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Development of a terbium-based coordination polymer nanoprobe for determination of clonazepam in exhaled breath condensate

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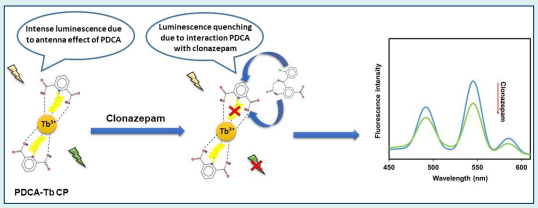
Abstract

Introduction: Clonazepam is a long-acting benzodiazepine utilized for conditions such as seizures, panic disorder, and other neurological/psychiatric disorders. It functions by increasing GABA effects, which leads to reduced neural activity. Careful dosage titration and monitoring are necessary due to potential side effects, including drowsiness, impaired motor coordination, memory loss, and dependence.



Methods: In this work, an optical probe based on a terbium (Tb) coordination polymer was developed for the quantification of clonazepam in the exhaled breath condensate (EBC). The principle of the probe was based on the interaction of clonazepam with pyridine-2,6-dicarboxylic acid -Tb coordination polymer nanoparticles, which dynamically quenches the Tb^{3+} luminescence through a collisional mechanism, as confirmed by the linear Stern-Volmer plot and the increased quenching efficiency at higher temperatures. As the increase in response intensity is proportional to clonazepam concentration, a method was offered for its determination in EBC samples.

Results: This method presented a linear relationship with clonazepam concentration in the range of 0.001-1.5 $\mu g.mL^{-1}$ with a limit of detection of 0.0002 $\mu g.mL^{-1}$ and the intra-day and inter-day relative standard deviation of 1.8%, and 2.7%, respectively. The validated method was used for clonazepam analysis in patients receiving this medication. The recovery values, ranged from 90% to 104%, served as an indicator of the method's accuracy, and confirmed that the measured signal was attributable specifically to clonazepam with high reliability.

Conclusion: By being faster, simpler, and cost-effective than traditional techniques, this method presented a valuable tool for therapeutic drug monitoring.

Introduction

Clonazepam is a long-acting benzodiazepine, anticonvulsant, and antianxiety drug widely used in neurological and psychiatric disorders such as seizures, panic disorder, mania, and REM sleep behavior disorder. The action mechanism of this drug is to increase serotonin synthesis and enhance GABA-A receptors; this drug increases the frequency of opening of chloride channels in neurons, leading to hyperpolarization and decreased neural activity, leading to anticonvulsant, sedative, and antianxiety effects.[1] The dosage and administration of the drug depend on the type of disorder, with treatment starting at a low dose and gradually increasing. To avoid side effects, gradual dose increases and careful monitoring of treatment are essential. Clonazepam use causes side effects such as drowsiness, impaired motor coordination, confusion, memory loss, and dependence. Regular monitoring is necessary to reduce the risk of cognitive

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Research Highlights

What is the current knowledge?

- Clonazepam is a well-established long-acting benzodiazepine used to treat various neurological and psychiatric conditions.
- Therapeutic drug monitoring of clonazepam is important due to potential side effects and the risk of dependence, necessitating careful dosage adjustments.

What is new here?

- A simple fluorometric probe utilizing a terbium coordination polymer has been developed for quantifying clonazepam.
- This method allows for the detection and measurement of clonazepam in EBC sample in the concentration range of 0.001-1.5 µg.mL-1
- The developed probe offers a faster, simpler, and cost-effective approach to therapeutic drug monitoring of clonazepam compared to existing traditional techniques.

and motor impairment, depression, and potential abuse.[2]

Different analytical methods have been reported to measure clonazepam level in various biological samples, including reverse phase high performance liquid chromatography (RP-HPLC)-UV,[3] liquid chromatography tandem mass spectrometry (LC-MS/MS),[4] gas chromatography-mass spectrometry (GC-MS),[5] electrochemical sensor,[6,7] colorimetric sensor,[8] and spectrofluorometric methods.[9,10] Despite its high accuracy and separation power, the chromatographic method requires complex, time-consuming and expensive sample preparation steps. Electrochemical sensors have been proposed as a rapid and sensitive. However, these methods suffer from insufficient selectivity. This creates a demand for affordable and user-friendly platforms to detect clonazepam in biological samples.

In recent decades, nanomaterials have provided sensitive, selective, and rapid platforms for drug detection. Coordination polymers (CPs), as a class of nanomaterials, are hybrid materials composed of metal ions and organic ligands that are used in chemical and biological sensing, catalysis, drug delivery, and imaging due to their high stability and unique optical properties. Their optimized design allows the fabrication of highly sensitive, selective, and fast-response fluorescence sensors for the visual and real-time detection of analytes.[11] Terbium (Tb) stands out among the lanthanides for luminescence-based sensing due to a unique combination of advantageous photophysical properties. These include a long-lived emission with an exceptionally narrow and intense spectral band, high photochemical stability, and the ability to tune its excitation wavelength through strategic ligand selection.[12] Furthermore, its significant charge and ionic radius allow for direct coordination with analytes. Furthermore, Tb emission parameters are highly responsive and susceptible to changes in the local chemical environment upon interaction with target molecules,[13] making it an ideal center for constructing responsive sensing platforms. Herein, the ligand pyridine-2,6-dicarboxylic acid (PDCA) was chosen to synthesize Tb-based CPs (Tb-CPs). PDCA acted as an effective tridentate chelator, facilitating the formation of a stable and well-defined polymeric network. To the best of our knowledge, this work represented the first application of a CP-based fluorescent probe for the detection and determination of clonazepam. A particularly innovative aspect of this study was its focus on analyzing exhaled breath condensate (EBC). It acted as a promising and non-invasive alternative biological sample[14] minimized patient discomfort and clinical complexity. This approach therefore, targeted a critical need for convenient monitoring in critical care settings.

Materials and Methods

Reagents and solutions

All consumables were of the analytical grade and deionized water was obtained from Shahid Ghazi Pharmaceutical Company (Tabriz, Iran). In this study, Tb (III) chloride hexahydrate ($TbCl_3 \cdot 6H_2O$, Acros Organics, USA), PDCA (Merck, Germany) and Tris-HCl (Merck, Germany) were used. The standard stock solution of clonazepam (Sobhan Pharmaceutical Company, Iran) was prepared by dissolving a certain amount of the drug in ethanol, and working solutions were prepared daily by diluting the stock solution in ultrapure water.

Apparatus and instruments

Fluorescence spectra were recorded with a FP-750 spectrofluorometer (Jasco, Japan) with a bandwidth of 5 nm in the excitation and emission directions. Data control and processing were performed with the Windows-based Spectra Manager TM software. The shape, size, and elemental composition of the nanoparticles were examined by scanning electron microscopy (SEM) and energy dispersive X-ray spectroscopy (EDX) MIRA III model (TESCAN, Czech Republic), and the hydrodynamic size and size distribution of the particles in solution were determined by a dynamic light scattering (DLS) instrument (Philips, Leiden, The Netherlands). The pH of the solutions was adjusted with a digital pH meter model 744 (Metrohm Ltd., Switzerland) and the materials were weighed with an electronic analytical balance model AB204-S (Mettler Toledo, Switzerland).

Synthesis of Tb-PDCA CP nanoparticle (NPs)

For synthesis of Tb-PDCA CP NPs, 200 µL of 75 $mmol \cdot L^{-1}$ PDCA solution and 100 µL of 5.36 $mmol \cdot L^{-1}$ Tb solution were added to 5 mL of 48 $mmol \cdot L^{-1}$ Tris–HCl buffer (pH 7.4) and the resulting mixture was stirred for 30 min at room temperature using a magnet on a magnetic stirrer to perform the coordination reaction and form CP NPs.[15]

Sample preparation

EBC samples were collected using a laboratory-made device and used directly for analysis without the need for

prior preparation.[16] The samples used in the optimization and calibration phase were collected from healthy volunteers, while for the real-world sample study, patients under mechanical ventilation were selected as subjects and tracheal aspirates were collected from the ventilator waste. All participants provided written informed consent under a protocol approved by the Tabriz University of Medical Sciences as IR.TBZMED.REC.1404.299.

General procedure

At this stage, 150 µL of EBC sample (either real or clonazepam spiked) was poured into a microtube and then 10 µL of citrate buffer (0.1 mol.L^{-1}) with a pH of 5.0 was added. After that, 45 µL of the synthesized Tb-PDCA CP NPs were added to the solution and the total volume was adjusted to 400 µL with ultrapure water. Fluorescence intensity was measured at $\lambda_{em} = 546$ nm ($\lambda_{ex} = 374$ nm). The analytical signal, ΔF, was defined as $F_0 - F$, where F and F_0 are the fluorescence intensities with and without clonazepam, respectively. All measurements were performed in triplicate at room temperature, and the mean value was reported.

Results and Discussion

Characterization of PDCA -Tb CP

The morphology of the Tb-PDCA CP NPs was characterized by SEM analysis. Fig. 1A presents the SEM images of the probe before and after the addition of thiopental. DLS analysis, shown in Fig. 1B, indicated a primary particle size distribution of less than 100 nm. Successful synthesis was also verified using EDX spectroscopy and spectrofluorimetry. The EDX spectrum (Fig. 1C) confirmed the expected chemical composition, including the elements C, N, O, and Tb. Further confirmation was provided by the fluorescence spectrum (Fig. 1D), which, under 374 nm excitation, displayed three characteristic emission peaks at 492, 546, and 587 nm, consistent with the formation of the probe.

Mechanism of clonazepam sensing

In this system, the detection element was a Tb-based CP. This material was architecturally defined by Tb^{3+} ions acting as central metallic nodes, which were structurally connected into an expansive, ordered network through bridging organic ligands of PDCA. When photoexcited at a wavelength of 374 nm, the Tb-PDCA CP displayed the signature green luminescence of Tb ions, with distinct emission peaks at 492, 546, and 587 nm (Fig. 2). The intense luminescence was primarily driven by an "antenna effect," whereby the PDCA ligands effectively absorbed incident light and subsequently transferred the energy to the Tb^{3+} ions, sensitizing their characteristic emission.

The analytical capability of the resulting Tb-PDCA CP probe was evaluated using the benzodiazepine drug clonazepam as a model analyte. Upon introducing clonazepam to an aqueous suspension of the Tb-PDCA CP probe, a pronounced and concentration-dependent quenching of the characteristic Tb luminescence was observed (Fig. 2). The photoluminescence intensity decreased as the concentration of clonazepam increased. This direct, proportional "turn-off" response established the system as a highly sensitive and quantitatively reliable fluorescent platform for the specific detection of clonazepam.

To elucidate the molecular mechanism underlying the fluorescence quenching, a Stern-Volmer analysis

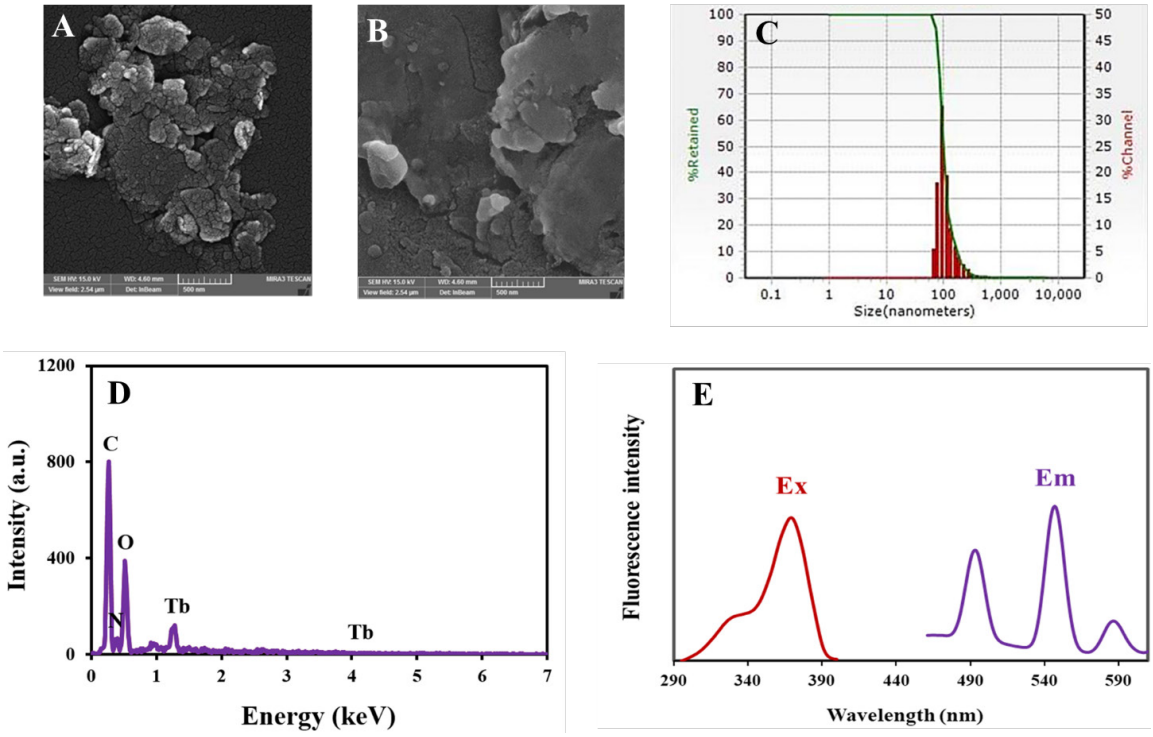


Fig. 1. Characterization findings: (A) SEM image of Tb-PDCA CP NPs before (left) and after clonazepam addition (right); (B) DLS analysis, (C) EDX analysis and (D) excitation/emission spectrum of synthesized Tb-PDCA CP NPs.

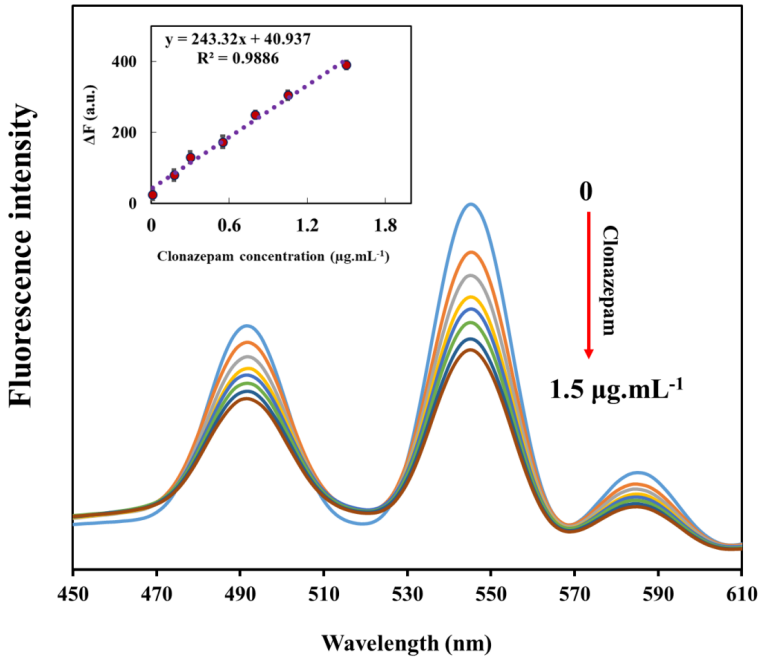


Fig. 2. The fluorescence spectra of PDCA-Tb CP-based probe in the absence and presence of clonazepam at different concentrations (0.001-1.5 μg.mL^{-1}).

was performed. This analytical approach quantified the interaction between a luminescent probe and a quencher by measuring the reduction in emission intensity as a function of increasing quencher concentration. The data were processed according to the classical Stern-Volmer equation,

$$\frac{F_0}{F} = 1 + K_{sv}[Q] \quad (1)$$

Where F_0 and F represent the steady-state luminescence intensities in the absence and presence of the quencher (clonazepam), [Q] is the quencher concentration, and K_{SV} is the Stern-Volmer quenching constant, which quantitatively reflects the quenching efficiency. So, the characteristics of the Stern-Volmer plot and the temperature-dependent behavior of K_{SV} serve to distinguish between the two quenching modalities: static quenching, which involves the pre-association of the fluorophore and quencher into a non-emissive ground-state complex, and dynamic (collisional) quenching, which occurs through diffusive encounters that deactivate the fluorophore's excited state.

In this work, the quenching of the Tb-PDCA CP NPs emission by clonazepam was conclusively identified as a dynamic process. This assignment was based on two experimental findings. First, the Stern-Volmer plot (F_0/F versus [Q]) yielded a linear correlation across the studied concentration range (Fig. 3), consistent with a single, homogeneous quenching process. Second, and most diagnostically, the calculated K_{SV} values exhibited a positive temperature dependence, increasing with rising temperature from 15 up to 35 ^{0}C. This behavior was a symbol of dynamic quenching, as elevated thermal energy accelerated molecular diffusion, thereby increasing the frequency of collisional encounters between the quencher

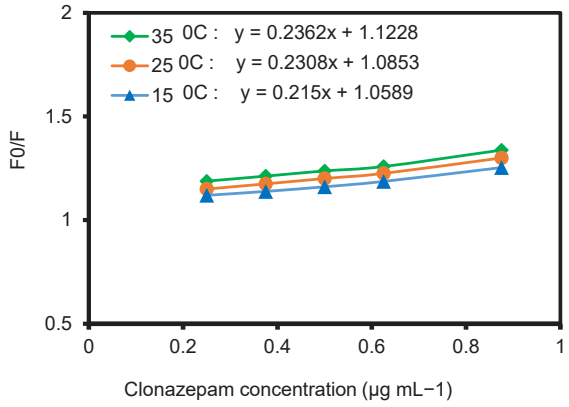


Fig. 3. Stern–Volmer plot for the quenching of Tb-PDCA CP NPs based probe by clonazepam.

and the luminescent centers. It should be noted that the spectrofluorometer's cell holder was equipped with a thermostat that provided temperature control ranging from 0 to 40 °C.

The structural features of clonazepam directly enabled this efficient collisional quenching mechanism. The aromatic and electron-deficient nature of clonazepam may allow for weak non-covalent interactions (*e.g.*, π-π stacking or electrostatic interactions) with the aromatic rings of the PDCA ligands or the nanoparticle surface. This proximity could facilitate photoinduced electron transfer from the excited state of the probe to clonazepam, providing a non-radiative decay pathway, enhancing the quenching efficiency and leading to the observed concentration-dependent attenuation, or "turn-off," of the characteristic green luminescence (Scheme 1).

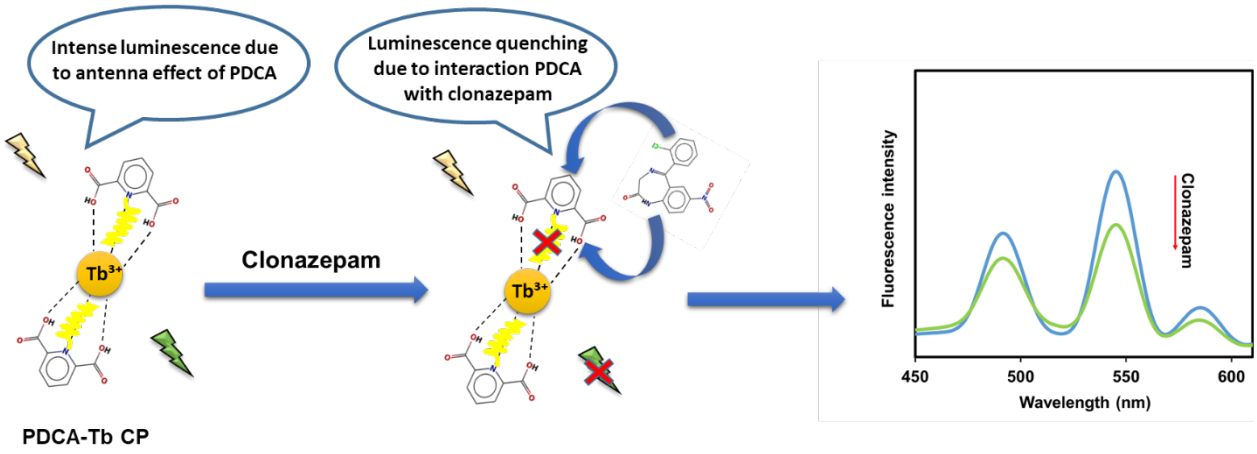
Optimization of reaction conditions

Preliminary experiments showed three variables with a significant effect on the probe's fluorescence response: the

pH of the solution, the amount of Tb-PDCA CP NPs used, and the incubation time for the interaction. To provide a consistent basis for this optimization, a standardized clonazepam concentration of 0.3 $\mu g.mL^{-1}$ was chosen as the analyte concentration. The first parameter was the solution's pH and measurements of the fluorescence response were taken across a pH spectrum from 3.0 to 9.0. The results revealed a pH-dependent pattern; as the pH increased up to 5.0, the intensity of the fluorescence signal (ΔF) increased and when the pH was raised above this optimal point, a gradual decrease in the fluorescence signal was noted, as depicted in Fig. 4A. This drop is likely due to changes in the protonation state of the probe leading to the deactivation of the Tb-PDCA system under basic conditions. Thus, a pH of 5.0 was chosen as the optimal condition. Next, the amount of Tb-PDCA CP NPs was investigated in a range from 10 to 60 µL. The result, presented graphically in Fig. 4B, demonstrated that the fluorescence response increased with greater volumes of Tb-PDCA CP up to 45 µL and reached a plateau at higher amounts. This means all binding or sensitizing sites were occupied, so adding more nanoparticles no longer increased the fluorescence signal. So, the optimum response was achieved at a volume of 45 µL. Following this, a final analysis was performed to determine the impact of incubation time on the analytical response. The nanoprobe's response toward clonazepam was tracked over a series of time intervals, from immediate measurement to periods longer than 20 minutes. The findings (Fig. 4C) presented that the nanoprobe's signal maintained notable stability for up to 20 minutes, exhibiting no considerable loss in the intensity or change in the spectral properties. This suggested that the system achieved equilibrium almost immediately after mixing, eliminating any requirement for more incubation period to achieve the signal.

Analytical performance of the developed method

To study the analytical performance of the optimized nanoprobe for quantifying clonazepam, its concentration-



Scheme 1. Schematic illustration of the quenching of Tb-PDCA CP NPs-based probe by clonazepam.

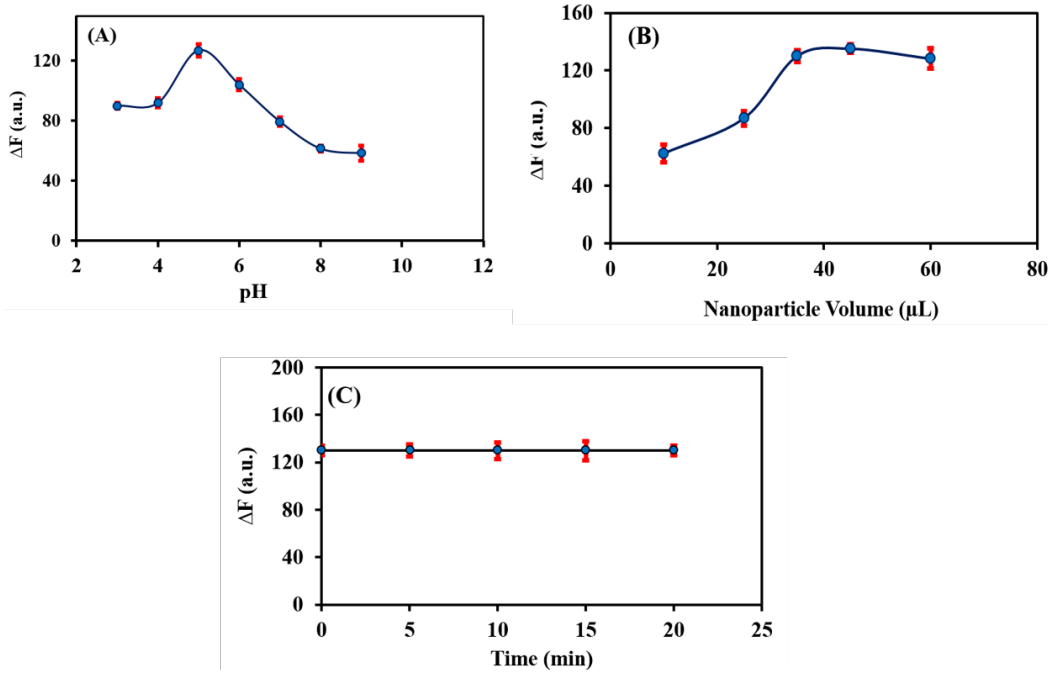


Fig. 4. Impact of (A) pH, (B) the volume of PDCA-Tb CP NPs, and (C) time on the probe response.

dependent fluorescence behavior was analyzed in EBC samples. This investigation was performed using the previously determined optimal parameters to guarantee that the performance valuation accurately represented the probe's efficacy in a relevant application setting. Known concentrations of clonazepam were introduced into a series of EBC samples, and the corresponding changes in the system's fluorescence intensity (ΔF) were recorded. The nanoprobe produced a correlated and linear analytical response to clonazepam across a concentration range of 0.001-1.5 μg.mL^{-1}. This linear relationship was visually displayed in the inset of Fig. 2. Linear regression analysis produced the equation $\Delta F = 243.32\ C_{Clonazepam} + 40.937$. In this context, ΔF was defined as ($F_0 - F$), where F_0 represents the initial fluorescence intensity at 546 nm without clonazepam, and F was the intensity recorded after the analyte adding. The strength and reliability of this linear correlation were confirmed by a high coefficient of determination ($R^2 = 0.9886$). The method's sensitivity was further characterized by calculating the limit of detection (LOD) using the formula $3S_b/m$, where S_b was the standard deviation of multiple blank measurements and m was the slope of the calibration curve. The LOD was determined to be 0.0002 μg·mL^{-1}. Additionally, the precision of the analytical method was evaluated through a replicate study. For five replicate analyses of an EBC sample containing 0.3 μg·mL^{-1} of clonazepam, the relative standard deviation (*RSD*) was 1.8%. The inter-day precision for the same concentration was calculated to be 2.7%.

Table 1 provided a comparative analysis of the performance features of the validated method against other reported techniques for clonazepam determination in various matrices. While sophisticated chromatographic methods like HPLC and GC are robust and trusted for laboratory use, they typically demand extensive sample preparation, advanced instrumentation, and specialized personnel. Electrochemical methods, on the other hand, often face limitations in selectivity against interferences and long-term reproducibility, potentially compromising reliability in complex samples. Optical methods offer a notable alternative, characterized by their portability, capacity for miniaturization, and suitability for rapid, on-site analysis which are key attributes for point-of-care diagnostics. The data in Table 1 indicated that our method achieved a linearity range and LOD comparable to, and in some instances better than, previously reported methods for this drug. This superior performance, particularly at lower concentrations, underscored the method's high efficiency.

Interference study

In order to investigate the selectivity of the probe towards clonazepam, the effect of other drugs, either over-the-counter, taken concurrently or structurally related, such as pantoprazole, aspirin, ibuprofen, losartan, chlordiazepoxide, alprazolam, amoxicillin, acetaminophen, propranolol, diltiazem, naproxen, oxazepam, and diazepam, was studied in a solution with a constant concentration of 0.3 μg.mL^{-1} and under optimal conditions. The data illustrated in Fig. 5 demonstrated that the probe's response remained mostly unaffected by the presence of the tested pharmaceuticals, except in the cases of oxazepam and diazepam. These results verified an acceptable level of specificity for the precise quantification of clonazepam. The observed interference from oxazepam and diazepam was likely due to their structural similarity to clonazepam, which compromised the accuracy of the clonazepam determination. To address these constraints, separation or extraction techniques could be employed prior to analysis. The exploration of such a strategy represented a potential avenue for future research aimed at enhancing the probe's specificity.

Determination of clonazepam in real EBC samples

The Tb-PDCA CP NPs-based fluorescent probe was applied to real-world analysis by determining clonazepam concentrations in the EBC of three medicated patients collected from the ventilator waste (results in Table 2). The reliability of these values and the potential for matrix interference were checked using a standard addition method, wherein samples were spiked with known quantities of clonazepam at two concentration levels (0.01

Table 1. Comparison of the performance features of the validated method for clonazepam determination with other reported techniques

Method	Probe	Sample	LOD (μg.mL^{-1})	Linear range (μg.mL^{-1})	Reference
RP-HPLC-UV	-	Aqueous samples	-	0.1-30	[3]
LC-MS/MS	-	Urine		0.002-0.3	[4]
GC-MS	-	Pericardial fluid	0.02	0.04-0.25	[5]
Electrochemical sensor	Multi-wall carbon nanotube molecularly imprinted polymer	Drinking water, urine, blood, tablet	0.27	0.52-22.8	[6]
Electrochemical sensor	FeCu-LDH@MXene nanocomposite	Pharmaceutical ampoules, plasma	0.03	0.2-134	[7]
Colorimetric sensor	DH-1,6-NAPY-8-CN-AgNPs	Plasma	0.0009	0.015-24.0	[8]
Spectrofluorimetry	Polydopamine nanoparticles	Plasma	-	0.05-1.0 1.0-15.0	[9]
Spectrofluorimetry	Nitrogen-doped carbon dots	Aqueous samples	3.8×10^{-6}	1.5×10^{-5}-0.002	[10]
Spectrofluorimetry	Tb-PDCA NPs	EBC	0.0002	0.001-1.5	This work

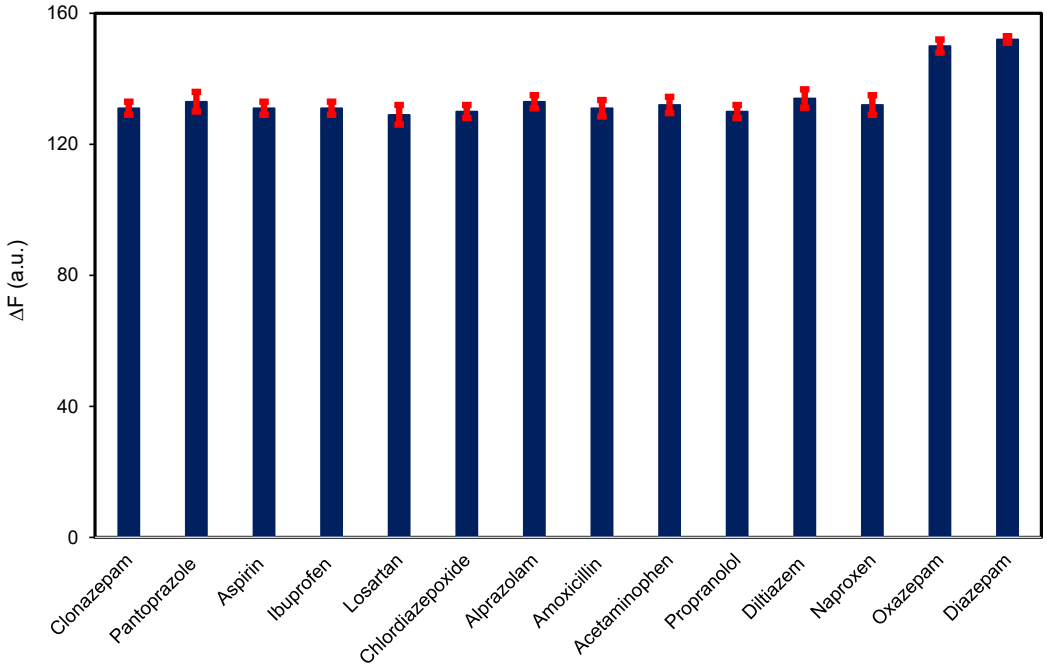


Fig. 5. The selectivity study in the presence of interferences with a concentration of 0.3 μg.mL^{-1} (clonazepam concentration = 0.3 μg.mL^{-1}).

and 0.1 μg.mL^{-1}). As shown in Table 2, the calculated recovery percentages fell within the acceptable range of 90% to 104%, confirming accuracy of the probe's response toward clonazepam in this biological matrix.

Conclusion

This study developed a Tb-PDCA CP NPs-based fluorescence sensor for detecting clonazepam in EBC. The probe's fluorescence intensity decreased proportionally with rising clonazepam concentrations. A linear response was established over the range of 0.001 to 1.5 μg·mL^{-1}, with a LOD of 0.0002 μg·mL^{-1}. The method proved reliable, showing low variability (intra-day RSD: 1.8%; inter-day RSD: 2.7%). Compared to traditional chromatographic methods, this approach was quicker, simpler, and more economical, making it a promising tool for therapeutic drug monitoring to avoid toxicity or under-dosing. Future efforts should involve validation with larger patient groups to ensure consistency across different physiological states. This work highlighted the potential of nanomaterial probes and advanced EBC as a practical medium for non-invasive diagnostics.

Table 2. Determination of clonazepam concentration in EBC of medicated patients and recovery experiments

Sample	Added (μg.mL^{-1})	Found (μg.mL^{-1})	Recovery (%)[a]
	-	0.005	-
1	0.01	0.014	90.0
	0.1	0.1134	99.4
	-	0.007	-
2	0.01	0.0174	104.0
	0.1	0.118	100.6
	-	0.003	-
3	0.01	0.0129	99.0
	0.1	0.11	97.1

[a] Recovery (%) = [clonazepam concentration in samples (after spiking - before spiking)/Added] × 100.

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Authors' Contribution

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Supervision: Abolghasem Jouyban.
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Visualization: Maryam Khoubnasabjafari, Vahid Jouyban-Gharamaleki.
Writing-original draft: Zahra Najafzadeh.
Writing-review & editing: Elaheh Rahimpour, Abolghasem Jouyban.

Competing Interests

The authors declare that they have no competing interests.

Data Availability Statement

All data generated or analyzed during this study are included in this published article.

Declaration of AI-assisted Tools in the Writing Procedure

The author(s) declare that Generative AI was not used in the creation of any figures included in this manuscript. During the preparation of this work the authors used DeepSeek AI Tool in order to improve the writing process and to enhance the readability and language of the manuscript. After using this tool/service, the authors reviewed and edited the content as needed and takes full responsibility for the content of the published article.

Ethical Approval

Ethical approval for this study was guided by the Declaration of Helsinki. Prior to participation, the study's objectives and procedures were explained to all individuals, with guarantees provided regarding the

confidentiality and anonymity of their information. Written informed consent was obtained following this explanation. In the case of illiterate participants, interviewers read the consent form aloud in full prior to obtaining their consent. All participants were advised of their right to withdraw at any stage of the research. All participants provided written informed consent under a protocol approved by the Tabriz University of Medical Sciences as IR.TBZMED.REC.1404.299.

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