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Development and validation of a green spectrofluorimetric method for brivaracetam determination using N-doped graphene quantum dots: Mechanistic insights and bioanalytical applications

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ABSTRACT

Brivaracetam is an important antiepileptic drug, and its sensitive and selective determination in biological matrices is crucial for therapeutic drug monitoring and pharmacokinetic studies. However, the currently available analytical methods suffer from limitations such as low sensitivity, high environmental impact, and complex sample preparation. In this work, a highly sensitive and selective spectrofluorimetric method was developed for the determination of brivaracetam in human plasma and pharmaceutical formulations. The method is based on the interaction between brivaracetam and N-doped graphene quantum dots (GQDs) leading to fluorescence quenching. The quenching mechanism was investigated using density functional theory calculations, Stern-Volmer analysis and thermodynamic studies. A static quenching process was observed indicating complex formation between the analyte and nanomaterial. Different factors affecting the quenching efficiency were carefully optimized such as pH, GQDs concentration, and incubation time. The proposed method was validated according to the ICH M10 guidelines and showed excellent linearity in the concentration range of 0.1–2.5 μg/mL with a limit of detection of 0.033 μg/mL. Furthermore, the method displayed good precision and accuracy, and selectivity in the presence of common plasma/formulation excipients. Hence, the developed method was successfully applied for the determination of brivaracetam in pharmaceutical formulations as well as pharmacokinetic monitoring in human plasma samples. The "greenness", "blueness" and "whiteness" of the proposed method was also evaluated using the AGREE, BAGI and RGB12 metrics, respectively, confirming its eco-friendly, high practicability and sustainable nature transcending the reported conventional analytical techniques, posing the developed method as a promising analytical tool for brivaracetam determination.

1. Introduction

The accurate determination of brivaracetam in biological matrices and pharmaceutical formulations presents significant analytical challenges in clinical practice. Brivaracetam, a novel antiepileptic drug, acts by selectively binding to synaptic vesicle protein 2A (SV2A), demonstrating improved efficacy and tolerability compared to earlier generation anticonvulsants [1,2]. Given the chronic nature of epilepsy treatment and the need for personalized dosing regimens, reliable therapeutic drug monitoring of brivaracetam is essential for optimizing patient outcomes and minimizing adverse effects.

Current analytical methods for brivaracetam quantification

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primarily rely on chromatographic techniques such as LC-MS/MS and HPLC-UV [3–6]. For example, a study by Iqbal et al. developed a UPLC–MS/MS method for the analysis of brivaracetam in plasma using gradient mobile phase combination of acetonitrile and 0.1 % formic acid in water with application to pharmacokinetic studies in rat plasma [4]. Another study by Mansour et al. reported an HPLC-UV method for the determination of brivaracetam in human plasma and pharmaceutical formulations using a mobile phase made out of (70:30 v/v) mixture of 1 % of triethylamine in water and acetonitrile [6]. While these methods provide adequate selectivity, they present several significant limitations. The requirement for extensive sample preparation procedures, substantial organic solvent consumption, and sophisticated instrumentation makes them resource-intensive and potentially inaccessible in routine clinical settings. Furthermore, these methods often generate considerable analytical waste, raising environmental concerns about their long-term sustainability in clinical laboratories.

Recent advances in nanomaterial-based sensing platforms have opened new possibilities for developing more sustainable analytical methods. Among these, quantum dots (QDs) have emerged as a promising fluorescent nanomaterial for various analytical applications due to their unique optical properties, tunable emission wavelengths, and high photostability [7]. In particular, graphene quantum dots (GQDs) have garnered significant attention for their potential in fluorescence-based sensing and detection, owing to their excellent biocompatibility, ease of synthesis, and facile surface functionalization [8]. Furthermore, doping graphene quantum dots with heteroatoms, such as nitrogen, can further enhance their photophysical characteristics and analytical performance [9]. Hence, N-doped graphene quantum dots represent an attractive platform for the development of sensitive and selective fluorescent assays particularly for bioanalytical applications [10,11]. However, selectivity remains a challenge for the detection of specific analytes, and further investigations are needed to fully understand the mechanisms governing the fluorescence modulation. Surface functionalization can play a crucial role in enhancing the selectivity of N-GQDs towards target analytes [12]. Among different strategy for functionalization, the use of ionophores such as sodium tetraphenyl borate have shown to enhance the binding affinity towards positively charged drugs thus improving the detection limits and selectivity [13,14].

In addition to the analytical aspects, the assessment of the "greenness" of the analytical method is also of paramount importance to ensure the sustainability and environmental friendliness of the developed analytical procedures [15]. GQDs have also demonstrated great potential as green, fluorescent probes with minimal toxicity compared to traditional semiconductor quantum dots [16]. Furthermore, spectrofluorimetry as a technique is generally considered a greener analytical approach compared to other instrumental techniques due to the reduced consumption of reagents and solvents as well as lower energy requirements [17,18]. Hence, the development of spectrofluorimetric methods based on functionalized N-GQDs represents a promising approach towards the realization of "greener" analytical protocols. In this context, the literature reported the use of N-doped graphene quantum dots for the sensitive and selective detection of many pharmaceutical analytes [19,20], however, studies focusing on the determination of the antiepileptic drug brivaracetam using N-GQDs have not been reported yet.

Therefore, the present work aims to develop a green, sensitive, and selective spectrofluorimetric method for the determination of brivaracetam in human plasma and pharmaceutical formulations using N-doped graphene quantum dots as the fluorescent probe. The spectral characteristics of the N-GQDs-brivaracetam system will be investigated, and the sensing mechanism will be elucidated through quantum chemical calculations, Stern-Volmer analysis, and thermodynamic studies. The factors affecting the fluorescence quenching process will be systematically evaluated, and the optimized method will be validated according to the ICH M10 guidelines for bioanalytical method validation. Furthermore, the developed method will be applied to analyze brivaracetam in pharmaceutical formulations and to study the pharmacokinetic profile of the drug. Additionally, the "greenness", "blueness" and "whiteness" of the analytical method will be assessed using different metrics, to evaluate the environmental impact, analytical practicability and sustainability of the proposed spectrofluorimetric approach in comparison with the reported literature.

2. Experimental

2.1. Materials

Brivaracetam reference standard with certified purity (99.26 %) was obtained from the Egyptian Drug Authority, Cairo, Egypt. The materials used for the synthesis of N-doped functionalized graphene quantum dots, including citric acid as the carbon source, urea, and sodium tetraphenyl borate, were purchased from Sigma Aldrich (St. Louis, MO, USA). Britton Robinson buffer solutions were prepared from acetic acid, boric acid and phosphoric acid of analytical grade purchased from Merck (Darmstadt, Germany). HPLC grade acetonitrile used for sample preparation was supplied by Sigma Aldrich (St. Louis, MO, USA). The pharmaceutical formulation containing brivaracetam, Brivafutal,® manufactured by Future Pharmaceutical Industries in Egypt, was obtained from the local pharmacy in Cairo, Egypt. Each tablet is claimed to contain 100 mg of brivaracetam.

2.2. Instrumentation

The spectrofluorimetric measurements were performed on a Jasco FP-6200 spectrofluorometer equipped with a 150 W Xenon lamp as an excitation source and a photomultiplier tube detector. Slit widths for both excitation and emission were set to10 nm. The pH measurements were carried out using a calibrated Jenway 3510 pH meter. The UV measurements were conducted on a Shimadzu 1800 UV–Vis spectrophotometer. Dynamic light scattering (DLS) measurements were performed using a Malvern Zetasizer Nano ZS90 instrument to determine the particle size of the N-GQDs. Transmission Electron Microscopy (TEM) analysis was conducted using A JEM-2100 transmission electron microscope operated at 200 kV to obtain the morphology of the N-GQDs. Fourier-transform infrared (FT-IR) spectra were recorded on a Nicolet iS5 FT-IR spectrometer in the wavenumber range of 4000–400 cm^{-1} using the KBr pellet technique.

2.3. Reagents and standard solutions

The N-GQDs were synthesized by a hydrothermal treatment of 0.3 g of citric acid and 0.7 g of urea in 25 mL of distilled water at 180 °C for 2 h. The obtained colloidal solution was then dialyzed against deionized water to remove any unreacted precursors and subsequently dried under vacuum to obtain the N-GQDs powder. To functionalize the prepared N-GQDs, 50 mL of 10^{-2} M sodium tetraphenyl borate was added to 50 mL of 10^{-2} M brivaracetam solution and the mixture was allowed to react to form ion pair complex. The precipitate was allowed to stand for 24 h with its mother liquor then, filtered, washed and dried. Twenty-five mg of the dried complex was mixed with 75 mg of the N-GQDs powder and dispersed in 50 mL of water *via* sonication for 20 min to yield a 2 mg/mL of the functionalized N-GQDs. The Britton-Robinson (B-R) buffer solutions employed in this study were freshly prepared in the pH range 3–10 using 0.04 M mixtures of phosphoric acid, acetic acid, boric acid and the pH was adjusted using 0.2 M sodium hydroxide to achieve the desired pH.

A stock solution of brivaracetam (100 μg/mL) was prepared by dissolving 10 mg of the drug into 100 mL of distilled water. This solution was further diluted with the same solvent to obtain working standard solution (10 μg/mL) that was used for calibration and all other experimental purposes.

2.4. Construction of the fluorescent probe

At optimized conditions, 1.5 ml of the 2 mg/mL functionalized N-GQDs was combined with various volumes of the brivaracetam working standard solution in 10 mL volumetric flasks. Then 1.5 mL of B-R buffer at pH 8.0 was added and allowed to stand for 5 min. The volume was made up to the mark with distilled water to give the desired concentration of brivaracetam (0.1–2.5 μg/mL). The emission spectra of the resultant solutions were scanned between 220 and 600 nm upon excitation at 339 nm and the fluorescence intensity at 440 nm was recorded (F). A blank solution was prepared in the same manner except that brivaracetam was replaced with distilled water and the fluorescence intensity was measured under the same conditions (F0). A calibration curve was constructed by plotting the change in fluorescence intensity (F0/F) against the corresponding brivaracetam concentration and the regression equation was computed.

2.5. Theoretical calculations

Density functional theory (DFT) calculations using Gaussian 09 software were performed to elucidate the interaction mechanism between brivaracetam and the functionalized N-GQDs and to identify the possible sites of interaction. The basis set used was B3LYP/6-31G (d) for geometry optimization. The results were visualized using GaussView6 program. The thermodynamic parameters including Gibbs free energy change (ΔG°), enthalpy change (ΔH°), and entropy change (ΔS°) were calculated from the Stern-Volmer quenching constant (Ksv) determined at different temperatures to understand the nature of the quenching process.

2.6. Bioanalytical method validation

The developed spectrofluorimetric method was validated according to the ICH M10 guidelines for bioanalytical method validation in terms of linearity, range, accuracy, precision, limits of detection and quantification, selectivity, matrix effect and robustness [21]. The sample preparation protocol was optimized to ensure efficient and selective extraction of brivaracetam from human plasma. A simple protein precipitation technique was adopted using acetonitrile as the protein-precipitating agent where 2 mL of acetonitrile was added to 5 mL of human plasma spiked with brivaracetam, vortexed for 1 min and centrifuged at 4000 rpm for 10 min. The supernatant was collected, dried and then redissolved in 2 mL of distilled water and the fluorescence intensity of the final extract was measured under the optimized conditions.

Standard calibration curve was constructed using spiked human plasma samples in the concentration range of 0.1–2.5 μg/mL brivaracetam. Linearity was determined using the relative fluorescence intensity (F0/F) against the corresponding brivaracetam concentrations. The back calculated concentrations of the calibration samples as well as the coefficient of determination (r2) were assessed. Limits of detection and quantification were determined based on the residual standard deviation of the regression line and the slope.

The accuracy and precision of the method were evaluated by analyzing quality control samples at four concentration levels (0.1, 0.3, 1.75 and 2.0 μg/mL) corresponded to the lower limit of quantification, low, medium and high concentrations of the standard curve. The selectivity of the method was evaluated through competitive experiments in which the fluorescence responses of the N-GQDs were recorded in the presence of common interfering substances at concentrations 10 times higher than that of brivaracetam. These substances included pharmaceutical excipients, ions, structurally related compounds, common amino acids and pooled plasma samples. Furthermore, the matrix effect was evaluated by analyzing three different batches of blank human plasma spiked with two concentration levels of brivaracetam *i.e* low and high QC and the % recovery and CV% in each concentration were determined. Robustness of the method was examined by introducing small deliberate changes in the experimental parameters such as pH of the buffer solution (±0.2), N-GQDs concentration (±0.1 mL), and reaction time (±0.5 min), the % recovery ± SD was calculated.

2.7. Application to pharmaceutical formulation and pharmacokinetic studies

The validated spectrofluorimetric method was applied for the determination of brivaracetam in commercial Brivafutal ® tablets. Ten tablets were accurately weighed, finely powdered and an amount equivalent to 10 mg of brivaracetam was extracted with 100 mL water. The resulting solution was sonicated for 10 min, filtered and the required dilutions were made with water. The fluorescence intensity of the sample solutions was measured under the optimized conditions and the brivaracetam content was calculated using the regression equation.

The developed method was further applied to a pharmacokinetics study of brivaracetam in healthy human volunteers after oral administration of a single 100 mg dose. All the volunteers provided written informed consent prior to participation and the study was approved by the Ethics Committee in the Faculty of Medicine, Al-Azhar University, Damietta branch (Approval No: DFM-IRB00012367-24-12-0014). Blood samples were collected at different time points (0, 0.5, 1, 2, 4, 6, 8, 12, 18 and 24 h) after drug administration and the plasma was separated by centrifugation. The validated spectrofluorimetric method was used to determine the brivaracetam concentration in the plasma samples, and the pharmacokinetic parameters, including C_{max}, T_{max}, $t_{1/2}$, $AUC_{0\text{-}t}$, and $AUC_{0\text{-}\infty}$, were calculated using non-compartmental analysis with the PK solver tool [22].

2.8. Green, blue and white analytical chemistry assessment

Three metrics were used to assess the greenness, blueness and whiteness of the developed spectrofluorimetric method to ensure the principles of green chemistry, analytical practicability and sustainability: 1) Analytical GREEnness metric approach (AGREE): This metric is based on 12 principles of green analytical chemistry and evaluates the environmental, health, and safety aspects of the method [23]. 2) Blue applicability grade index (BAGI): This metric quantifies the blueness (or degree of user-friendliness) of the analytical method [24]. 3) RGB12: This metric evaluates the whiteness or overall sustainability of the analytical method [25]. Comparison of the proposed method with the conventional analytical methods such as HPLC and LC-MS/MS has been also performed to ensure its advanced analytical characteristics in terms of greenness, blueness and whiteness.

3. Results and discussion

3.1. Characterization and spectral characteristics of the functionalized N-GQDs

The functionalized N-doped graphene quantum dots (N-GQDs) were analyzed using various techniques, including dynamic light scattering (DLS), transmission electron microscopy (TEM), UV–Vis spectroscopy, and fluorescence spectroscopy. DLS measurements indicated an average hydrodynamic diameter of 3.45 ± 0.60 nm (Fig. 1A), suggesting the formation of small, uniformly distributed nanoparticles. TEM imaging confirmed the presence of well-dispersed, quasi-spherical N-GQDs with an average size of 3.63 ± 0.65 nm (Fig. 1B), aligning closely with the DLS results. The FT-IR analysis revealed the presence of key functional groups characteristic of the N-GQDs, including C═O, C–N, and N–H, indicating successful nitrogen doping and surface functionalization. Specifically, the analysis showed peaks in the range of 3400–3200 cm^{-1} corresponding to O–H and N–H stretching vibrations, peaks in the range of 2900–2800 cm^{-1} attributed to C–H stretching, peaks around 1650 cm^{-1} assigned to C═O stretching, and various C–N, C–O, and C–C

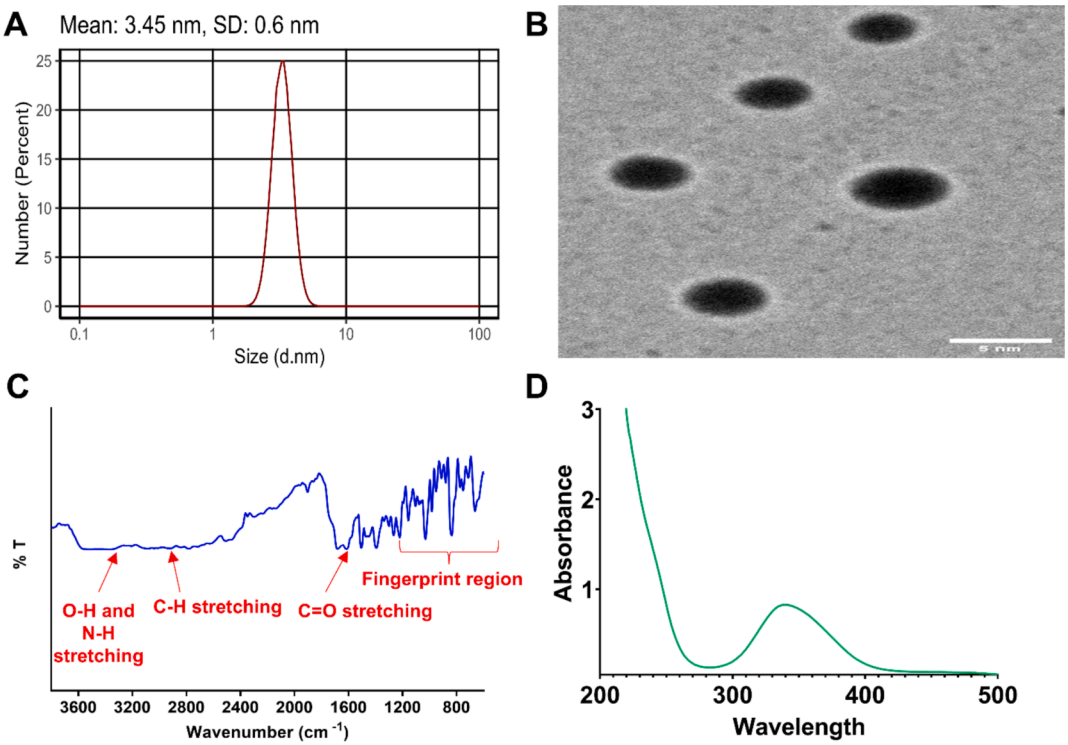

Fig. 1. Characterization of N-doped graphene quantum dots (N-GQDs). (A) Dynamic light scattering (DLS) analysis showing the size distribution of N-GQDs with a mean diameter of diameter of 3.45 ± 0.60 nm, indicating the uniformity and small size of the synthesized N-GQDs. (B) TEM image of N-GQDs, revealing well-dispersed, quasi-spherical nanoparticles (scale bar: 5 nm). (C) FT-IR spectrum of N-GQDs showing characteristic functional groups: O-H and N-H stretching (3400–3200 cm^{-1}), C–H stretching (2900–2800 cm^{-1}), C═O stretching (around 1650 cm^{-1}), and fingerprint region (<1500 cm^{-1}) containing various C–N, C–O, and C–C vibrations. (D) UV–Vis absorption spectrum of N-GQDs showing characteristic absorption peak around 340 nm.

vibrations in the fingerprint region below 1500 cm^{-1}(Fig. 1C). The UV–Vis spectrum of the N-GQDs showed a broad absorption band in the UV–visible region with a maximum absorption at around 340 nm (Fig. 1D). This peak can be attributed to the n – π* transitions of the carbonyl groups present in the N-GQDs as well as N-doping of the graphene structure which also facilitate the creation of new energy levels and introduction of defects that can result in the observed broad absorption.

Regarding the fluorescence properties, the excitation spectrum peaked at 339 nm, closely matching the absorption maximum. Upon excitation at this wavelength, the N-GQDs displayed a strong emission peak at 440 nm, manifesting as a bright blue fluorescence under UV light (365 nm) (Fig. 2A). This pronounced Stokes shift of approximately 100 nm is characteristic of N-GQDs and can be attributed to the quantum confinement effect and the presence of oxygen and nitrogen-containing functional groups on the surface. Upon addition of brivaracetam, a significant quenching of the fluorescence intensity of the N-GQDs was observed, owing to the interaction between the analyte and the N-doped QDs (Fig. 2B). Such a quenching process can be exploited for the sensitive detection of brivaracetam.

3.2. *Mechanisms of sensing of brivaracetam using N-GQDs*

The observed fluorescence quenching of the N-GQDs upon the addition of brivaracetam can be attributed to various mechanisms. When N-GQDs absorb photons, electrons are excited from the ground state to higher energy levels, creating electron-hole (e^{-}–h^{+}) pairs that can relax through multiple pathways. In the absence of brivaracetam, radiative recombination dominates, resulting in photoluminescence at 440 nm. This process is enhanced by N-doping, which introduces new

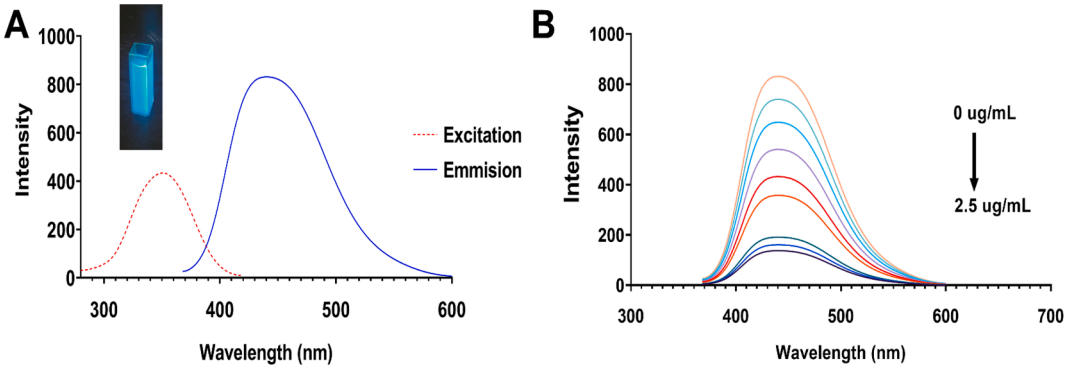

Fig. 2. (A) Excitation and emission fluorescence spectra of the functionalized N-GQDs demonstrated a pronounced fluorescence emission peak at a maximum wavelength (λ_{max}) of 440 nm when excited at 339 nm. (B) Fluorescence emission spectra resulting from the quenching response of the functionalized N-GQDs upon exposure to varying concentrations of brivaracetam.

energy levels and defects in the graphene structure. However, the interaction with brivaracetam creates additional non-radiative pathways through photoinduced electron transfer and/or static and dynamic quenching mechanisms.

To understand these interactions, we conducted both theoretical calculations and experimental analyses. DFT calculations provided insights into the electronic structure of brivaracetam and its potential interactions with N-GQDs. The frontier molecular orbitals, Highest occupied molecular orbital (HOMO) and lowest unoccupied molecular orbital (LUMO) (Fig. 3A and B), revealed a significant energy gap (6.601 eV), indicating the relatively low reactivity of brivaracetam and supporting the observed fluorescence quenching mechanism. The electrostatic potential (ESP) surface plot (Fig. 3C) demonstrated a high electron-rich region around the two carbonyl groups, facilitating hydrogen bonding and electrostatic interactions with N-GQDs. Additionally, a positive charge distribution around the amino group suggests its involvement in electrostatic interactions with the negatively charged carboxyl groups of the N-GQDs.

To elucidate the quenching mechanism experimentally, a comprehensive Stern-Volmer analysis was conducted at different temperatures (Fig. 4A and B). The results strongly indicate a predominantly static quenching process. The Stern-Volmer constant (K_{SV}) values were found to be 2.39×10^5, 2.13×10^5, and $1.83 \times 10^5 M^{-1}$ at 298, 303, and 313 K, respectively (Table S1). These high K_{SV} values suggest a strong interaction between brivaracetam and N-GQDs. Notably, both K_{SV} in addition to the binding constant (Ka) decreased with increasing temperature, which is characteristic of static quenching. The Stern-Volmer plot (Fig. 4A) shows a decreasing slope with increasing temperature, further confirming the static quenching mechanism. The modified Stern-Volmer plot (Fig. 4B) exhibited linear relationships, suggesting a single class of quenching process. These findings collectively support the formation of a non-fluorescent ground-state complex between brivaracetam and N-GQDs. It is worth noting that the inner filter effect has been excluded as a potential quenching mechanism based on the negligible absorption of brivaracetam at either the excitation (339 nm) or emission (440 nm) wavelengths of the N-GQDs (Fig. S1). This lack of spectral interference provides strong evidence that the observed fluorescence quenching is due to direct interaction between brivaracetam and the N-GQDs rather than any secondary optical effects.

Thermodynamic parameters were also calculated to gain deeper insights into the interaction forces (Table S1). Negative ΔG° values (−30.39 to −31.15 kJ/mol) across the temperature range indicate a spontaneous binding process. The negative ΔH° value (−15.15 kJ/mol) confirms the exothermic nature of the interaction, while the positive ΔS° value (51.10 (J/mol K^{-1}) suggests an entropy-driven process, likely due to the release of water molecules upon complex formation. The Van't Hoff plot (Fig. 4C) corroborates these findings, showing a linear relationship between ln Ka and 1/T. The binding constants (Ka) were determined to be 2.11×10^5, 1.89×10^5, and 1.57×10^5 M^{-1} at 298, 303, and 313 K, respectively, further supporting the strong and spontaneous nature of the interaction.

3.3. Analytical method development and validation

Different parameters affecting the fluorescence quenching process such as pH, buffer volume, N-GQDs concentration and incubation time were optimized to achieve maximum sensitivity and selectivity for brivaracetam detection (Fig. 5). Different pH values ranging from 3 to 10 were evaluated, and pH 8.0 was found to provide the optimal fluorescence response (Fig. 5A). This comes in line with previous reports suggesting that the quenching efficiency is enhanced in a neutral to slightly basic medium due to the deprotonation of the N-GQDs and the formation of hydrogen bonds with the target analytes [11]. Increasing the pH above 9 or decreasing the pH to acidic region resulted in decreased fluorescence quenching. This could be attributed to disruption of hydrogen bonding or potential competition from buffer ions on the N-GQDs surface. The volume of the B-R buffer solution was also optimized, and 1.5 mL was found to be the ideal volume for maximum quenching response (Fig. 5B). Another important factor that can affect fluorescence quenching is the N-GQDs concentration. An optimum N-GQDs concentration of 0.03 mg/mL was used as further increase in the concentration did not result in enhanced quenching (Fig. 5C). Additionally, an incubation time of 5 min was found to be sufficient for the quenching to reach equilibrium (Fig. 5D).

The optimized method was validated according to the ICH M10 guidelines for bioanalytical method validation (Table 1). The linearity was evaluated in the range of 0.1–2.5 μg/mL, exhibiting an excellent linear relationship with a correlation coefficient (r^2) of 0.9992. Such range was below the reported therapeutic concentrations of brivaracetam in human plasma indicating the suitability of the proposed method for pharmacokinetic studies. The bake calculated concentrations were all within ± 15 % of the nominal values, indicating good linearity and accuracy. Furthermore, LOD and LOQ were determined to be 33 ng/mL and 100 ng/mL, respectively, demonstrating the high sensitivity of the developed method (Table 1).

Accuracy of the method was evaluated by recovery studies, where known amounts of brivaracetam were spiked into human plasma samples at 4 QC levels. The mean recovery ranged from 95.56 % to 103.55

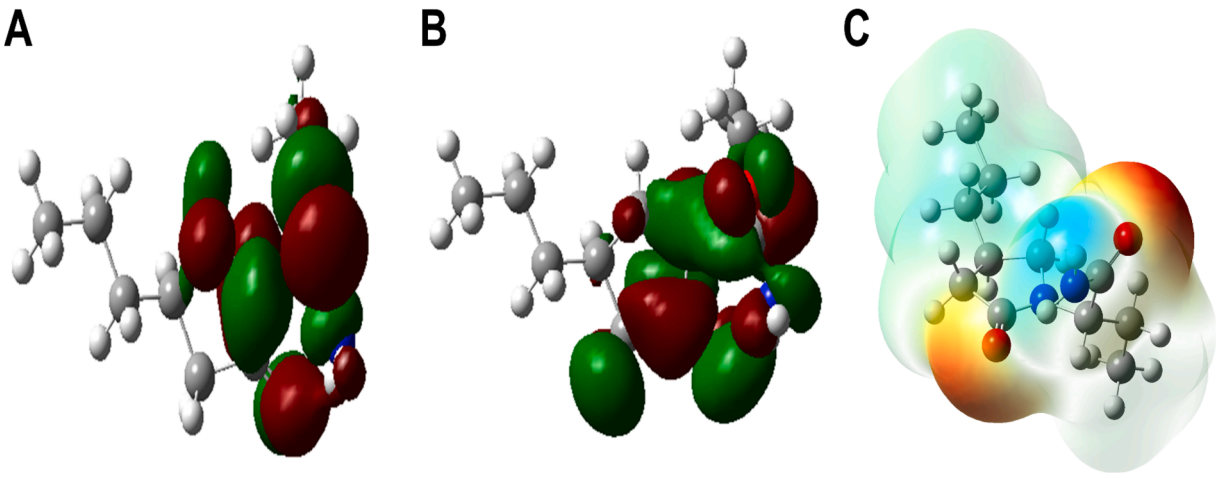

Fig. 3. Computational chemistry analysis of brivaracetam. (A) Highest occupied molecular orbital (HOMO) and (B) lowest unoccupied molecular orbital (LUMO), illustrating the electron density distribution in frontier orbitals. (C) Electrostatic potential surface, demonstrating the molecule's charge distribution in three-dimensional space.

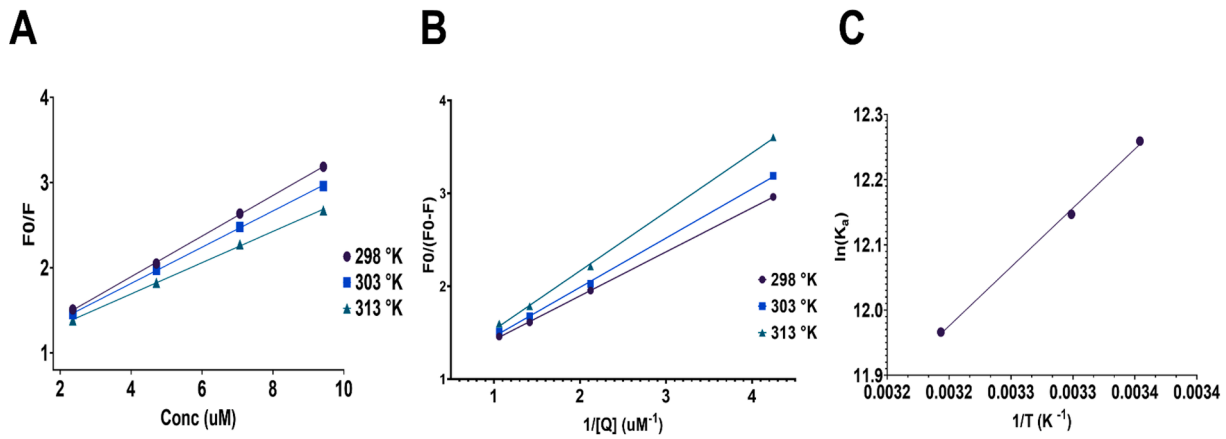

Fig. 4. Investigation of the quenching mechanism of functionalized N-GQDs by brivaracetam. (A) Stern-Volmer plots showing the relationship between F0/F and brivaracetam concentration at different temperatures (298 K, 303 K, and 313 K). The decreasing slope with increasing temperature suggests a static quenching mechanism. (B) Modified Stern-Volmer plots displaying F0/(F0-F) versus the inverse of brivaracetam concentration (1/[Q]) at different temperatures. The linearity of these plots confirms a single type of quenching interaction and validates the applicability of the Stern-Volmer equation to this system. (C) Van 't Hoff plot showing the temperature dependence of the binding constant (Ka).

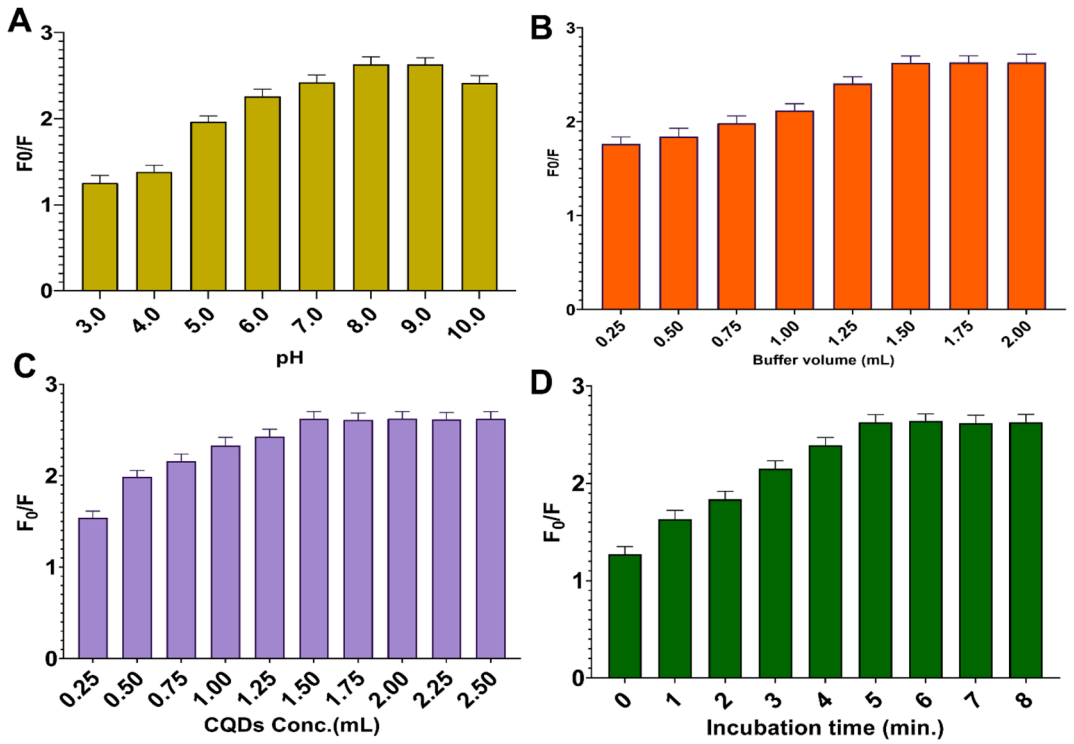

Fig. 5. Analysis of key parameters affecting the fluorescence quenching of N-GQDs by brivaracetam. The graphs show the influence of (A) buffer pH, (B) buffer volume, (C) N-GQD concentration, and (D) incubation time on the quenching process. Error bars represent the standard deviation from triplicate experiments.

%, well within the acceptable range of 85–115 % as per ICH guidelines (Table 2). The intra-day and inter-day precisions, expressed as percent relative standard deviation (RSD%), were found to be less than 4 %, respectively, indicating good precision. These results demonstrate the suitability of the developed method for the quantitative determination of brivaracetam in human plasma.

Selectivity of the developed method was comprehensively evaluated by analyzing the response to brivaracetam compared to various potential interfering compounds. As shown in (Fig. S2), the method demonstrated high selectivity, with brivaracetam showing a normalized fluorescence response (F0-F)/F0 of approximately 0.62, while all tested interferents exhibited negligible responses (<0.05). The tested compounds included structurally related drugs (piracetam, valproic acid, carbamazepine, phenytoin), common ions (K^+, Ca^{2+}, Mg^{2+}, Na^+, Cl^-, SO_4^{2-}, PO_4^{3-}), and biological molecules present in plasma (tryptophan, tyrosine, glutamic acid, albumin, glucose). Even in pooled plasma samples, the interference was minimal, demonstrating the method's robustness in complex biological matrices. This high selectivity can be attributed to the specific interaction between brivaracetam and the functionalized N-GQDs, as supported by the DFT calculations and thermodynamic analysis. The matrix effect was evaluated at two concentration levels of QC samples, which were found to be within the acceptable limits and ranged from 94.36 % to 105.19 %, with CV%< 7 %, indicating minimal matrix effect (Table S2). Finally, the robustness of the method was demonstrated by systematically varying the critical parameters, such as pH, N-GQDs concentration and incubation time,

Table 1
Statistical parameters of the calibration curve of brivaracetam using the developed method.

Parameter	Brivaracetam
Linearity range (μg/mL)	0.1–2.5
Intercept (a)	0.9671
Slope (b)	1.1127
Coefficient of determination (r2)	0.9992
SE of intercept (Sa)	0.0139
SE of slope (Sb)	0.0099
SD of residuals	0.0112
LOD (μg/mL)	0.0333
LLOQ (μg/mL) [a]	0.1

[a] Lower limit of quantitation.

Table 2
Accuracy and precision results for the determination of brivaracetam in human plasma by the proposed method.

Concentration (μg/mL)	Within-run		Between-run	
	Accuracy (% R ± SD) [a]	Precision (RSD%)	Accuracy (% R ± SD) [a]	Precision (RSD%)
0.1	95.56 ± 2.355	2.465	97.50 ± 3.448	3.536
0.3	96.91 ± 2.578	2.660	102.49 ± 3.025	2.952
1.75	101.34 ± 3.465	3.419	103.37 ± 2.457	2.377
2.0	103.55 ± 2.439	2.355	101.68 ± 2.510	2.468

[a] Average of three determinations.

with no significant changes in the analytical response (Table S3).

3.4. Application to pharmaceutical formulations and pharmacokinetics

The validated N-GQDs based fluorescence quenching metho was successfully applied for the determination of brivaracetam in pharmaceutical formulations (Table 3). The results obtained were in good agreement with the labeled claim, with mean recovery values of 100.49 ± 1.186 %, indicating the accuracy and reliability of the proposed method for quality control analysis. Furthermore, the recovery results were compared with reported chromatographic method [6] in terms of mean and variance using student's *t*-test and F-test, respectively, which revealed no significant difference (Table 3), confirming the reliability of the developed fluorescence method.

The validated method was also applied to a pharmacokinetic study of brivaracetam in human plasma. The mean plasma concentration–time profile revealed typical absorption, distribution, and elimination phases following an oral dose of 100 mg brivaracetam (Fig. 6). The key pharmacokinetic parameters, such as C_{max}, $t_{1/2}$, $AUC_{0\text{-}t}$, and $AUC_{0\text{-}\infty}$, were calculated and found to be in good agreement with the reported values [26], indicating the suitability of the developed method for accurate quantification of brivaracetam in biological matrices (Table 4). In detail, the time to reach maximum plasma concentration (T_{max}) was observed

Table 3
Quantitative analysis of brivaracetam in the commercial Brivafutal ® tablets by the proposed method and statistical comparison with the reported method.

	N-GQDs probe	Reported method
)%Recovery ± SD) [a]	100.49 ± 1.186	99.73 ± 1.120
Variance	1.405	1.255
t-test (2.306)[b]	0.326	
F-test (6.388)[b]	1.120	

[a] Average of five determinations.
[b] The values in parenthesis are tabulated values of "t "and "F" at (P = 0.05).

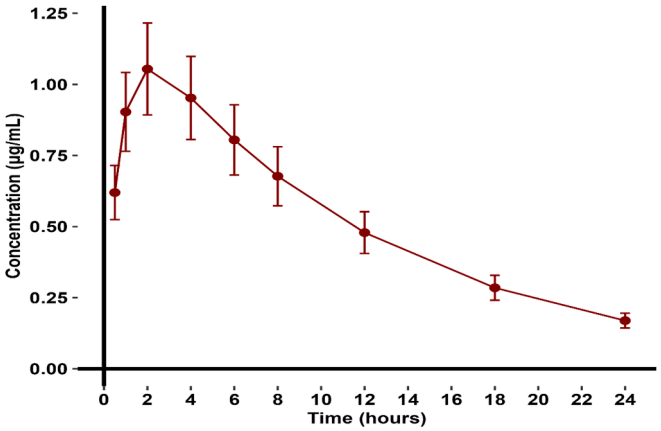

Fig. 6. Mean plasma concentration–time profiles of brivaracetam following a single oral dose of 100 mg as analyzed using the developed method.

Table 4
PK parameters for brivaracetam using the proposed method following administration of a single oral dose of 100 mg to human subjects.

Parameters	Mean Values
T_{max} (h)	2
$t_{1/2}$ (h)	8
C_{max}(μg/mL)	1.054
Elimination constant (1/h)	0.087
AUC $_{0\rightarrow t}$ (μg/mL.h)	12.723
AUC $_{0\rightarrow \infty}$ (μg/mL.h)	14.677
MRT $_{0\text{-inf\_obs}}$ (h)	12.170

at 2 h post-dose, indicating relatively rapid absorption of brivaracetam, consistent with its known properties as a small, lipophilic molecule. The maximum plasma concentration (C_{max}) reached 1.054 μg/mL, demonstrating significant systemic exposure within the therapeutic range. The elimination half-life ($t_{1/2}$) of 8 h and elimination rate constant of 0.087 h^{-1} indicate a moderate rate of drug clearance, supporting twice-daily dosing regimens. The area under the curve from time zero to the last measurable concentration (AUC $_{0\rightarrow t}$) was 12.723 μg/mL·h, while the AUC extrapolated to infinity (AUC $_{0\rightarrow\infty}$) was 14.677 μg/mL·h. The mean residence time (MRT $_{0\text{-inf\_obs}}$) of 12.170 h is consistent with the observed half-life and previous literature [26]. These pharmacokinetic parameters align well with previously reported data for brivaracetam, validating the accuracy and reliability of our proposed analytical method.

3.5. Greenness, blueness and whiteness assessment of the developed method in comparison with the reported literature

Initial assessment of the developed fluorescence quenching method for brivaracetam determination based on N-GQDs revealed several attributes in line with the principles of green analytical chemistry. Firstly, the use of N-GQDs as the sensing material, which are derived from sustainable carbon sources, contributes to the greenness of the method. Secondly, the absence of organic solvents during analytical method development further enhances the environmental benignity, as most of the reported methods for brivaracetam quantification involve the use of hazardous organic solvents. In addition, the sample preparation step is minimal, although acetonitrile precipitation was used, it is a relatively less toxic solvent in comparison to the traditionally used ones. Moreover, the method utilizes simple and inexpensive instrumentation, such as a fluorescence spectrophotometer, which is widely available in most analytical laboratories. However, in order to assess the overall greenness, blueness and whiteness of the developed method, a more systematic evaluation using established metrics, such as AGREE, BAGI and

RGB12 has been performed and benchmarked against the reported chromatographic methods.

The AGREE score for the presented fluorescence quenching method was calculated to be 0.7 (Fig. 7A), indicating a "greener" profile compared to the reported UPLC-MS/MS and HPLC-UV methods for brivaracetam quantification (Fig. 7B and 7C), which had AGREE scores ranging from 0.6 to 0.66. The most significant contributions to the improved greenness came from the use of N-GQDs, the reduction in organic solvent consumption, and low energy requirements of the fluorescence technique. On the other hand, UPLC-MS/MS [4] and HPLC-UV [6] methods generally involve more complex sample preparation, higher solvent consumption, and increased energy demands, which negatively impact their greenness scores. In addition to the AGREE assessment, the BAGI score was determined to be 72.5 for the N-GQDs based fluorescence method (Fig. 7D), indicating a "blue" profile, whereas the reported chromatographic methods had BAGI score of 75 (Fig. 7E and F) suggesting a comparable analytical blueness.

Another generalized metric is the RGB12 algorithm, which considers analytical performance of the method (red), environmental impact (green), and practicality (blue). The combination of red, green, and blue provides an overall whiteness score. The RGB12 score for the N-GQDs based fluorescence method was calculated to be 93.1 (Fig. 7G), which is higher than the reported HPLC-UV and UPLC-MS/MS methods with scores ranging from 76 to 79.7 (Fig. 7H and I), indicating a superior whiteness profile. In detail, the method's red score (97.5) suggests robust analytical capabilities, including suitable scope of application, precision, and accuracy, comparable to established chromatographic techniques. Notably, its green score (96.3) exceeds both UPLC-MS/MS

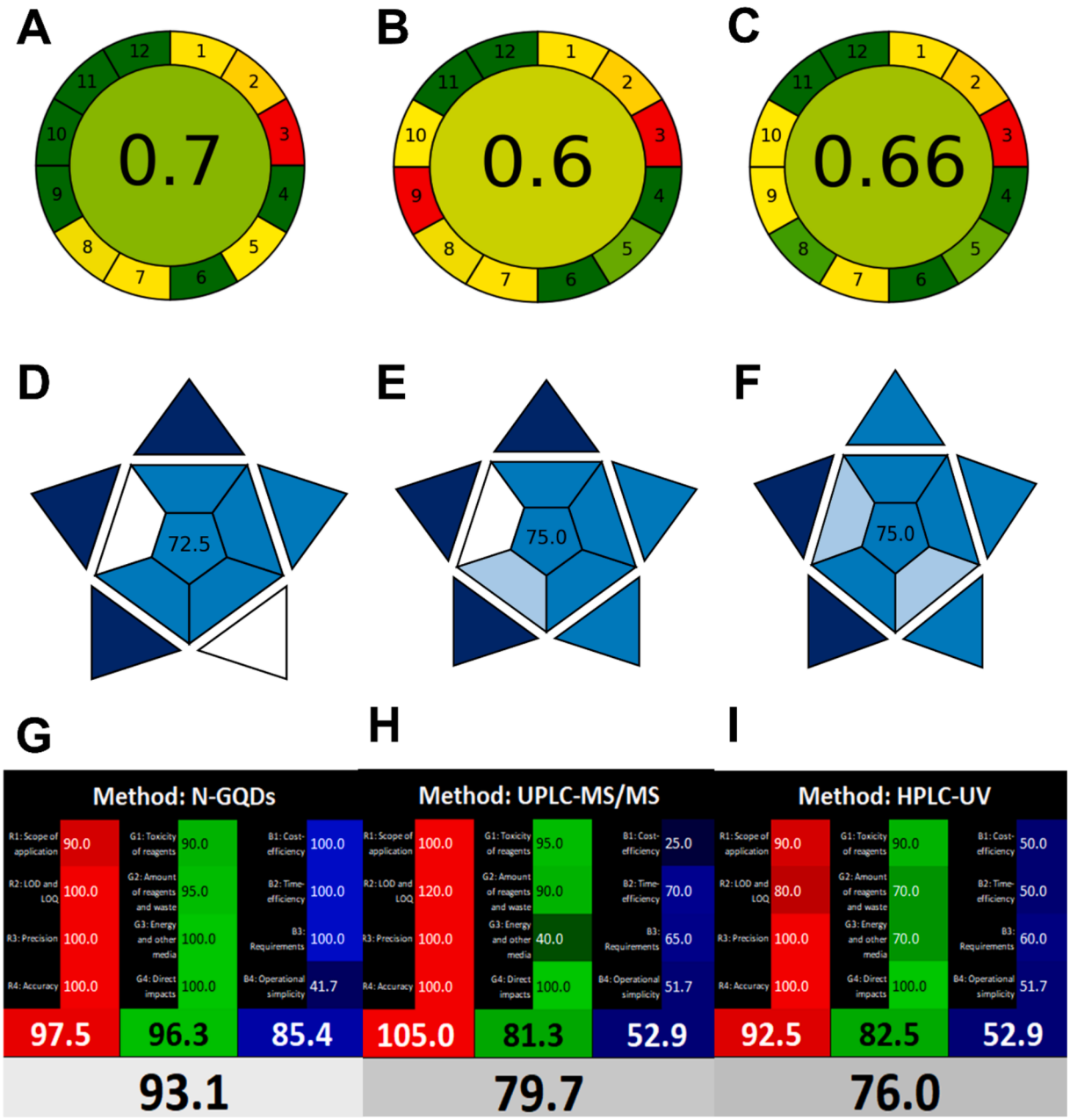

Fig. 7. Greenness assessment of (A) the developed N-GQDs method, (B) the reported UPLC-MS/MS and (C) the reported HPLC-UV chromatographic methods using the AGREE tool to determine their environmental impact. Blueness assessment of (D) the developed N-GQDs method, (E) the reported UPLC-MS/MS and (F) the reported HPLC-UV chromatographic methods using the BAGI tool to determine their analytical practicability. Whiteness assessment of (G) the developed N-GQDs method, (H) the reported UPLC-MS/MS and (I) the reported HPLC-UV chromatographic methods using the RGB12 algorithm to determine their sustainability.

(81.3) and HPLC-UV (82.5), reflecting enhanced environmental compatibility attributed to the utilization of N-GQDs, reduced organic solvent usage, and lower energy requirements. The method also demonstrates improved practicality, as evidenced by its blue score of 85.4, higher than the 52.9 scored by both chromatographic methods, indicating advantages in cost-efficiency, time-effectiveness, and operational simplicity.

These findings collectively suggest the potential of the N-GQDs based fluorescence method as an environmentally conscious and analytically viable alternative to traditional chromatographic techniques for brivaracetam quantification. The method's adherence to green analytical chemistry principles, combined with its maintenance of analytical standards and practical benefits, positions it as a potentially valuable approach for pharmaceutical and clinical applications, potentially contributing to the advancement of sustainable analytical methodologies in drug analysis.

3.6. Comparison with reported methods

Several analytical methods have been reported in the literature for the determination of brivaracetam in various matrices (Table S4). The present fluorescence-based method offers distinct advantages when compared to previously reported techniques. Mohamed et al. developed an UHPLC-MS/MS method with a linear range of 0.10–10 μg/mL and LOD of 0.05 μg/mL, requiring sample preparation through acetonitrile precipitation [3]. While sensitive, this method demands sophisticated instrumentation and organic solvents for sample preparation. Similarly, Iqbal et al. reported an UPLC-MS/MS method with improved sensitivity (LOD: 0.26 ng/mL) but required liquid–liquid extraction using *tert*-butyl methyl ether [4].

Simpler approaches have been explored, such as the HPLC method by Mansour et al., which achieved simultaneous determination of brivaracetam with piracetam and carbamazepine [6]. However, their method showed relatively higher LOQ (2.3 μg/mL for brivaracetam) compared to our fluorescence-based approach (LOD: 0.033 μg/mL). Several groups have focused on the chiral separation of brivaracetam isomers using specialized columns and complex mobile phases. For instance, Baksam et al. developed a chiral HPLC method with LOD of 0.3 μg/mL [27], while Choppari et al. achieved separation using an immobilized polysaccharide chiral column [28].

The present fluorescence-based method offers several advantages over these reported techniques: (1) superior sensitivity with LOD of 0.033 μg/mL, (2) simpler sample preparation, (3) more environmentally friendly approach as confirmed by AGREE metrics, and (4) cost-effective instrumentation compared to LC-MS/MS methods. Additionally, our method uniquely employs N-doped GQDs, providing both sensitivity and selectivity through specific molecular interactions, as supported by theoretical calculations.

4. Conclusion, limitations and future directions

In conclusion, the present work describes the development and validation of a novel fluorescence quenching method for the determination of brivaracetam in human plasma and pharmaceutical formulations, utilizing N-doped GQDs as the sensing platform. The method demonstrated satisfactory linearity, selectivity, accuracy, and precision in accordance with ICH M10 guidelines. The spectral characteristics, quenching mechanisms, and thermodynamic parameters were systematically investigated, providing insights into the sensing process. The method was successfully applied to the analysis of brivaracetam in commercial tablets and for the determination of pharmacokinetic parameters in human plasma samples, yielding results consistent with previously reported data.

While the developed method exhibits several advantages in terms of greenness, cost-effectiveness, and operational simplicity compared to conventional chromatographic techniques, it is important to acknowledge certain limitations. Firstly, a comparison of the pharmacokinetic results with a gold standard technique such as HPLC-MS/MS was not performed due to the unavailability of the analytical platform, which would have provided a more comprehensive evaluation of the method's analytical performance. Furthermore, incorporating a more selective material such as molecularly imprinted polymers for brivaracetam could potentially improve the selectivity and sensitivity of the assay. Future research could focus on expanding the applicability of the N-GQDs-based fluorescence method to the analysis of other antiepileptic drugs or other bioactive compounds. Additionally, further investigations into the long-term stability and reusability of the N-GQDs sensing platform would strengthen the practical utility of the method.

CRediT authorship contribution statement

Reem M. Alnemari: Writing – original draft, Methodology, Investigation, Formal analysis, Conceptualization. **Maram H. Abduljabbar:** Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Yusuf S. Althobaiti:** Writing – review & editing, Resources, Methodology, Investigation, Data curation. **Sameer Alshehri:** Writing – review & editing, Methodology, Investigation, Formal analysis. **Farooq M. Almutairi:** Writing – review & editing, Validation, Investigation, Formal analysis. **Humood Al Shmrany:** Writing – review & editing, Validation, Investigation, Formal analysis. **Eid Semer Alatwi:** Writing – review & editing, Validation, Investigation. **Ahmed Serag:** Writing – review & editing, Writing – original draft, Visualization, Formal analysis, Data curation. **Atiah H. Almalki:** Writing – review & editing, Resources, Project administration, Funding acquisition, Conceptualization.

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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. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jphotochem.2025.116357.

Data availability

Data will be made available on request.

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