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出版情報：Talanta Open. 6, pp.100139-, 2022-12. Elsevier
バージョン：

Talanta Open 6 (2022) 100139

Contents lists available at ScienceDirect

Talanta Open

journal homepage: www.sciencedirect.com/journal/talanta-open

High - throughput spectrofluorimetric approach for one-step, sensitive, and green assays of alfuzosin hydrochloride using a 96-well microplate reader: Application to tablet formulations and human urine

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ARTICLE INFO

Keywords:
Alfuzosin hydrochloride
Spectrofluorimetry
Calcein
Microplate
Native fluorescence
Greenness evaluation

ABSTRACT

In the current study, a novel fluorescence-based microplate methodology was introduced for the one-step determination of alfuzosin hydrochloride, with an assessment of the eco-friendliness of the two developed methods. The development of rapid analytical methods with high sensitivity, selectivity, and low cost is a growing trend. In this context, we propose two simple and rapid spectrofluorimetric methods for the quantitative analysis of alfuzosin in the form of active pharmaceutical ingredient, tablets, and urine samples. The first method relies on a direct assay of the native fluorescence of alfuzosin in water, whereas the second method exploits the quenching effect of alfuzosin on calcein dye for its assay at emission wavelengths of 390 and 520 nm, respectively. The obtained results showed high sensitivity with good linearity (> 0.999) over the concentration ranges of 0.75–12.5 and 10–1200 ng/mL, respectively. The eco-friendly features of the proposed methods were proven by greenness evaluation studies employing the National Environmental Methods Index, analytical Eco-scale score, and Green Analytical Procedure Index as three assessment tools. It was concluded that both methods are efficient analytical tools from a green perspective and are promptly applicable for the alfuzosin assay, while the first method demonstrated 10-fold higher sensitivity. In addition, ever-increasing miniaturization as handling large numbers of micro-volume samples simultaneously and reagents in the proposed methods have enabled them to be safer alternatives for rapid routine analysis in quality control units.

1. Introduction

Alfuzosin hydrochloride (ALF), a selective alpha 1-adrenoceptor antagonist, is defined as (2*RS*) -*N*- [3- [(4-Amino-6,7 dimethoxyquinazolin-2-yl)methylamino]propyl] tetrahydrofuran-2-carboxamide hydrochloride [1]. Its action as a vasodilator may be less frequent, as it acts more selectively on the smooth muscle tone within the prostate and bladder neck, causing relaxation of these muscles. Consequently, this effect results in symptomatic relief of benign prostatic hyperplasia, a common progressive disease encountered in aging men, within weeks [2]. Therefore, after oral administration of ALF, it alleviates symptoms of urinary obstruction by reducing outflow resistance and enhancing bladder emptying.

Several methods have been reported for the determination of ALF either alone or in combination, such as spectrophotometry [3, 4], spectrofluorimetry [5–7] with stability-indicating studies [8] and flow injection analysis [9]. High-performance liquid chromatography methods with UV [10, 11], MS/MS [12], and fluorescence [13] detections, UPLC [14], chiral HPLC [15] and HPTLC [16] have also been applied. Electrochemical methods such as potentiometry [17] and voltammetry [18] have also been developed for ALF determination in biological fluids and conductometric measurements of ALF in pharmaceuticals [19].

Most of the methods reported for ALF assay are time-consuming in both sample preparation and analysis, require high amounts of reagents and solvents, or require sophisticated and expensive instrumentation. Therefore, time-, cost-saving, and simple approaches for drug analysis without any hazardous ecological impact are usually desirable choices for routine analysis in quality control units. In addition, these analytical methods should achieve high-throughput and validation criteria such as high sensitivity, accuracy, precision, and robustness.

Fluorometry is one of the most convenient and inherent analytical

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https://doi.org/10.1016/j.talo.2022.100139
Received 30 May 2022; Received in revised form 14 August 2022; Accepted 18 August 2022
Available online 19 August 2022

techniques in pharmaceutical analysis due to its simplicity, high sensitivity, and availability. As a result, fluorometry is used in most laboratories of quality-controlled clinical units. Thus far, spectrofluorimetric methods reported for ALF estimation required at least 1.0 mL of each sample in a cuvette for classical measurements, producing large amounts of waste. Moreover, some earlier spectrofluorimetric studies involved time-consuming sample preparation steps such as derivatization, extraction steps to increase sensitivity, and the use of hazardous solvents and reagents. To the best of our knowledge, this is the first study to develop a new fluorescence-based microplate assay for evaluation of ALF in 96-microwell plates which incorporates the advantages of both spectrofluorimetry and microplate readers.

A microplate reader offers the advantages of automation and high-throughput properties, enabling the simultaneous measurement of numerous samples (up to 96 or 384) in one reaction vessel. The efficiency, simplicity, and safety of the plate reader enable its use as an economical tool in quality control laboratories. The use of a microplate reader can shorten the analysis time, offering the shortest read times within a few seconds for a 96-well plate for measuring intensity. The approach also decreases reagent and solvent consumption, and waste production, since the capacity of most commonly used microplates is up to 300 μL for 96-wells, while 384-well and 96-well half-area plates require less volumes up to 100 μL capacity [20]. Moreover, this allows for safer handling of the samples, especially biological samples, as microplate readers are equipped with many features such as shaking facility for mixing the sample with the reagents and controlling temperatures up to 42°C. As a result, microplate assays have received considerable attention to detect and estimate many pharmaceuticals [21–25].

In a fluorimetric microplate reader, excitation of fluorophores occurs after the absorption of light emitted by external source at a certain wavelength, such as a UV xenon flash lamp. Then, the fluorescence emission as a molecular process occurring due to the spontaneous emission of radiation from the electronically excited substrate is measured. Fluorescence emission usually occurs at longer wavelengths due to the Stokes shift phenomenon as the excited state energy can be dissipated during non-radiative processes, such as vibrational relaxation, internal conversion and non-radiative relaxation, and intersystem crossing [26]. Flat black bottom microplates are mainly used for spectrofluorometric measurements as they show minimal background signals above 380 nm, with prevention of cross-talk between the wells. Thus, these plates are well suited for fluorimetric assays of not only fluorophore dyes but also intrinsic fluorescence, such as the native fluorescence of ALF in water emitting fluorescence at 390 nm. Fluorescent probes are typically employed because of their stable molecular derivatives with high sensitivity, specificity, and quantum efficiency. Calcein dye, a popular calcein green dye and its analogs of a polyanionic derivate of fluorescein with different colors, are a class of organic fluorescent bio-macromolecule dyes. Calcein green ($C_{30}H_{26}N_2O_{13}$) is chemically known as bis [N, N-bis (carboxymethyl) aminomethyl] fluorescein. This versatile dye is typically used for various applications and in vitro visualization because of its high molecular stability, quantum efficiency, good purity, and affordable cost.

Calcein was originally used for titrimetric analysis as a metallochromic indicator for calcium [27, 28] and other non-metallic species such as lactic acid [29]. The fluorescence of calcein complexes was also been utilized for flow injection [30], fluorimetric [31] analysis of metals, formation of macromolecular fluorescent sensors for the discrimination of beta-lactam antibiotics [32], and quantification of biologically relevant carboxylates [33]. The detection of biologically important compounds such as cysteine [34]and ATP [35] was also been reported. Chemical quenching of calcein was also applicable for quantifying pharmaceuticals, such as cefixime [36] and cefotaxime [37] spectrofluorimetrically, as well as in chemiluminescence systems for estimating azathioprine [38]. Calcein at higher concentrations is a self-quenched fluorophore utilized for bulk self-quenching assays such as permeability assays [39, 40] and electroporation studies [41]. Many other reported researches, too numerous to be mentioned here, also invested the fluorescence and chelating properties of this dye for application in the medical and biological fields. These properties facilitated the establishment of a new and simple fluorescence-based microplate assay for ALF based on its quenching effect on calcein fluorescence.

To date, no method has been developed for the spectrofluorimetric assay of ALF using a microplate reader to achieve better sensitivity in a short analysis time. Collectively, this study proposed a new and simple spectrofluorimetric microplate assay of the cited drug, not only in pure form and tablets but also in human urine. The native fluorescence of the drug (method I) and its quenching effect on fluorogenic dyes, such as calcein, due to the formation of a complex between them at neutral pH (method II) were exploited for its estimation without the use of hazardous chemicals.

2. Experimental

2.1. Instrumentation

An Infinite® M Plex 200 PRO MicroPlate Reader (Tecan, Japan) equipped with a UV xenon flash lamp, Quad 4 Monochromators™ system, and NEC computer with i-control™ software (2.0) was used for all fluorescence assays. Greiner microplates with 96 well, Fluotrac with flat black bottom [GRE96fb_Chimmy well] were employed for all measurements with 5.0/10.0 and 20 nm bandwidths for the emission and excitation monochromators, respectively. Thus, the minimal Stokes shift for wavelength settings in the microplate apparatus (Eq. 1), is not less than 35 nm to avoid any overlap between the excitation and emission wavelengths and to attain the best detection limit and S/N ratio:

$$\text{Minimumdistancerule} = \text{Bw(Ex)} + \text{Bw(Em)} + 5\text{nm} \quad (1)$$

A top-detected mode was applied with adjustable Z-focus, complete with integrated background correction to optimize the signal-to-noise ratio for every assay automatically. Fluorescence intensity (FI) was measured using UV and red-sensitive photomultiplier tubes (PMTs) converting the light signal emitted by a sample after its multiplication into an electric signal and then quantified in relative fluorescence units (RFU). FI is not an absolute measurement like absorbance to be compared between fluorescence readers of different manufacturers because it depends on every characteristic of the instrument and there are neither standardization of PMT nor RFU scale. Hence, FI is usually relative to other measurements, to a reference measurement taken by an instrument, or to the gain settings.

Accordingly, the detector gain (detector amplification factor) is a critical parameter that provides better data resolution. The incorrect selection of fluorescence gain has a negative effect on the quality of the data. A high fluorescence gain provides large amplification for bright samples, resulting in saturation of the detector and overflow of the data, causing unusable data. However, a lower gain for the dim samples results in indistinguishable signals from background noise. Therefore, the modification of the gain affects the positions and not the width of the dynamic range. To avoid these issues on microplate readers, the fluorescence gain in fluorescence intensity measurements is automatically adjusted by selecting the optimal option by reading the whole plate and automatically selecting the highest reading. The gain is also either determined through calculation from a specific well of the expected highest concentration, or manual adjustment relying on trial or predefined values of the fluorescence gain.

Microplate readers also possess an internal heating system which has been utilized to control the temperature and maintain the temperature of the microplates at 25°C. Shaking action for mixing reagent with the analyte for 1 min, and settle time of 300 mS after shaking was also applied. A lag time of 0 μs and an integration time of 20 μs were set for normal fluorescence measurements, and the step size was set at 10 for a

reasonable time of scanning.

The number of flashes, which is the number of measurements per well with the top probe vertical offset, was manually adjusted to 7 flashes to optimize the reading time with higher quality data.

A LAQUA (HORIBA Scientific) pH/ion meter F-72, vortex mixer (Vision Scientific KMC-1300 V), centrifuge (Himac CF16RN), and ultrasonic bath sonicator (AS ONE®, Japan) were used.

2.2. Materials and reagents

2.2.1. Calcein preparation

HEPES (2-[4 - (2-hydroxyethyl) piperazin-1-yl] ethanesulfonic acid) buffer, calcein (Dojindo, Japan), and 5N sodium hydroxide solution (Wako, Japan) were used. A 1 mM calcein solution was prepared by dissolving 0.0623 g of calcein powder, with the help of vortex, in 100.0 mL of 50 mM aqueous HEPES adjusted to pH 7.2 with 5N sodium hydroxide solution. Dilution of the stock solution of calcein was performed with the same solvent to obtain a 0.1 mM working solution. The prepared solutions of calcein were kept refrigerated and protected from light and showed stability for up to 10 days.

2.2.2. Chemicals and reagents

Pure sample of ALF was generously provided by Eva Pharma, Egypt (lot no. 1422R118) with purity of 100.80% according to the comparison method [1]. Prostetrol® modified release tablets, a product of Eva Pharma with batch no. (10)190238 containing 10 mg of ALF per tablet, were purchased from the Egyptian market to be analyzed using the proposed methods. Drug free human urine sample was taken from a healthy volunteer.

Analytical grade reagents, solvents of highest purity (Wako, Japan), and freshly obtained distilled water from a Milli-Q water purification system were used throughout the experiment.

2.3. Standard stock solutions

A stock solution (40 μg/mL) was prepared by dissolving 4 mg of the pure drug in 2.0 mL of methanol and diluting it to 100.0 mL with distilled water in a 100.0 mL volumetric flask. Further dilution with distilled water was performed to prepare 0.1 and 5.0 μg/mL of individual working solutions for methods I and II, respectively. The prepared solutions showed stability without any alteration for at least two weeks if refrigerated at 4°C and were brought to room temperature before use.

2.4. General procedure

2.4.1. Construction of calibration graphs

Aliquots of working solutions containing different concentrations of the cited drug were transferred into a 2.0 mL volumetric flask and diluted with distilled water. From each prepared solution, 200 μL (method I) and 160 μL (method II) were transferred to microplate wells. Then, 40 μL of calcein was added for method II, wherein 200 μL was the final volume of the assay solution per well. A simple one-step homogenous assay of a 96-well microplate containing 0.75–12.5 and 10–1200 ng/mL of the drug for methods I and II, respectively, was performed. The fluorescence intensities were recorded at $\lambda_{ex}/\lambda_{em}$ of 250/390 and 250/520 nm against reagent blanks for both methods, respectively. The native fluorescence intensity of ALF (FI) and the reduced fluorescence intensity of calcein (ΔFI) related to the quenching effect of the drug were plotted against the concentration to construct the calibration curve of each method.

2.4.2. Assay of pharmaceutical formulations

Ten Prostetrol® tablets were finely pulverized, and an equivalent amount to 2 mg/mL of ALF was accurately weighed and transferred into a 10.0 mL volumetric flask. The flask content was dissolved in 9.0 mL of methanol by sonication for 30 min in an ultrasonic bath. The final volume was adjusted with the same solvent, mixed well and then settled for approximately 15 min before filtering the supernatant [42]. Further dilution was also made with bi-distilled water to obtain 0.1 and 5.0 μg/mL solutions of ALF containing nearly the same volume of methanol in the standard stock solution of a pure drug as methanol negatively affected method II. Aliquots from this solution were subjected to spectrofluorimetric measurements, as described under the '' construction of calibration curves''. A standard addition technique was applied, and the recovery of each concentration was calculated using the corresponding regression equations to assess the accuracy of the proposed methods.

2.4.3. Procedure for spiked urine sample

Urine samples (5.0 mL of urine sample collected from healthy individuals) were placed into centrifugation tubes, spiked with 250 μL ALF of standard stock solution, and then mixed well to nearly simulate the concentration excreted unchanged in the human urine. Then, methanol was added to adjust the volume to 10.0 mL, followed by centrifugation at 1000 rpm for 15 min to precipitate the protein, and the blanks of drug-free urine were similarly treated. Aliquots of the clear supernatant were filtered using a Millex® HA filter unit with a 0.45 μm pore size and diluted with distilled water to suit the linearity range of the proposed methods. General procedures were applied and each concentration was measured against the related blank.

3. Results and discussion

The fluorophore can be affected by its surrounding physical and chemical environments, such as the pH, ionic strength, diluting solvent, temperature, time, and reactive species. Therefore, trials were conducted to optimize the assay of the cited drug by studying different factors to enhance the fluorescence intensity. Measurements were performed in triplicates, hence the added error bars represent the standard deviation of triplicated measurements.

3.1. Fluorescence spectra

ALF contains a fluorophore group, 4- amino quinazoline moiety, which exhibits fluorescence emission at 390 nm in distilled water after excitation at 250 nm (Fig. 1A).

In addition, calcein, an anionic organic fluorescent dye, was chosen to form the dye analyte complex because of its stability in water and high quantum yield[33]. The excitation spectra of pea green calcein solution in 50 mM HEPES (pH 7.2) alone and after the addition of the drug were scanned from 230 to 550 nm at an emission wavelength of 590 nm, which exhibited three characteristic excitation peaks at 250, 300, and 500 nm (Fig. 2A). The absorption spectrum obtained using the microplate reader for 0.1 mM of calcein also showed two distinct peaks at 250 and 490 nm (Fig. S1). The fluorescence emission spectrum of calcein depicted in Fig. 2 shows a single maximum emission peak at 520 nm. It was observed that 250 nm was the suitable excitation working wavelength as it manifested high gain compared to other excitation peaks with high linearity and a reasonable range. Upon adding the studied drugs to calcein buffered at pH 7.2, the location of the emission peak did not change. However, a gradual decrease in the fluorescence intensity of calcein was observed. In addition, the difference between the emission spectra of calcein before and after drug addition (ΔFI) indicated the extent of reactivity between calcein and ALF. Therefore, greater reactivity would show a greater difference. The fluorescence of calcein was quenched on forming a non-fluorescent ground state complex between the ionizable groups of the calcein anionic probe and cationic analyte through electrostatic attraction. Therefore, the emitted FI was related to the uncomplexed fluorophore. Hence, the above principle was reasonably applicable for quantifying ALF in tablets and biological fluids, such as urine.

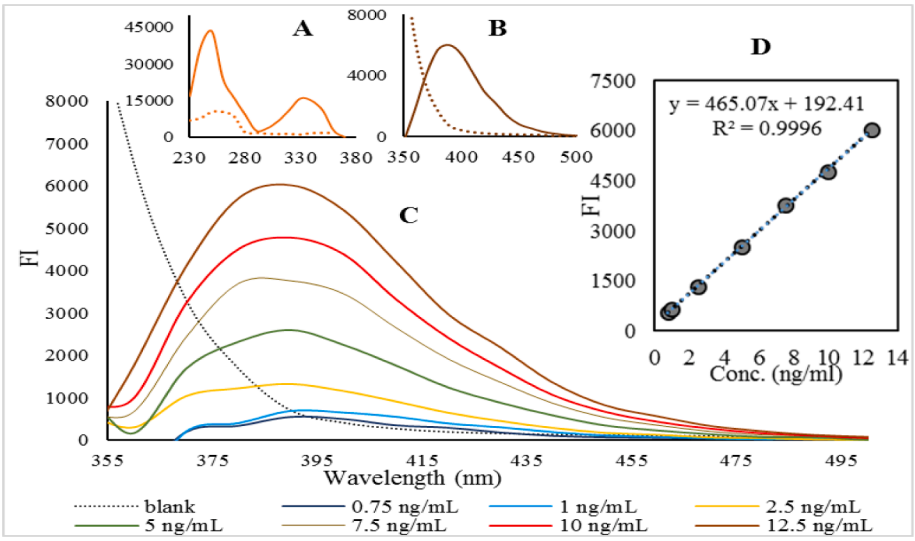


Fig. 1. **(A)** Excitation; **(B)** Emission spectra of water blank (····) and 12.5 ng/mL of ALF (–); **(C)** Emission spectra of different concentrations of ALF in distilled water (0.75–12.5 ng/mL) at 390 nm after excitation at 250 nm; **(D)** The calibration plot.

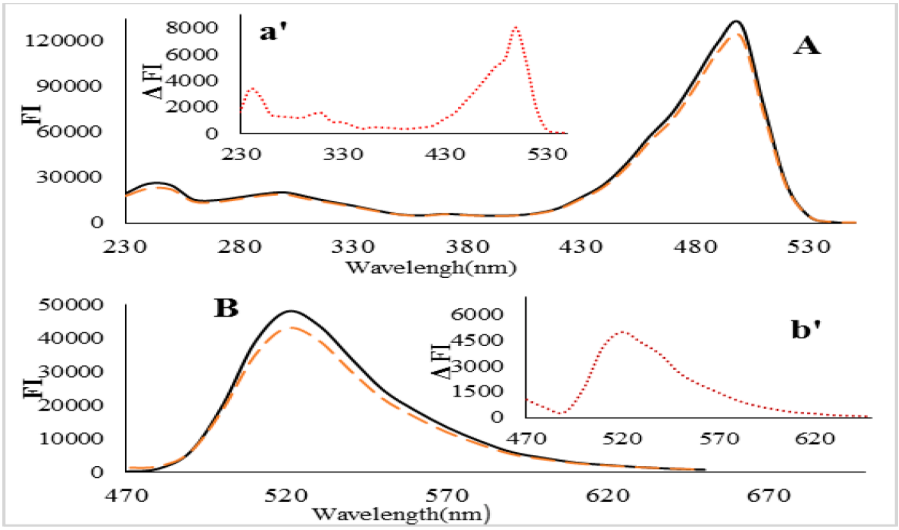


Fig. 2. **(A)** Excitation; **(B)** Emission spectra of 0.1 mM calcein (40 μL) after dilution with distilled water (–) and after complexation with 1200 ng/mL of ALF (- -) at $\lambda_{ex}/\lambda_{emi}$ = 250/520 nm; **(a'& b'):** The difference excitation and emission spectra, (ΔFI) depicts the extent of quenching effect of ALF on calcein (...).

3.2. Influencing factors on the fluorescence intensity of the system

3.2.1. Effect of pH and buffers

The influence of pH from 2.8 to 10 on the native fluorescence of ALF and its quenching effect on calcein was studied and is given as FI (for method I) and ΔFI (for method II), which were plotted as a function of pH in Fig. S2. In addition, the impact of the addition of 0.1 M HCl and 0.1 M of NaOH on the native fluorescence was also tested.

For method I, the addition of the buffer, acid or base did not improve the native fluorescence of ALF. However, the FI decreased upon using basic buffer and 0.1 M NaOH with a bathochromic shift to 420 nm. Therefore, the optimum FI of ALF was attained in distilled water without adjusting the pH.

For method II, calcein at neutral pH (7.2) showed higher solubility and a strong fluorescence signal, exhibiting a high reactivity with ALF (Fig. S2). Thus, the results confirmed that the maximum fluorescence intensity of calcein was observed at pH 6.5–8.5 and decreased at lower and higher pH values, as reported in previous studies [43]. Therefore, the calcein working solution was prepared at a neutral pH to achieve a perfectly adequate medium for the reaction. Several buffers with neutral pH were also tested, such as phosphate-buffered saline (PBS), glycerol (5%) in HEPES-buffered saline, and monopotassium phosphate-disodium phosphate (KH_2PO_4– Na_2HPO_4). It was found that aqueous 50 mM HEPES (pH 7.2) was the most suitable buffer for further investigations.

3.2.2. Effect of time and temperature

The reaction time of the calcein-ALF system and the stability of ALF native fluorescence were studied at room temperature for 40 min and away from light in method II. The native FI remained stable for at least 40 min. The formed complex was immediately stabilized after shaking with stable ΔFI values for 15 min, then a slight decrease in the ΔFI was observed (Fig. S3). Therefore, all spectrofluorimetric measurements were performed within a few seconds.

The impact of different temperatures on the native fluorescence of ALF and its reaction with calcein was carried by adjusting the apparatus at 25, 30, 35, and 40°C. As a result, the fluorescence intensity did not change while the temperatures were varied for method I. Meanwhile, a

gradual decrease in ΔFI was noticed for method II (Fig. S4). Consequently, the ALF analysis was completed at room temperature (25°C) within a few seconds.

3.2.3. Effect of the diluting solvent

The selection of an appropriate diluting solvent for the spectrofluorimetric measurements was studied by evaluating different solvents (i.e., distilled water, acetone, acetonitrile, methanol, and ethanol) to enhance the emitted fluorescence intensity. Nevertheless, organic solvents (e.g., acetone) should not be used at higher concentrations because they can damage polystyrene microplates. Furthermore, the tested organic solvents reduced the FI of calcein dye (especially with acetone), negatively affecting the quenching efficiency in method II. Fig. S5 indicates that bi-distilled water was the most suitable diluting solvent for both the methods.

3.2.4. Effect of additives

The addition influence of 0.1 M KCl and different organized media such as 1 % (w/v) of sodium dodecyl sulfate, beta-cyclodextrin and 1 % (v/v) of triton X-100 were investigated for method I and II. Neither of these additives enhanced fluorescence intensity (Fig. S6).

3.2.5. Effect of calcein concentration and volume for method II

The effect of the calcein concentration on this quenching system was studied to determine the optimum concentration for the ALF assay. The intensity of fluorescence emission was examined over a concentration range of 0.01–1 mM at 520 nm after selection of 250 nm as the working excitation wavelength. Upon increasing the concentration of calcein relative to ALF, the ΔFI values initially increased up to 0.2 mM followed by a plateau and then a decrease (Fig. 3A). There was also an increase in the blank response. However, the linearity of FI calcein gradually decreased at concentrations above 0.1 mM and then lost (Fig. 3B). The changes in the spectrofluorimetric signals indicated that a complex was formed between calcein and ALF. Meanwhile, the non-monotonic response of the emitted fluorescence of calcein (FI) was related to the effect of concentration-mediated self-quenching, which is widely known for other xanthene dyes [32]. Therefore, the optimum concentration of calcein selected for further experiments was 0.1 mM to promote higher intensities while avoiding the self-quenching effect of the calcein fluorophore. 0.4 μL of 0.1 mM calcein was adequate for obtaining the best results (Fig. S7).

3.2.6. Stoichiometry of the formed complex for method II

The stoichiometry of the formed complex was determined using the continuous variation (Job's) method [44]. Equimolar solutions of 0.1 mM of calcein and ALF were prepared. Calcein concentrations ranging from 0.0125 to 0.0625 mM (125, 100, 90, …., 25 μl) and 0.0625 to 0.0125 mM of the drug (25, 50, 60,….., 125 μl) were added to the microplate wells and made up to 200 μl with distilled water. The formed complexes were measured using a one-step spectrofluorimetric procedure after shaking at 250/520 nm for $\lambda_{exci}/\lambda_{emi}$ against the reagent blanks. The results showed a (1:1) binary complex adduct of the drug with calcein dye (Fig. 4). The ratio of 1:1 was the most suitable explanation for complex formation with regard to drug stoichiometry (Scheme 1).

3.3. Mechanism of calcein quenching

Method II relies on quenching the native fluorescence of calcein dye through complex formation with ALF. Quenching or such decreases in the emitted fluorescence intensity can occur using different methods divided into two main categories: stable (static) and dynamic quenching mechanisms. The quenching mechanism was investigated using Stern-Volmer law [26, 45] represented in Eq. (2) as follows:

$$I^{\circ}/I = 1 + K[Q] \quad (2)$$

where I° and I are the emission intensities of calcein in the absence and presence of different concentrations of a quencher [Q], respectively, K is the Stern-Volmer quenching constant, and denotes the association constant of complex formation for the static quenching type.

A Stern-Volmer plot was constructed by plotting I°/I versus the concentration of the quencher (ALF) (Fig. S8). The obtained linear curve indicated that the system followed the Stern-Volmer law and underwent a single quenching mode either static or dynamic. Otherwise, an upward nonlinear curvature could be observed when both quenching types occur for the same fluorophore [26]. A linear regression equation was obtained as $I^{\circ}/I = 4 \times 10^{7}$ [Q] + 1.0089, $R^2 = 0.9993$ and intercept of nearly one. The association constant (K) of the formed complex obtained from the slope of the plot is equal to 4×10^{7} M^{-1}, suggesting the relative stability of the formed complex.

Dynamic and static quenching can be distinguished by their differing dependences on viscosity and temperature or preferably by measuring the lifetime [26]. It is believed that calcein quenching proceeded in a static mechanism, as the fluorophore and quencher possess a point of interacted to form a new compound. The ALF targeted here contains a

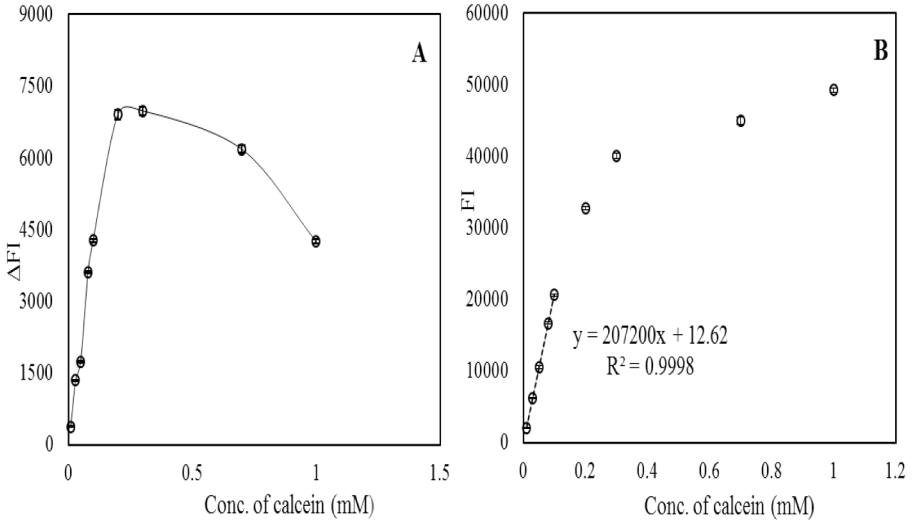


Fig. 3. (**A**) Effect of calcein concentrations on the quenching effect of 0.8 μg/mL ALF as a difference emission intensity (ΔFI) related to the formed complex and (**B**) Fluorescence emission intensity (FI) of different concentrations of calcein prepared in 50 mM HEPES buffer (pH 7.2).

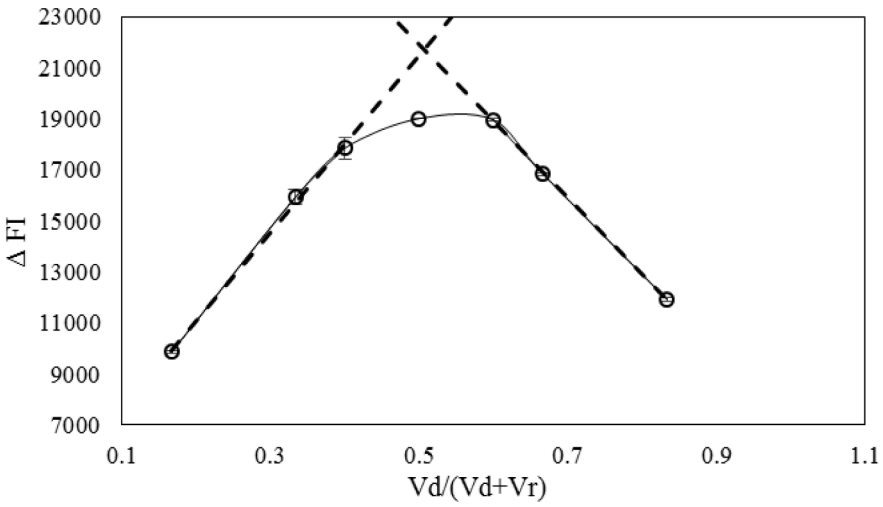
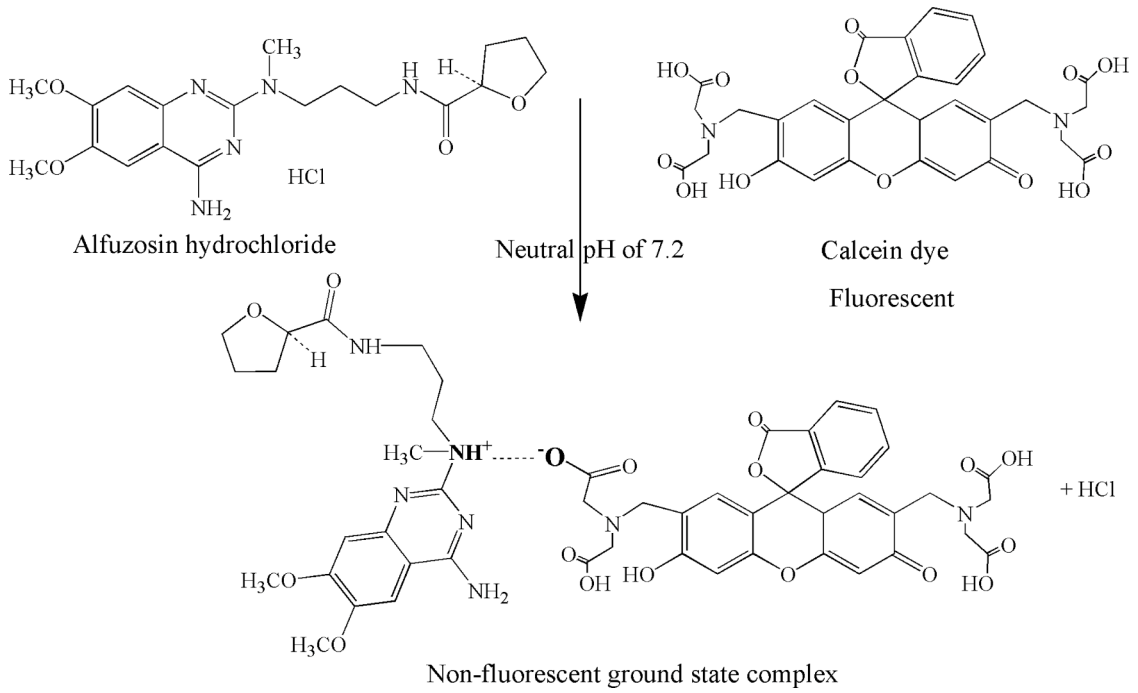


Fig. 4. Continuous variation plot for 1 × 10^{-4} mM of **ALF**and 1 × 10^{-4} M of calcein dye.



Scheme 1. The proposed mechanism of calcein quenching for ALF quantification.

tertiary amino group in its side chain that can interact with one of the carboxylate groups in calcein, which is a better hydrogen bonding acceptor (Scheme 1). A non-fluorescent ground state complex was formed, where it might absorb the incident light and return immediately to the ground state without photon emission.

Moreover, the quenching effect with regard to the temperature shows low amounts of static quenching at higher temperatures due to the dissociation of weakly bound complexes [26]. In contrast, dynamic quenching occurs in larger amounts as it depends on diffusion, and higher temperatures enhance collisions between molecules, resulting in a larger diffusion coefficient and an increase in quenching efficiency. Based on this fact, Fig. S4 shows a gradual decrease in the value of ΔF during temperature elevation from 25 to 40°C. Thus, it could be concluded that fluorescence quenching of calcein by the cited drug belonged to the static type. In addition, examination of the absorption spectra of a fluorophore before and after quenching represents further evidence for the static type. This deduction can be made since the effect of collisional quenching is only on the excited state of the fluorophore, resulting in no changes in the absorption spectrum. Meanwhile, the occurrence of static quenching is related to the formation of a ground-state complex accompanied by the perturbation of the absorption spectrum of the fluorophore, as shown in Fig. S1. The aqueous solution of ALF did not exhibit any absorbance in the visible region. Once a complex formed between ALF and calcein, the absorbance intensity decreased gradually with increasing ALF concentration at 490 nm (Fig. S1).

3.4. Method validation

The developed methods were validated according to ICH guidelines [46] with regard to the following parameters.

3.4.1. Linearity and ranges

Under the studied conditions, the calibration curves were

constructed by plotting the native fluorescence (FI) of ALF or ΔFI values after quenching of calcein with the studied drug against concentrations in the range of 0.75–12.5 or 10–1200 ng/mL, respectively (Figs. 1 and 5). Statistical analysis of the data showed linearity with high values of correlation coefficients of 0.9996 and 0.9998 for methods I and II, respectively (Table 1).

3.4.2. Sensitivity

The limits of detection and quantification (LOD and LOQ) were calculated according to the ICH guidelines [46]: LOD = 3.3 σ/s and LOD = 10 σ/s, where, σ is the standard deviation of the replicated blank responses and s is the slope of the calibration plot. Table 1 lists the values of LOD and LOQ, indicating the sensitivity of the proposed methods.

3.4.3. Accuracy and precision

The accuracy of the proposed methods was investigated using nine samples of three different concentrations of the pure drug selected to cover the low, medium, and high ranges of the calibration graph. The mean percentage recoveries of these ALF concentrations were calculated (Table 1).

Intra-day and inter-day precisions were also examined by analyzing three different concentrations of the pure drug by triplicate determinations on the same day (intra-day) and on three different days (inter-day). The relative standard deviation (RSD%) and percentage relative error (Er%) were calculated using Eq. (3) that follows:

$$Er(\%) = [(\text{ found } - \text{ added })/\text{ added }] \times 100 \quad (3)$$

The data listed in Table 1 confirm the high precision of the proposed methods.

3.4.4. Specificity

The assay for ALF in urine and its commercial tablets confirmed the specificity of the proposed methods. The selectivity was also evaluated by studying the interference effect of some common excipients, such as silica, polyethylene glycol 200, sucrose, starch, and sodium chloride, which are widely found in tablets dosage form. The excipients were added to the drug at the same concentration (10^{-5} M) and analyzed under optimum conditions using general procedures. Table S1 shows sufficiently good recoveries with no significant interference, while silica and starch exhibited minimal interference, which could be eliminated through filtration. The selectivity was also examined by application of the proposed methods to certain drugs of phosphodiesterase-5 inhibitors such as tadalafil and vardenafil that co-administered with the studied drug for symptomatic treatment of lower urinary tract symptoms attributed to BPH. The obtained results under the optimized conditions indicated higher selectivity of method I by lack of FI at λ_{em} of 390 nm, while method II exhibited minimal interference.

Table 1
Regression and analytical performance data for spectrofluorimetric estimation of ALF using method I and II.

Parameters	Method I	Method II
Linearity range (ng/mL)	0.75- 12.5	10-1200
Intercept	192.41	394.44
Slope	465.07	3.3191
Correlation Coefficient(r^2)	0.9996	0.9998
LOD (ng/mL)	0.23	3.196
LOQ (ng/mL)	0.70	9.685
Accuracy		
(Mean recovery% ± Er%)	98.73-1.269	100.80+0.798
	100.70+0.698	98.11-1.891
	99.32-0.676	101.29+1.288
Precision (RSD %)		
Repeatability [a]	1.582	1.988
Intermediate precision [b]	1.666	1.566

[a]The intra-day and [b] the inter-day RSD (n=9) of determining 2.5, 5.0 and 10.0 or 10, 800 and 1200 ng/mL of ALF by method I and II within the same day and in three successive days.

3.4.5. Robustness

The influence of deliberate variations in one spectrofluorimetric parameter up or down around the nominal level was examined while keeping all others constant. The studied variables on 10 and 1200 ng/mL of ALF for methods I and II, respectively, showed negligible influence on the results where the RSD (%) was less than ± 2% (Table S2).

3.5. Statistical analysis

Statistical examination of the results of the proposed methods was performed using the Student's t-test and variance ratio F-test at the 95% confidence level. The results obtained were compared with those of the

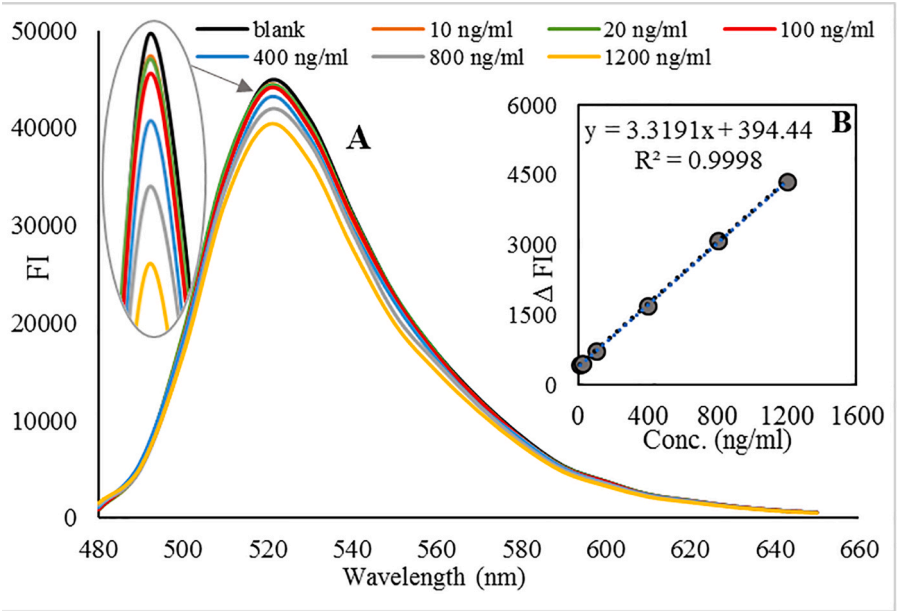


Fig. 5. **(A)** Emission spectra of 0.1 mM calcein (40 μL) after dilution with distilled water as a blank and after complexation with different concentrations of ALF (10–1200 ng/mL) at 250/520 for $\lambda_{ex}/\lambda_{emi}$; **(B)** Calibration plot between these different concentrations versus their quenching effect as ΔFI.

pharmacopeial potentiometric method for the ALF assay [1] and exhibited no significant differences (Table S3).

3.6. Analytical applications

The results obtained from the spectrofluorimetric methods using a microplate reader showed excellent feasibility for the assay of ALF in Prostetrol® tablets and urine samples. Approximately 11 % of the daily ALF dose is excreted in the urine as an unchanged drug [2]. Hence, the sensitivity of the proposed methods enabled the in vitro estimation of ALF in spiked samples of human urine. The recovery of the target analyte and the standard deviation (SD) values confirmed the suitability of applying the proposed procedures for fast routine analysis (Table S4).

Compared with cuvette spectrofluorimetric assays, the analytical performance of the two proposed methods in terms of LOD and linearity range were in line with those of reported methods based on either native fluorescence or fluorogenic probes (Table 2). However, the microplate assays were superior to the reported methods in terms of simplicity, rapidity, and environmental friendliness, while simultaneously maintaining their efficiency for analytical applications in different matrices at the same time. It is noteworthy that microplate-based methods could handle up to 96 samples with a small volume of 200 μL, which is 5–10 times smaller than that required for cuvette-based measurements. In addition, labor-intensive procedures such as solid-phase extraction for the preconcentration of alfuzosin to enhance its sensitivity were not required for sample preparation. The microplate reader could perform triplicate measurements of 24 samples within nearly one minute for fluorescence intensity indicating the advantage of rapidity. At the same time, scanning with a step size of 10 took about 8 min, which reduced not only the time of analysis but also energy consumption. Moreover, the greenness assessment described in our study with detailed results focused on the environmental impacts of the procedures using the microplate reader methodology to prove that the proposed methods were user-friendly and a better alternative.

3.7. Greenness profile of the proposed method

The developed methods were assessed from a green perspective view to determine their impact on the environment. The assay-based microplate reader mostly complies with green analytical chemistry (GAC) principles because the miniaturization of analytical techniques reduces the volume of samples, reagents, and analytical waste compared to classical techniques.

To deepen this view, the greenness profile of the developed methods was established using three assessment tools related to GAC: NEMI, analytical Eco-Scale, and GAPI methods. The applied tools introduced easier and more visible information on the analytical procedure to facilitate its evaluation.

NEMI labeling was initially employed as one of the oldest tools used for qualitative greenness assessment of analytical methodologies. The circular NEMI pictogram encompasses four fields reflecting four different criteria of the analytical procedures: persistence, bioaccumulation potential, toxicity (PBT), hazardous chemicals, corrosiveness, and waste. Hence, when the required criterion of each field is met by the developed method, the field is filled with green [51]. Adopting these criteria, green-colored quadrants in the pictograms are shown in Table 3 due to satisfaction with their requirements by the applied methods, except for the quadrant of hazardousness due to methanol usage, regardless of the minute amount per sample. However, environmental assessment can be easily read from a glance at the NEMI symbol, but generalized information can only be obtained with no significance to the quantity of chemicals, waste, or energy consumption.

The analytical Eco-Scale approach was applied to obtain a more comprehensive assessment in a more quantitative way, where a numerical score was given for the applied method. The Eco-Scale score is the subtracted result of the total penalty points from the basis of 100 points (ideal green analysis). The penalty points of the developed analytical procedure are assigned to the type and amount of chemical reagents, consumed energy, occupational hazards, and waste amount and its treatment. Then, the score is ranked on a scale where the green analysis method is deemed excellent if the score is higher than 75, acceptable if the score is higher than 50, and inadequate if it is less than 50 [52]. Table 3 demonstrates the calculation of the analytical Eco-Scale scores in detail, which assessed the greenness profile of the proposed methods more clearly than that obtained by NEMI. Both methods scored higher than 75, so they ranked as excellent green methods, showing the superiority of method I with an Eco-scale score of 87 as opposed to 82 for method II due to the low consumption of chemicals.

A recent tool known as GAPI for detailed greenness assessment was also applied, a specific symbol with five pentagrams involving 15 zones representing five main categories: sample handling, general method type, sample preparation, reagents/solvents used, and instrumentation. The GAPI approach evaluates 15 criteria covering every step in the whole analytical procedure using a color code: green, yellow, and red, signifying low, medium, and high ecological impacts, respectively [53]. Visual inspection of the GAPI pentagram for the proposed methods mostly showed green and yellow shaded zones, indicating their lower impact on the environment (Table 3). As expected, both methods exhibited similarity in all pentagrams except aspect 10 due to similar sample preparation steps and instrumentation using a microplate reader, while the only difference was related to the reagents used in method II.

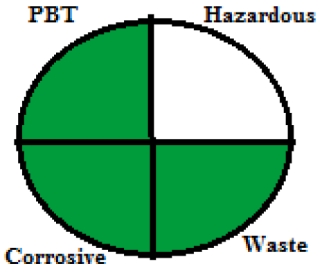
Overall, we attempted the greenness assessment described here to shed light on the merits of the applied method in terms of user-friendliness and safety for quantification of ALF for use in routine work and quality control purposes in pharmaceutical industries.

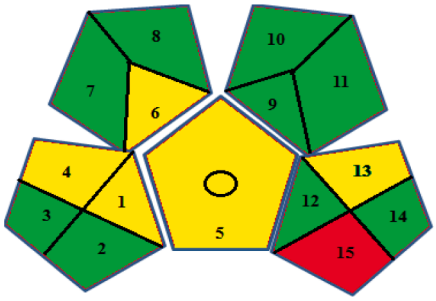
Table 2
Comparison of reported spectrofluorimetric methods for determination of ALF with the proposed methods.

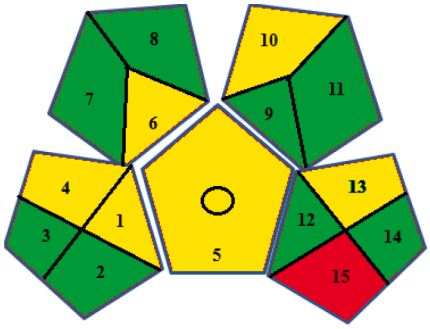
Matrix	Reagents	λ_{ex}/ λ_{em} (nm)	LOD (ng/mL)	Linearity (ng/mL)	Ref.
Tablets, human plasma and in the presence of its degradation products	-Native fluorescence in deionized water	325/ 390	1.6	50.0–750.0	[8]
	-Fluorescamine	380/ 465	9.04	100.0–900.0	
Tablets, urine and plasma	Magnetic solid-phase extraction combined with mixed micelle cloud point extraction.	332/ 383	0.16	5.0–300.0	[47]
Tablets and human urine	Dansyl chloride	405/ 516	8	25.0–500.0	[48]
Tablets, plasma and urine	Polymeric ionic liquid-coated magnetic nanoparticles	332/ 383	0.035	0.5–45.0	[5]
Tablets and human plasma	Hantzsch reaction	415/ 480	8.9	70.0–900.0	[6]
Tablets and human plasma	Micellar system of polyoxyethylene 50 stearate	325/ 382	0.97	4.0–100.0	[49]
Human plasma	Orthophthalaldehyde with 2-mercaptoethanol	337/ 430	0.52	10.0–400.0	[50]
ALF with vardenafil hydrochloride in tablets, human plasma and urine	Distilled water and sodium dodecyl sulphate	265/ 380	0.26	1.0–16.0	[7]
Tablets and human urine	-Native fluorescence	250/ 390	0.23	0.75–12.50	Proposed methods
	-Calcein dye	250/ 520	3.2	10–1200	

Table 3
Greenness assessment of the proposed method by the analytical Eco-Scale, NEMI and GAPI approaches.

Analytical Eco–Scale score parameters	**Penalty points (PPs)**		**NEMI pictograms**
I–Reagents/word sign/no of pictograms			
Bi-distilled water/ - / 0	0		
Methanol/ danger/ 3	6		
Calcein / warning/ 1 (*Method II*)	1		
5 M NaOH/ danger/ 2 (*Method II*)	4		
HEPES/-/ 0 (*Method II*)	0		
	Method I	Method II	
	∑= 6	∑=11	
II–Instruments			**GAPI pictograms***
a-Energy (kWh per sample)			Method I
Spectrofluorimetric microplate reader	0		
pH meter	0		
Vortex mixer	0		
Sonicator	0		
Centrifuge	0		
b–Occupational hazards (Hermetic sealing or not)	3		
c–Waste			
Waste amount (mL or gm)	1		
Waste treatment	3		
	∑= 7		
	Method I	Method II	
Total PPs	13	18	Method II
Analytical eco–scale score = 100- total PPs	87	82	
	Excellent green analysis		







* Red zones depict high ecological impact; yellow zones represent lower impact; and green zones represent more safe effect to the environment.

4. Conclusion

We employed two fluorescence-based microplate assays of alfuzosin hydrochloride by measuring its native fluorescence and quenching effect on calcein. The main goal of our comprehensive study was to broaden the horizon for utilizing microplate readers for rapid quantitative assays of active pharmaceutical ingredients, manifesting its suitability for application in quality control units. To the best of our knowledge, this is the first report to perform two spectrofluorimetric methods based on microplate reader for estimation of alfuzosin hydrochloride with a detailed evaluation of their green-profile. These adapted studies for the 96-well microplate format are believed to be a potential step forward in simple, rapid, and cost-saving assays of pharmaceutical compounds such as ALF in the pharmaceutical formulations and biological fluids. Moreover, the presented data, which ascertained the higher sensitivity of the native technique with a detection limit of 230 pg/mL at a higher speed of analysis and without needing any pre-treatment, were in favor of our method over previously reported methods. Overall, the proposed green methods were validated according to ICH guidelines for the routine analysis of the studied drug with convenient precision and accuracy, conferring a strong candidate for replacing more elaborate procedures or more sophisticated and costly equipment.

CRediT authorship contribution statement

Youstina M. Metias: Conceptualization, Formal analysis, Methodology, Investigation, Validation, Writing – original draft. **Mervat M. Hosny:** Supervision, Writing – review & editing. **Magda M. Ayad:** Supervision. **Noritada Kaji:** Conceptualization, Resources, Supervision, Writing – review & editing.

Declaration of Competing Interest

The authors hereby declare no competing interests.

Acknowledgement

The authors acknowledge the financial support offered to Youstina

Mekhail Metias from the Ministry of Higher Education of the Arab Republic of Egypt (joint scholarship).

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.talo.2022.100139.

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