

Article

Preventing Drug-Facilitated Sexual Assault: A Smartphone-Readout Lateral Flow Assay for GBL Detection in Water and Saliva

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Abstract

The rising prevalence of Drug-Facilitated Sexual Assault (DFSA) underscores the urgent need for rapid, sensitive, and selective analytical tools. Among the most concerning substances are γ -hydroxybutyric acid (GHB) and its precursor γ -butyrolactone (GBL), both of which pose significant forensic challenges due to their rapid metabolism and narrow detection windows. While detection methods for GHB have advanced, the monitoring of GBL remains less explored. This study reports the extended application of a fluorescein-based chemosensor previously validated for GHB for the selective detection of GBL in aqueous media and synthetic saliva. The probe operates via fluorescence quenching proportional to GBL concentration, achieving detection limits well below toxicological thresholds. Mechanistic investigations reveal that the recognition process is driven by an acid–base equilibrium and the reversible opening of the fluorescein lactone ring, with the 2-aminonaphthoxazole moiety playing a pivotal role. The sensor exhibits high selectivity against common DFSA-related interferents. Furthermore, the system was integrated into a portable lateral flow assay coupled with a smartphone-based readout, providing a cost-effective and user-friendly platform. Meeting the WHO “ASSURED” criteria, this methodology represents a promising tool for forensic, clinical, and preventive applications against chemical submission.

Keywords: drug-facilitated sexual assaults; chemosensor; naphthoxazole core; γ -butyrolactone; lateral flow assay



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1. Introduction

The abuse of illicit substances has constituted a persistent global challenge for several decades, representing a serious threat to public security, essential healthcare services, and the stability of modern societies [1,2]. According to the United Nations Office on Drugs and Crime (UNODC), both the absolute number of users and the proportion of the world’s population engaging in drug use have shown a steady growing trend [3]. The most recent assessments in this field emphasize that the rapid proliferation of new psychoactive substances (NPSs), characterized by their wide availability, pharmacological

effects at low doses, and short detection windows, provokes the need to develop new analytical approaches that are rapid, sensitive, and selective for the reliable identification of illicit drugs [4–6]. These analytical challenges are further underscored by the rising incidence of Drug-Facilitated Sexual Assault (DFSA) and drug-impaired driving. The use of sedative or amnestic substances to facilitate criminal acts poses a severe societal risk, with DFSA cases reported in approximately 15–20% of sexual assault investigations. The prevalence of such incidents, frequently involving the surreptitious spiking of beverages, demands the development of robust, on-site detection tools to safeguard individuals in social environments [7,8].

Within the spectrum of substances associated with DFSA, γ -hydroxybutyric acid (GHB) is among the most frequently encountered, primarily due to its distinctive pharmacokinetic properties. Upon oral administration, GHB displays a rapid onset of action, typically within 15–30 min, with effects that may persist for approximately 6–8 h. Its forensic detection poses significant analytical challenges, as GHB undergoes extensive and rapid metabolic degradation, resulting in a narrow detection window within biological matrices [9,10]. On the other hand, γ -butyrolactone (GBL) has long been used in industrial applications such as cleaning solvents; however, when misused in a DFSA context, it is rapidly metabolized to GHB in the body after ingestion [11]. Consequently, the terms “knockout drops” and “date-rape drug” can be used interchangeably to refer to both GHB and GBL [12]. Since the conversion of GHB into GBL and vice versa in spiked drinks is relatively slow and pH-dependent, the formation of an equilibrium with a GBL:GHB 1:2 ratio has been reported [13]. In recent years, while several chemosensors for the detection of GHB in beverages and biological fluids have been published [14–17], there remains a notable scarcity of sensors capable of detecting GBL in beverages or saliva [18,19]. Therefore, and in line with our previous research on chemosensors for the detection of drugs used in DFSA cases [20–23], in this work, we present the application of a chemosensor for the detection of GBL integrated into a lateral flow system to provide rapid and reliable measurements in synthetic saliva.

2. Experimental Section

2.1. Materials and Instruments

All reagents employed in the synthesis were acquired from Sigma-Aldrich (Madrid, Spain) and used without further purification. The drugs tested, GBL, GHB, methcathinone, ketamine, diazepam, and (-)-scopolamine hydrobromide trihydrate, were purchased from Sigma-Aldrich with the required prior authorization from the Agencia Española del Medicamento y Productos Sanitarios (AEMPS). Synthetic saliva for pharmaceutical research was purchased from Sigma-Aldrich. All solvents were ACS reagent-grade or higher quality and used as received. DMSO was purchased from Scharlab S.L. (Barcelona, Spain). ^1H NMR and ^{13}C NMR spectra were recorded on Bruker Avance 300 MHz spectrometers (Billerica, MA, USA), with all signals referenced to the corresponding residual solvent peak. All photophysical analyses were carried out in air-equilibrated DMSO at 298 K, unless otherwise specified. UV–Vis absorption spectra were recorded with PerkinElmer Lambda 40 (Waltham, MA, USA) or Shimadzu UV-2600 (Kyoto, Japan) spectrophotometers using quartz cells with a path length of 1.0 cm. Mass spectrometry measurements were carried out using an AB SCIEX TripleTOFTM 5600 LC/MS/MS System (Framingham, MA, USA), equipped with two gas sources (both at 35 psi), operated at 450 °C with an ion gas voltage of 5500 V. For the smartphone-based approach, a 3D box was printed with black PLA using an Ultimaker 3 printer (Zaltbommel, The Netherlands). LEDs and optical filters were purchased from Thorlabs. Pictures were taken with a smartphone, and intensity values were retrieved via the integrated density function using ImageJ software (version 1.54g).

OriginPro (version 9.0) was used for titration plotting. Glass fiber strips and blank cellulose paper (Whatman Grade 17 CHR) were obtained from Whatman® (Maidstone, UK).

2.2. Synthesis of Probe 1

Fluorescein-5-isothiocyanate (0.486 mmol, 1 equiv.) was mixed with 2-aminonaphthoxazol (0.641 mmol, 1.3 equiv.) in 8 mL of THF. Then, NEt₃ (0.641 mmol, 1.3 equiv.) was added, and the mixture was stirred overnight (the solution turned orange). The following day, H₂O₂ (35%, 0.972 mmol, 2 equiv.) and tetrabutylammonium iodide (0.005 mmol, 0.01 equiv.) were added. The mixture was stirred for 16 h at room temperature (the solution turned maroon). The solvent was removed under reduced pressure, and the crude product was washed several times with cold CH₂Cl₂. The product was isolated as a maroon powder after drying under reduced pressure (65% yield). ¹H NMR (300 MHz, DMSO-d₆): δ (ppm) 8.58 (d, *J* = 2.2 Hz, 1H), 8.04–7.96 (m, 5H), 7.45 (m, 2H), 7.32 (d, *J* = 8.3 Hz, 1H), 6.69–6.64 (m, 4H), 6.55 (dd, *J* = 8.5, 2.3 Hz, 2H). ¹³C NMR (75 MHz, DMSO-d₆): δ (ppm) 168.34, 159.29, 158.64, 151.63, 146.56, 142.08, 139.82, 130.98, 129.45, 128.82, 127.38, 127.24, 127.15, 125.10, 124.47, 124.23, 123.97, 112.61, 112.34, 109.41, 104.49, 101.89, 66.68. HRMS: *m/z* calculated for C₃₁H₁₉N₂O₆ [M + H]⁺: 515.1243; found: 515.1238.

2.3. Fluorescence Titration of 1 with GBL Solutions in Water/Synthetic Saliva

In a 1 mL quartz cell (1.0 cm path length), the probe (50 μM of 1 with 2 equiv. of sodium acetate (NaOAc) in DMSO:H₂O 98:2) was mixed with increasing amounts of GBL aqueous solutions (25 mM and 100 mM) until reaching the saturation point (range: 0 to 0.7 mM). In all cases, the optical change occurred immediately. For synthetic saliva titrations, the same procedure was followed using GBL dissolved in synthetic saliva (range: 0 to 1 mM).

2.4. Interference Studies in Real Saliva

In a 1 mL quartz cell (1.0 cm path length), the probe (50 μM of 1 with 2 equiv. of NaOAc in DMSO:H₂O 98:2) was mixed with 10 μL of real saliva from 1 individual (male volunteer) after the ingestion of different beverages. The response was instantaneous in all cases.

2.5. Lateral Flow Assay for GBL Detection in Synthetic Saliva

Glass fiber strips (4.5 × 0.5 cm) were prepared. A solution of the probe (4 μL, 1 mM of 1 with 2 equiv. of NaOAc in DMSO:H₂O 98:2) was deposited at the center of the strip (Zone B, shown later on) and 4 μL of GBL solution in synthetic saliva at various concentrations was deposited 1 cm from one end (Zone A). The strips were dipped into 1 mL of THF, and the flow was developed for 3 min. Fluorescence emission was measured using the 3D-printed smartphone holder (490 nm LED, USB-OTG-powered) with a long-pass filter (>500 nm) for collecting the probe emission. The fluorescence quenching for each GBL concentration was calculated as the ratio between the fluorescence in Zone C and the blank.

2.6. Lateral Flow Assay Selectivity Studies in Synthetic Saliva

Glass fiber strips (4.5 × 0.5 cm) were prepared. A solution of the probe (4 μL, 1 mM of 1 with 2 equiv. of NaOAc in DMSO:H₂O 98:2) was deposited at the center of the strip (Zone B, shown later on), and 4 μL of 25 mM solutions in synthetic saliva of different drugs (GBL, GHB, methcathinone, ketamine, scopolamine and diazepam) was deposited 1 cm from one end (Zone A). The strips were dipped into 1 mL of THF, and the flow was developed for 3 min. Fluorescence emission was measured using the 3D-printed smartphone holder (490 nm LED, USB-OTG-powered) with a long-pass filter (>500 nm) for collecting the probe emission. The fluorescence quenching for each drug was calculated as the ratio between the fluorescence in Zone C and the blank.

2.7. Synthesis of Derivatives **2a**, **2b** and **3**

For **2a** (see Scheme S1 in the Supplementary Materials): Fluorescein (1.5 mmol, 1 equiv.) was mixed with NEt_3 (7.5 mmol, 5 equiv.) in 25 mL of THF. Acetic anhydride (7.5 mmol, 5 equiv.) was added dropwise, and the mixture was stirred for 2.5 h at room temperature. The solvent was removed under reduced pressure, and the crude product was washed several times with cold EtOH. The product was isolated as a white solid (98% yield). ^1H NMR (300 MHz, DMSO-d_6) δ (ppm) 8.07–8.05 (d, $J = 7.6$ Hz, 1H), 7.85–7.73 (m, 2H), 7.4 (d, $J = 7.7$ Hz, 1H), 7.28 (d, $J = 1.9$ Hz, 2H), 6.97–6.87 (m, 4H), 2.28 (s, 6H). ^{13}C NMR (75 MHz, DMSO-d_6) δ (ppm) 168.85, 168.40, 152.15, 152.04, 150.81, 136.02, 130.58, 129.13, 125.46, 125.03, 124.16, 118.63, 116.16, 110.49, 81.03, 20.85. HRMS: m/z calculated for $\text{C}_{24}\text{H}_{16}\text{O}_7$ $[\text{M} + \text{H}]^+$: 417.0974; found: 417.0982.

For **2b** (see Scheme S1 in the Supplementary Materials): Fluorescein (0.6 mmol, 1 equiv.) in 30 mL of EtOH was treated with concentrated sulfuric acid (1.4 mL) added dropwise and stirred at reflux for 20 h. The solvent was removed under reduced pressure, and the crude product was dissolved in CH_2Cl_2 and washed several times with a saturated aqueous solution of NaHCO_3 . The organic phase was dried over anhydrous magnesium sulfate, filtered, and evaporated under reduced pressure. The product was isolated as an orange powder without further purification (90% yield). ^1H NMR (300 MHz, DMSO-d_6) δ (ppm) 8.21 (d, $J = 7.6$ Hz, 1H), 7.9–7.8 (m, 2H), 7.5 (d, $J = 7.7$ Hz, 1H), 7.03 (d, $J = 7.8$ Hz, 2H), 6.89–6.82 (m, 4H), 3.4 (q, $J = 7.6$ Hz, 2H), 0.84 (t, $J = 7.5$ Hz, 3H). ^{13}C NMR (75 MHz, DMSO-d_6) δ (ppm) 164.84, 159.47, 156.86, 133.26, 133.06, 130.91, 130.74, 130.43, 130.31, 129.82, 121.27, 115.24, 109.58, 102.95, 61.01, 15.13, 13.32. HRMS: m/z calculated for $\text{C}_{22}\text{H}_{17}\text{O}_5$ $[\text{M} + \text{H}]^+$: 361.1076; found: 361.1076.

For **3** (see Scheme S2 in the Supplementary Materials): Probe **1** (0.1 mmol, 1 equiv.) was treated under the same conditions as **2b**. The product was isolated as an orange powder without further purification (20% yield). ^1H NMR (300 MHz, DMSO-d_6) δ (ppm) 7.91–7.88 (m, 5H), 7.73 (s, 3H), 7.48 (s, 2H), 7.45–7.39 (m, 5H), 3.77 (q, $J = 4$ Hz, 2 H), 1.11 (t, $J = 4.5$ Hz, 3H). ^{13}C NMR (75 MHz, DMSO-d_6) δ (ppm) 167.35, 154.97, 154.02, 147.33, 143.99, 131.02, 130.89, 129.76, 128.39, 128.18, 127.60, 127.38, 126.01, 125.49, 125.14, 124.86, 123.74, 109.15, 105.86, 105.69, 61.61, 15.61.

3. Results and Discussion

3.1. Synthesis and Spectroscopic Properties of **1**

The novel methodology developed herein to detect GBL in water and oral fluids is based on fluorogenic chemosensor **1** previously reported by S. Rodríguez et al. (Chart 1) [20]. Probe **1** was synthesized following the previously described procedure, with an optimized isolation approach, and its spectroscopic data are consistent with those previously described (see Figures S1–S6 in the Supplementary Materials Section) [22].

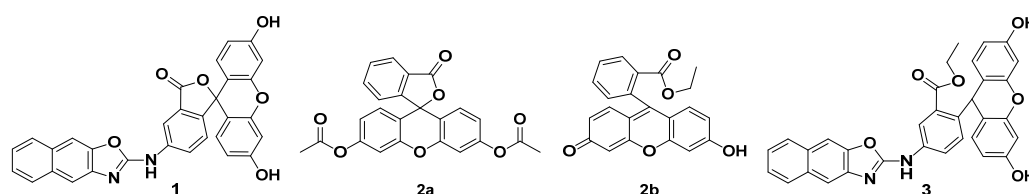


Chart 1. Chemical structure of probe **1** used for GBL detection.

A solution of compound **1** in DMSO exhibited the characteristic optical properties of the fluoresceine moiety. The UV-Vis spectrum of **1** (5×10^{-5} M in DMSO) displayed a prominent absorption band at $\lambda_{\text{max}} = 521$ nm ($\epsilon = 1886 \text{ cm}^{-1} \text{ M}^{-1}$) with a shoulder at ca. 490 nm, which corresponds to the maximum absorption band of the fluoresceine unit [24],

while the emission spectrum of **1** showed a maximum at $\lambda_{em} = 542$ nm when irradiated at $\lambda_{exc} = 521$ nm (see Figure 1).

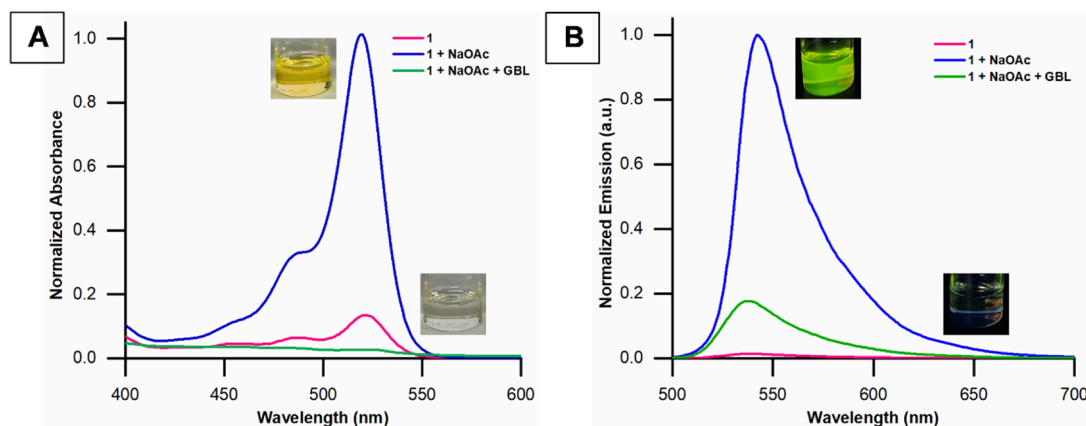


Figure 1. Normalized UV-Vis spectra (A) and emission spectra ($\lambda_{exc} = 521$ nm) (B) of compound **1** (50 μ M in DMSO:H₂O 98:2): probe alone (pink line), plus 2 equiv. of NaOAc (blue line), and plus 2 equiv. of NaOAc with 5 equiv. of GBL (green line).

3.2. Sensing Experiments in Solution

Building upon previous studies, we recorded the absorption and fluorescence spectra of a mixture of **1** with 2 equiv. of NaOAc in DMSO:H₂O 98:2 and 5 equiv. of GBL. As shown in Figure 1, the addition of 2 equiv. of NaOAc over **1** induced an alteration in its absorption spectrum, accompanied by a marked enhancement in the fluorescence emission intensity. This effect is attributed to the ability of NaOAc to activate both the fluorescein and the naphthoxazole moieties, thereby modulating the optical behavior of chemosensor **1**. The addition of 5 equiv. of GBL to this mixture led to a substantial fluorescence quenching, along with a sharp decrease in the absorption band and a hypsochromic shift in the emission maximum from 542 to 536 nm. These changes in both spectra can be observed with the naked eye, both under visible light (color change from yellow to colorless) and under a standard UV lamp (decrease in fluorescence intensity).

Prior to testing the probe in complex biological matrices such as oral fluids, the fluorescence response of a mixture of **1** (50 μ M in DMSO:H₂O 98:2) with 2 equiv. of NaOAc was investigated in the presence of increasing amounts of GBL. Figure 2 shows the linear intensity decrease in the fluorescence band as the concentration of GBL increases. The limit of detection (LOD) was determined from the plot of the % emission quenching at 542 nm (where % Quenching = $(1 - I_{sample}/I_{blank}) \times 100$) vs. GBL concentration, using the expression $LOD = 3.3 s_b/m$ (where s_b is the standard deviation of the blank and m is the slope of the titration curve) [25]. A LOD of 18.2 μ M for GBL was determined, a value much lower than that required to induce severe effects in a chemical submission victim, which is around 116–350 mM [26]. The fluorescence changes could be observed by the unaided eye under a common UV lamp (Figure 2C).

To study the practical applicability of the method, we explored the ability of the probe to detect GBL in oral fluids, as it would enable the development of a rapid and user-friendly test to help prevent DFSA. In this way, synthetic saliva samples were spiked with increasing quantities of a 25 mM aqueous solution of GBL. Fluorescence emission measurements of these samples after the addition of probe **1** and 2 equiv. of NaOAc showed a clear decrease in fluorescence intensity in the presence of increasing amounts of GBL, with a linear response in the 0–200 μ M concentration range (see Figure 3). The experimental data indicates a LOD for GBL of 32.7 μ M, which is lower than the expected concentration used in beverages to produce major adverse effects in a chemical submission victim [26].

The fluorescence changes were observed immediately with the naked eye under a common UV lamp. Finally, the recovery, accuracy, and analytical performances of the method [27] for sensing GBL in aqueous media and synthetic saliva were determined (see Tables S1–S3 in the Supplementary Materials Section).

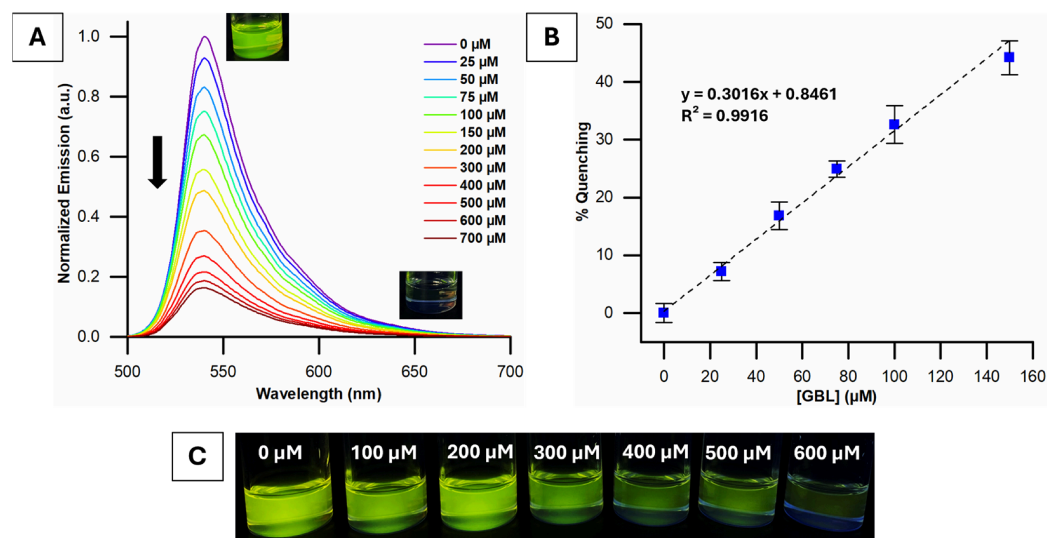


Figure 2. (A) Emission spectra ($\lambda_{\text{exc}} = 521 \text{ nm}$; $\lambda_{\text{em}} = 542 \text{ nm}$) of compound 1 (50 μM in DMSO:H₂O 98:2) in the presence of 2 equiv. of NaOAc and increasing concentrations of GBL in H₂O. (B) Calibration curve representing the % Quenching at $\lambda_{\text{em}} = 542 \text{ nm}$ ($\lambda_{\text{exc}} = 521 \text{ nm}$) vs. GBL concentration in H₂O. Error bars represent 3σ from three independent measurements. (C) Visible fluorescence decrease in probe 1 (with 2 equiv. of NaOAc) upon increasing GBL concentration under a standard UV lamp.

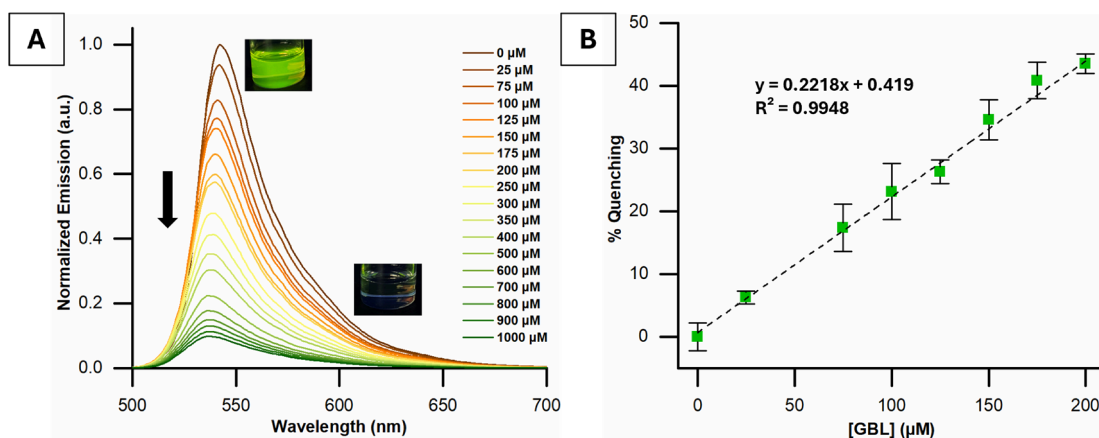


Figure 3. (A) Fluorescence emission spectra ($\lambda_{\text{exc}} = 521 \text{ nm}$; $\lambda_{\text{em}} = 542 \text{ nm}$) of compound 1 (50 μM in DMSO:H₂O 98:2) in the presence of 2 equiv. of NaOAc and increasing concentrations of aqueous GBL in synthetic saliva. (B) Calibration curve representing the % Quenching at $\lambda_{\text{em}} = 542 \text{ nm}$ ($\lambda_{\text{exc}} = 521 \text{ nm}$) vs. GBL concentration in synthetic saliva. Error bars represent 3σ from three independent experiments.

3.3. Mechanism Detection Proposal

In the present study, it is hypothesized that following the deprotonation of compound 1 [20] facilitated by the basic medium provided by the 2 equiv. of NaOAc and the subsequent addition of GBL to the reaction mixture and its concomitant conversion to GHB will result in a sufficient change in the medium's pH to promote the quenching of the fluorescence of the probe. To gain further insight into the detection mechanism and

to confirm that the observed fluorescence quenching response arises from an acid–base equilibrium involving the probe and GBL, a series of studies were conducted.

In an initial step, the pH-dependent response of the probe was evaluated across a range of buffer solutions. As shown in Figure 4, the emission intensity of probe **1** exhibited a strong correlation with the medium acidity, increasing progressively as the pH rose until reaching a plateau at pH 7, beyond which the variation in emission intensity became negligible. These experiments were conducted using the probe (50 μ M in DMSO:H₂O 50:50) in the presence of the following buffer systems: H₃PO₄/NaH₂PO₄ (pH 2 and 3), HOAc/NaOAc (pH 4, 5.01 and 6.02), NaH₂PO₄/Na₂HPO₄ (pH 7) and Tris HCl (pH 8 and 9).

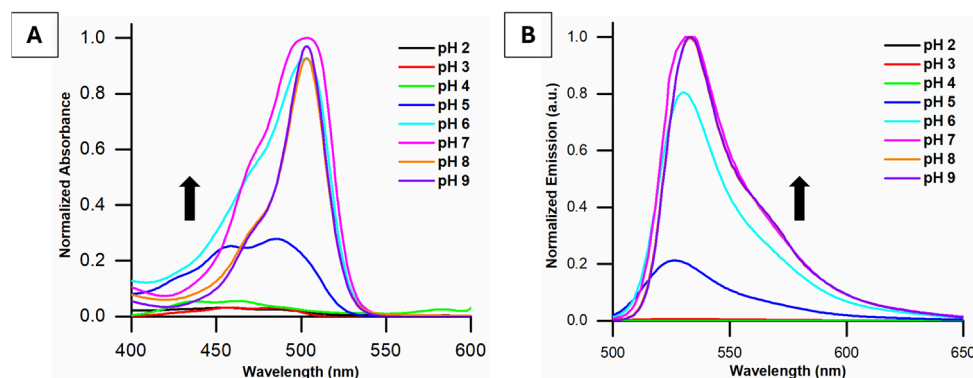


Figure 4. Normalized UV-Vis spectra (A) and emission spectra (B) of compound **1** (50 μ M in DMSO:H₂O 50:50) in the presence of excess buffers spanning different pH values.

After confirming the influence of pH on the fluorescence response, first, the pH-sensitive sites within the fluorescein scaffold of probe **1** were investigated. To this end, specific positions of the fluorescein free acid were derivatized (see Scheme S1 in the Supplementary Materials Section). Compound **2a**, featuring acetylated phenolic moieties, was synthesized by reacting fluorescein free acid with NEt₃ in THF, followed by the drop-wise addition of acetic anhydride. Upon solvent removal under vacuum and washing of the crude residue with cold EtOH, **2a** was isolated in 98% yield. To obtain compound **2b**, in which the fluorescein lactone ring is opened and esterified, the free acid was treated with concentrated H₂SO₄ in EtOH. After solvent removal, the residue was dissolved in CH₂Cl₂ and washed several times with saturated aqueous NaHCO₃ to obtain compound **2b** in 90% yield. The spectroscopic data for both derivatives were in agreement with previously reported values (see Figures S9–S14 in the Supplementary Materials Section) [28,29].

Compound **2a** is non-fluorescent under a common 254 nm UV lamp, whereas **2b** exhibits significant fluorescence, consistent with its structural configuration. The addition of 2 equiv. of NaOAc to **2a** resulted in a marked increase in fluorescence intensity over a two-hour period. This observation indicates that lactone ring opening within the fluorescein core still occurs, despite the protection of the hydroxyl groups, albeit with slower kinetics. Subsequent addition of an excess of GBL induced substantial fluorescence quenching, confirming that the sensing mechanism remains operative under these conditions. In contrast, treatment of **2b** with 2 equiv. of NaOAc did not induce any noticeable change in emission. Furthermore, the subsequent addition of GBL to this mixture failed to produce the fluorescence quenching previously observed for **2a**. These findings suggest that the reversible opening and closing of the lactone ring within the fluorescein moiety plays a pivotal role in the GBL detection mechanism (see Figure 5).

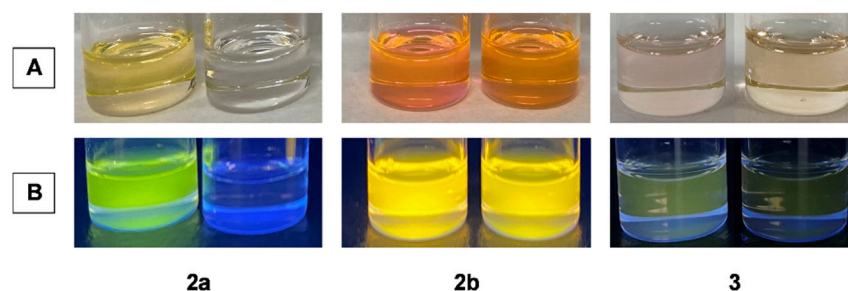


Figure 5. Optical changes in the compounds **2a**, **2b** and **3** (50 μ M in DMSO:H₂O 98:2) in the absence (left) and presence (right) of 2 equiv. of NaOAc with an excess of GBL (5 equiv.) in aqueous solution. Row (A): changes observed under ambient light. Row (B): changes observed under a standard UV lamp. The photograph of compound **2a** in the absence of GBL was taken 2 h after the addition of NaOAc, whereas in all other instances, images were recorded immediately upon mixing. The observed changes occurred instantaneously.

Having identified the functional position responsible for GBL detection in fluorescein free acid, compound **1** was derivatized at this site to evaluate the role of the 2-aminonaphthoxazole moiety in the recognition of this drug. For the synthesis of compound **3** (see Scheme S2 in the Supplementary Materials Section), **1** was dissolved in EtOH and treated with concentrated H₂SO₄ added dropwise, under the same conditions used to obtain derivative **2b**. Upon addition of 2 equiv. of NaOAc to compound **3**, a slight increase in fluorescence emission was observed. Subsequent addition of GBL to the reaction mixture quenched the fluorescence enhancement previously induced by the basic medium (Figure 5). These results confirm that both the 2-aminonaphthoxazole moiety and the fluorescein scaffold act as recognition units for the effective and selective detection of GBL.

3.4. Selectivity Studies

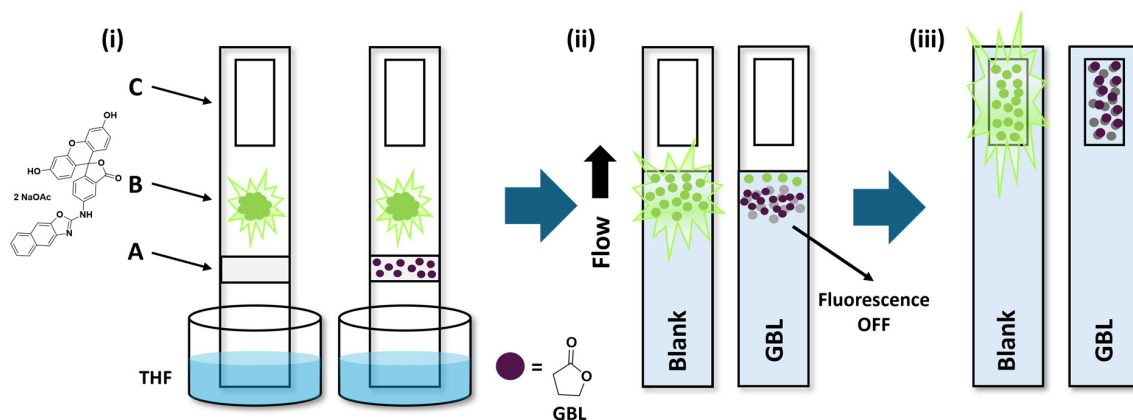
As an initial proof of concept to evaluate the practical applicability of this method for GBL detection in oral fluids, real saliva samples from a single volunteer were analyzed following the consumption of various commercial beverages, including coffee, cola, and lemon-flavored drinks. Figure S17 shows the response of compound **1** (in the presence of 2 equiv. of NaOAc) against real saliva samples after the ingestion of different beverages, as well as real saliva spiked with GBL. Notably, only the presence of GBL induced significant fluorescence quenching, whereas the other components of the oral matrix and ingested beverages produced no interference.

3.5. Lateral Flow Approach for GBL Detection

In the framework of point-of-care (POC) and point-of-need diagnostics [30], lateral flow assays (LFAs) [31] have emerged as highly effective platforms for the qualitative and quantitative detection of diverse analytes, ranging from biomarkers [32] and toxins [33] to chemical submission agents [34]. According to the ASSURED criteria established by the World Health Organization (WHO), ideal POC devices must be Affordable, Sensitive, Specific, User-friendly, Rapid and robust, Equipment-free, and Deliverable to end-users [35].

Building upon the results obtained in DMSO:H₂O mixtures, an LFA was developed for the sensitive and selective detection of GBL in synthetic saliva, with the aim of creating a rapid, low-cost, and user-friendly analytical platform. The LFA operates by capillary-driven migration of the liquid sample along a porous membrane. Glass fiber and cellulose paper were selected as supporting materials for an initial comparative analysis of their performance as solid supports. The fabricated strips (4.5 $\times$ 0.5 cm) comprise three functional zones: Zone A (sample application), Zone B (where the probe is deposited), and Zone C (the downstream detection region where the solvent front, THF, migrates and the optical

signal is measured). As illustrated in Scheme 1, the presence of GBL is expected to induce fluorescence quenching of the probe within Zone C. Following development, this reduction in intensity is monitored using a smartphone camera equipped with specialized optical accessories (see Figures S18 and S19 in the Supplementary Materials Section) [36].



Scheme 1. Design of the LFA using probe 1 (1 mM with 2 equiv. of NaOAc in DMSO:H₂O 98:2) on glass fiber paper. Zone A: sample application; Zone B: deposition of the probe; Zone C: detection region (i) Schematic of probe integration in Zone B and immersion in THF. (ii) Representation of negative (no quenching, left) and positive (fluorescence decrease, right) tests. (iii) Selective fluorescence quenching detected in Zone C only in the presence of GBL.

Optimal conditions were established by depositing a 4 μ L aliquot of the probe solution (1 mM of compound 1 with 2 equiv. of NaOAc in DMSO:H₂O 98:2) onto Zone B, followed by drying for 2 min. For GBL detection, 4 μ L of synthetic saliva spiked with GBL (25 mM) was applied to Zone A. Conversely, for blank controls, 4 μ L of synthetic saliva was deposited onto Zone A. After dipping the lower end in THF and allowing 3 min of development, the strips were placed in a custom holder for fluorescence measurements using a smartphone camera integrated into a 3D-printed case. This setup utilized a 490 nm LED for excitation and a long-pass filter (>500 nm) to capture the emission. Images were acquired under controlled conditions (ISO 100, 1/8 s exposure), and the fluorescence intensity was quantified using ImageJ software by extracting the integrated density from Zone C. The most significant contrast in fluorescence between the blank and the spiked samples was achieved using glass fiber strips, which were consequently selected for further experiments. While these strips exhibited high initial fluorescence in the absence of the analyte, the presence of GBL resulted in pronounced fluorescence quenching (see Figure 6).

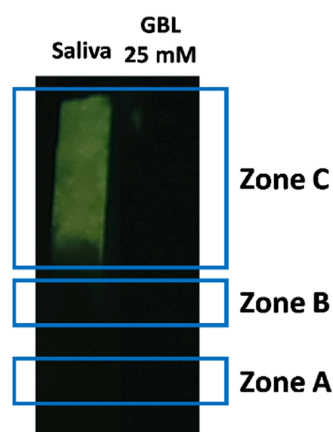


Figure 6. LFA strip image showing the smartphone-based readout for the determination of the fluorescence intensity of the probe in the absence (left) and presence (right) of GBL.

3.6. LFA Quantitative Response and Selectivity

The LFA response was subsequently evaluated against increasing concentrations of GBL in synthetic saliva (0.01 to 100 mM). As shown in Figure 7, a progressive decrease in fluorescence intensity was observed with increasing GBL concentration. A LOD of 0.512 mM was obtained, which surpasses previously reported chromogenic methods (see Table S4). Notably, this LOD is well below the concentration range typically found in spiked beverages intended for chemical submission (116–350 mM) [26].

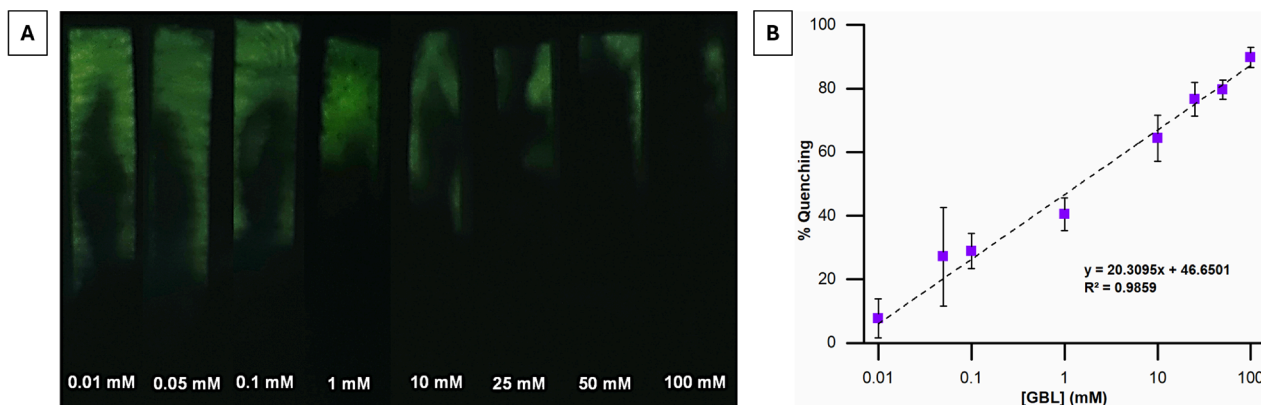


Figure 7. (A) Fluorescence quenching of probe 1 (1 mM with 2 equiv. of NaOAc in DMSO:H₂O 98:2) measured in Zone C of the LFA in the presence of increasing GBL concentrations in synthetic saliva (3 min development). (B) Representation of the % Quenching measured in Zone C of the LFA vs. GBL concentration. Error bars represent 3 σ from three independent experiments.

The selectivity of the system was also tested against other common drugs of abuse (methcathinone, diazepam, scopolamine, ketamine, and GHB). As illustrated in Figure 8, none of these substances (25 mM in synthetic saliva) induced significant fluorescence quenching after 3 min, confirming the high specificity of the probe for GBL under these assay conditions.

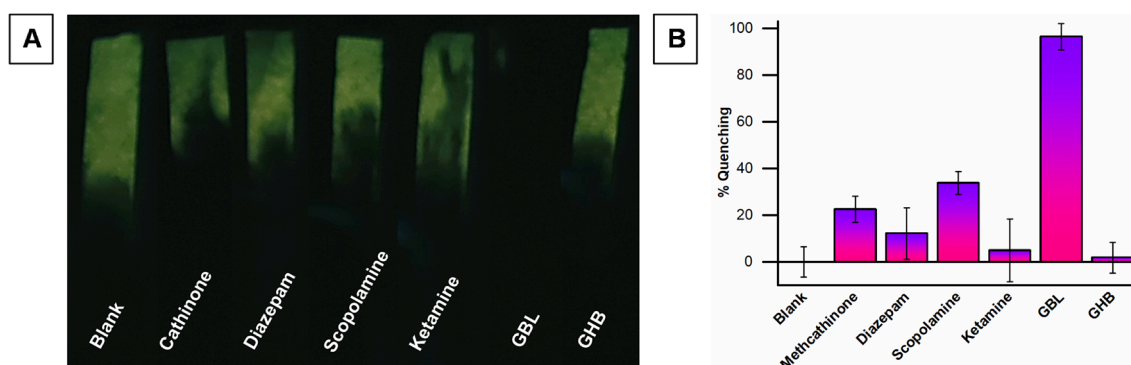


Figure 8. (A) Images of LFA strips showing probe fluorescence in Zone C. Contrast and brightness were enhanced by 20% for visual clarity; quantitative analysis was performed on raw images. (B) Selectivity study: % Quenching of fluorescence of probe 1 in the presence of various DFSA drugs (25 mM) measured in Zone C. Error bars represent 3 σ from three independent experiments.

4. Conclusions

In this work, we developed a novel fluorescein-based fluorescent chemosensor capable of detecting GBL with high sensitivity and selectivity in aqueous media and synthetic saliva. The probe demonstrated low detection limits, well below concentrations associated with severe intoxication, and exhibited strong pH-dependent optical responses linked to

fluorescein lactone ring dynamics. Importantly, the system showed high selectivity, with no interference from other drugs of abuse. Furthermore, the chemosensor was successfully integrated into a portable and low-cost lateral flow assay, enabling rapid, on-site detection of GBL using a smartphone-based readout. A LOD of 0.512 mM was obtained for GBL, which is well below the typical concentration range reported in beverages intended to induce severe adverse effects in victims of chemical submission (116–350 mM). Furthermore, the selectivity of the method was demonstrated against other submission drugs (methcathinone, ketamine and scopolamine) as potential interferents, yielding excellent results as these drugs did not induce fluorescence quenching. This platform fulfills the WHO ASSURED criteria, positioning it as a promising tool for forensic, clinical, and preventive applications in the context of chemical submission and public safety.

Supplementary Materials: The following supplementary information can be downloaded at <https://www.mdpi.com/article/10.3390/chemosensors14070169/s1>: Figure S1: ^1H NMR spectrum of compound **1** in DMSO- d_6 ; Figure S2: ^{13}C NMR spectrum of compound **1** in DMSO- d_6 ; Figure S3: HSQC NMR spectrum of compound **1** in DMSO- d_6 ; Figure S4: HMBC NMR spectrum of compound **1** in DMSO- d_6 ; Figure S5: COSY NMR spectrum of compound **1** in DMSO- d_6 ; Figure S6: Mass spectrometry (HRMS) data of compound **1**; Figure S7: Calibration curve representing the % Quenching at $\lambda_{\text{em}} = 542 \text{ nm}$ ($\lambda_{\text{exc}} = 521 \text{ nm}$) vs. GBL concentration in aqueous solution. Error bars represent 3σ from three independent experiments; Figure S8: Calibration curve representing the % Quenching at $\lambda_{\text{em}} = 542 \text{ nm}$ ($\lambda_{\text{exc}} = 521 \text{ nm}$) vs. GBL concentration in synthetic saliva. Error bars represent 3σ from three independent experiments; Table S1: Recovery and accuracy of the method for GBL detection in aqueous samples. Calculations were performed according to the method described by Rawat et al. [27]; Table S2: Recovery and accuracy of the method for GBL detection in synthetic saliva samples. Calculations were performed according to the method described by Rawat et al. [27]; Table S3: Analytical performance of the proposed method in water and synthetic saliva; Scheme S1: Synthesis of fluorescein free acid derivatives **2a** and **2b**; Figure S9: ^1H NMR spectrum of compound **2a** in DMSO- d_6 ; Figure S10: ^{13}C NMR spectrum of compound **2a** in DMSO- d_6 ; Figure S11: Mass spectrometry (HRMS) data of compound **2a**; Figure S12: ^1H NMR spectrum of compound **2b** in DMSO- d_6 ; Figure S13: ^{13}C NMR spectrum of compound **2b** in DMSO- d_6 ; Figure S14: Mass spectrometry (HRMS) data of compound **2b**; Scheme S2: Synthesis of the 2-aminonaphthoxazol derivative **3**; Figure S15: ^1H NMR spectrum of compound **3** in CDCl_3 ; Figure S16: ^{13}C NMR spectrum of compound **3** in DMSO- d_6 ; Figure S17: Quenching of emission ($\lambda_{\text{exc}} = 521 \text{ nm}$; $\lambda_{\text{em}} = 542 \text{ nm}$) of compound **1** (50 μM in DMSO: H_2O 98:2) with 2 equiv. of NaOAc (blank) and the same solution spiked with 8 equiv. of GBL in real saliva samples collected post-ingestion of various beverages; Figure S18: Illustration of the elution process on glass fiber strips for GBL detection using probe **1** (1 mM with 2 equiv. of NaOAc in DMSO: H_2O 98:2); Figure S19: Schematic representation of the smartphone-based fluorescence readout system, including the darkroom chamber, UV excitation source, and the mobile device for image capture and signal processing; Table S4: Comparison of the sensing properties of different optical selective probes for GBL.

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