenness of sample preparation, short analysis time, and high sensitivity of the presented approach render it especially suitable for quantifying DZP and its primary metabolite in human plasma. We highly endorse this established approach for routine therapeutic medication analysis of DZP, and it can also be modified for

Table 4
Demographic information of the male volunteers.

Sample Size (n)	**53**	
	mean	**SD**
Age	45.9	±10.7
Weight	78	±12.6
Length	173.8	±6.6
Start Age	18.6	±6.7
Daily Dose	9.3	±6.4
Daily Dosages	**n**	**%**
5 mg	32	60.38
10 mg	9	19.61
15 mg	2	2.56
17.5 mg	1	0.58
20 mg	6	32.26
25 mg	3	32.26

Table 3
Recovery results of the validation study.

	Patient plasma concentration (µg/mL)	Spiked amount (µg/mL)	Expected concentration (µg/mL)	Found amount (µg/mL)*	Recovery%*	Bias%*
NDZP	0.25					
	0.25	0.50	0.75	0.79	105.3	−5.3
	0.25	1.00	1.25	1.32	105.6	−5.6
	0.25	1.50	1.75	1.74	99.4	0.6
DZP	0.51					
	0.51	0.50	1.01	1.07	105.9	−5.9
	0.51	1.00	1.51	1.61	106.6	−6.6
	0.51	1.50	2.01	1.98	98.5	1.5

* Mean of three replicated analyses.

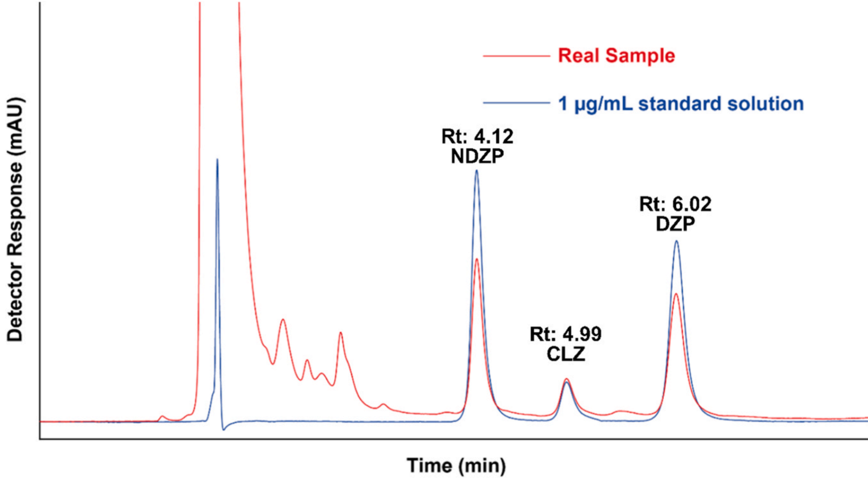

Fig. 3. Chromatograms of the analytical standard solution mixtures (1 μg/mL) and real sample.

monitoring overdose or poisoning in suicide cases. The suggested method is readily applicable in routine therapeutic and non-therapeutic DM of DZP. In addition to DM, this method is also highly beneficial for bioequivalence research, pharmacovigilance, and pharmacokinetic investigations. Due to the major variations noted in plasma drug and primary metabolite concentrations, the polymorphism frequencies of CYP2C19 and CYP3A4 subtypes involved in DZP metabolism will be assessed in our forthcoming clinical study.

CRediT authorship contribution statement

All authors contributed substantially to the conception and design of the data or the analysis and interpretation of the results. **KCT** contributed to conducting the analysis, interpreting the results, and writing the paper draft. **SOK** contributed to the study's design, interpreting the results, evaluating the results, and editing and reviewing the manuscript. **KCT** and **SOK** contributed equally to this work. **CHB** contributed to conducting the analysis and interpreting the results. **MD** contributed to collecting diagnosed patients' biological samples and clinical data. **IOI** contributed to collecting diagnosed patients' biological samples and clinical data. **DKA** contributed to the study's design, evaluation of the results, and editing and reviewing of the manuscript. **HSS** contributed to the study's design, evaluation of the results, and editing and reviewing the manuscript. **MG** contributed to the study's design, evaluation of the results, and editing and reviewing the manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jpba.2025.116805.

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