Received: 5 January 2022 | Revised: 18 February 2022 | Accepted: 23 March 2022

DOI: 10.1002/bio.4240

RESEARCH ARTICLE

LUMINESCENCE WILEY
The Journal of Biological and Chemical Luminescence

Quantitative analysis of favipiravir and hydroxychloroquine as FDA-approved drugs for treatment of COVID-19 using synchronous spectrofluorimetry: application to pharmaceutical formulations and biological fluids

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Funding information
Alexander von Humboldt foundation

Abstract

Coronavirus disease 2019 (COVID-19) is a contagious viral infection caused by coronavirus 2 (SARS-CoV-2) that causes severe acute respiratory syndrome. It has ravaged several countries and burdened many healthcare systems. As the process of authorizing a novel treatment for human use is extensive and involves multiple phases to obtain safety information and identify potential concerns. Therefore, the fastest and easiest choice was to use United States Food and Drug Administration (US FDA)-approved drugs such as favipiravir and hydroxychloroquine. For the simultaneous estimation of both medications, a simple synchronous spectrofluorimetric approach was established in which both drugs were measured at 372 and 323 nm, respectively in the presence of each other without interference at $\Delta\lambda$ 60 nm. The effect of various experimental conditions on synchronous fluorescence intensities were thoroughly investigated and optimized. The maximum synchronous fluorescence intensities were obtained at pH 5.4 using acetate buffer (0.2 M, 0.5 ml) and ethanol as a diluent. Excellent linearity ranges were obtained using 1.0–18.0 ng/ml and 10.0–120.0 ng/ml for favipiravir and hydroxychloroquine, respectively. The approach exhibited high sensitivity with detection limits down to 0.25 ng/ml and 1.52 ng/ml and quantitation limits down to 0.77 ng/ml and 4.62 ng/ml, respectively. Spiking human plasma samples with the studied drugs yielded high % recoveries, allowing a significant bioanalytical application. Moreover, the method was validated according to International Conference on Harmonization guidelines and further applied to commercial pharmaceutical preparations with good results.

KEYWORDS
biological fluids, COVID-19, favipiravir, hydroxychloroquine, synchronous fluorimetry

1 | INTRODUCTION

Coronavirus disease 2019 (COVID-19), a highly contagious viral illness caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), has had a serious disastrous effect on the world's demographics, actually resulting in more than 3.8 million deaths worldwide, making it the most significant global health crisis since the 1918 influenza pandemic. SARS-CoV-2 spreads quickly over the world after a first case of this primarily respiratory viral disease was identified in late December 2019 in Wuhan, Hubei Province, China.

SARS-CoV-2 quickly spread around the world, prompting the World Health Organization (WHO) to proclaim it a worldwide pandemic on 11 March 2020. COVID-19 has decimated numerous countries and overloaded many healthcare systems. Long-term shutdowns caused by the pandemic have led to the loss of livelihoods, which has had a ripple effect on the worldwide economy.[1] The rapid spread and devastating effects of COVID-19 prompted researchers throughout the world to discover antiviral drugs that could stop the virus from spreading and help patients recover faster.[2,3] Because the process for approving a novel drug for human use is lengthy and involves numerous phases to collect safety data and identify potential hazards, the simplest and fastest option was to use United States Food and Drug Administration (US FDA)-approved drugs such as favipiravir (FPV), remdesivir, lopinavir/ritonavir, azithromycin, chloroquine and hydroxychloroquine (HCQ).[4]

Favipiravir (Figure 1a) is a pyrazine carboxamide derivative (6-fluoro-3-hydroxy-2-pyrazine carboxamide) and purine nucleic acid analogue that is incorporated in place of guanine or adenine and thereby affects viral replication by inhibiting RNA-dependent RNA polymerase (RdRp).[4–6] FPV is given in the form of a prodrug. It has a high bioavailability (94%) and protein binding (54%) as well as a low distribution volume (10–20 L). After a single dose, it reaches C_{max} after 2 h. After multiple doses, both T_{max} and half-life increase. It has a short half-life (2.5–5 h), resulting in fast renal clearance in the

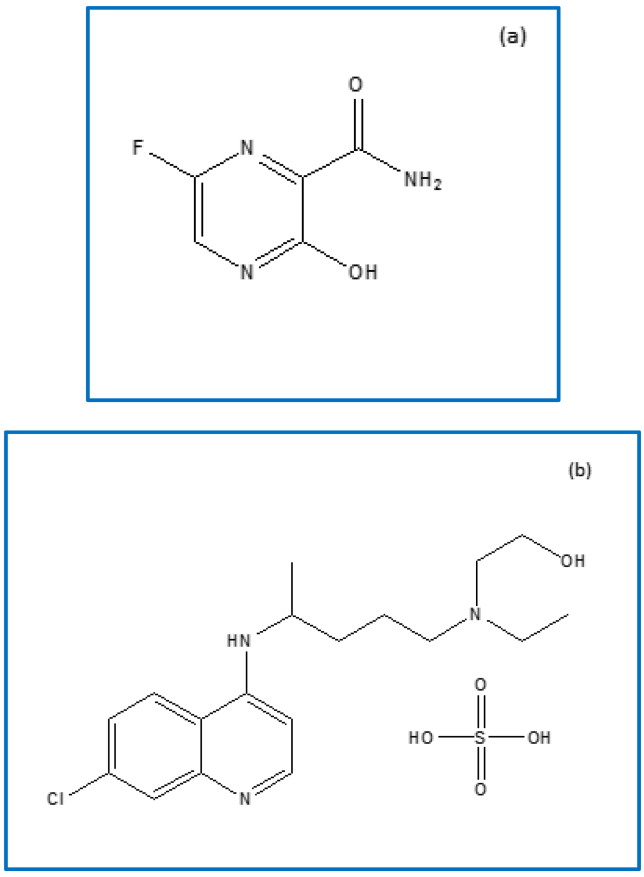


FIGURE 1 Chemical structure of (a) favipiravir and (b) hydroxychloroquine sulphate

hydroxylated form.[7] Different clinical studies have been conducted to determine the efficacy of FPV in the treatment of corona virus infections, and it was revealed that FPV increased viral clearance and improved chest computed tomography (CT) scans.[2,8] FPV was lately indicated for the treatment of COVID-19 infections in various countries, including Russia, Egypt, KSA, UAE, Italy, India, Japan, and Turkey.[9,10] Literature review reveals few reported methods for analysis of FPV such as spectrophotometry,[11] spectrofluorimetry,[3,12] liquid chromatography,[3,13–16] and electrochemical methods.[17,18]

Hydroxychloroquine sulphate (HCQ) (Figure 1b) has a long history of use in the prevention and treatment of malaria, as well as in the treatment of chronic inflammatory illnesses such as sarcoidosis, rheumatoid arthritis, and systemic lupus erythematosus.[19] HCQ is a derivative of chloroquine and was synthesized to impart high water solubility, lower adverse effects, drug–drug interactions, and toxicity when compared with its parent compound.[20] According to a literature review, HCQ can suppress coronavirus multiplication and fusion to the cell membrane.[21,22] In viral cells, HCQ works by preventing glycosylation of host receptors, proteolytic processing, and endosomal acidification. In addition, HCQ inhibits the synthesis of cytokines and reduces autophagy and lysosomal activity in host cells, all of which have immunomodulatory effects.[23,24] In general, hyphenated and electroanalytical techniques which including spectrophotometry,[25] high performance liquid chromatography,[26–29] gas chromatography mass spectrometry[30] potentiometry,[31] voltammetry,[32,33] are used to qualify and quantify HCQ. Both FPV and HCQ are reported in many guidelines for the treatment of COVID-19.[4] As a result, the purpose of this research was to measure both FPV and HCQ spectofluorimetrically in the biological fluids at the same time to monitor their therapeutic drug effects.

Emission spectrofluorimetry is affordable technology with good sensitivity and selectivity, allowing it to be used for quantitative investigation of a wide range of pharmaceutical substances. Selectivity issues might develop, however, when determining multicomponent materials with overlapped and broad bands emission spectra at the same time.[34] As a result, typical emission spectrofluorimetry may necessitate lengthy preseparation procedures before analysis. Other approaches, such as synchronous fluorescence spectroscopy (SFS) and/or derivative SFS, which entail careful adjustment of the experimental parameters, permit the simultaneous identification of these combinations by eliminating such spectrum overlap without the need for separation operations.[35,36] SFS is critical in the investigation of mixtures with overlapped spectra. Rather than scanning one of the excitation or emission wavelengths at a time, it scans both at the same time throughout the measurement. SFS outperforms traditional fluorescence spectroscopy in terms of sensitivity and selectivity, time and effort. Furthermore, it is capable of removing interference caused by Rayleigh scattering.[37] It also can be used to quantitatively determine several medicines in a single run due to its small, precise, and sharp spectrum.[36,38–41]

To date, no method for the simultaneous quantification of FPV and HCQ as co-administered drugs in biological fluids has been documented. This necessitates the establishment of a responsive

procedure for simultaneous estimation of both drugs for their therapeutic drug monitoring. So, our goal was to develop a modern sensitive, delicate, perceptive, and environmentally friendly procedure for simultaneous quantification of the two medications in their tablets and in biological fluids. It also took into account the requirement to employ a green methodology that was scientifically validated to remove the use of harmful toxic materials and solvents, while remaining environmentally friendly. The established SFS method allowed us to detect the substances indicated above with greater sensitivity and precision while causing the least amount of environmental damage. Furthermore, because it uses a process that is common in most research, the strategy is easy and inexpensive.

2 | EXPERIMENTAL

2.1 | Apparatus

- All of the experiment characteristics were carried out with an Agilent Cary Eclipse fluorescence spectrophotometer and an operating data system from Cary software. The Agilent voltage was set to 800 V with a smoothing factor of 20. The obtained data were saved as Asci. format and then the figures were generated using Microsoft Excel (2010).
- A Consort NV P-901 Belgium pH meter was calibrated and subsequently used for adjusting pH of studied buffer solutions.
- For biological samples, a Vortex IVM-300p from Gemmy Industrial Corporation in Taiwan was utilized for mixing, while the centrifugal force of Germany's version 2-16P was used for detachment.

2.2 | Materials and reagents

- Favipiravir pure material was generously donated by a national Pharmaceutical Company (EIPICo., 10th of Ramadan, Egypt).
- Hydroxychloroquine sulphate pure material was generously donated by EVA Pharma Co., Cairo, Egypt.
- Avipiravir® tablets; batch # 2107233 (200 mg FPV/tablet), product of EVA Pharma Co., Cairo, Egypt.
- Hydroquine® tablets; batch # LCE1042, (200 mg HCQ/tablet) product of Minapharm Pharmaceuticals, Heliopolis, Cairo, Egypt.
- Plaquenil® tablets; batch # BEG004, (200 mg HCQ/tablet) product of Sanofi Aventis, Cairo, Egypt.
- Hydroxytoid® tablets; batch # 2101204, (200 mg HCQ/tablet) product of EVA Pharma Co., Cairo, Egypt.
- Futarhomal® tablets; batch # 210115A, (200 mg HCQ/tablet) product of Future Pharmaceutical Industries (FPI), 1st Industrial Zone, Badr City, Cairo, Egypt.
- All pharmaceuticals were purchased from the local market.
- Organic solvents including acetonitrile, methanol, ethanol, n-propanol and acetone were all of high performance liquid chromatography (HPLC) grades and purchased from Fisher Scientific distributer in Egypt. Cetrimide, sodium dodecyl sulphate, carboxy methyl cellulose, Tween-80 and β-cyclodextrin were purchased from Sigma-Aldrich, Germany. Sodium acetate trihydrate, acetic acid 96.0%, sodium hydroxide, and boric acid were purchased from El-Nasr Pharmaceutical Chemicals, Cairo, Egypt.
- Plasma samples were purchased from the National Egyptian Blood Bank and stored in the refrigerator at −20°C until used after a mild thaw.

2.3 | Standard stock solutions of FPV and HCQ

FPV and HCQ stock solutions of concentration (100.0 μg/ml) were prepared separately in a 100 ml volumetric flask by dissolving 0.01g of each drug in methanol. For subsequent use, the stock solutions were stored at 4°C in the refrigerator.

2.4 | Working solutions of the studied drugs

Working solutions of concentration (1.0 μg/ml) of FPV and (5.0 μg/ml) of HCQ were prepared separately by transferring 1.0 ml and 5.0 ml, respectively from their standard stock solutions (100 μg/ml) to 100 ml volumetric flask, then completing to the final volume with methanol. The working solutions were stored in the refrigerator at 4°C for later use.

2.5 | Procedures

2.5.1 | Construction of calibration graphs

Within the limits of the concentration ranges listed in Table 1, separate aliquots of FPV and HCQ working standard solutions were transferred into two series of 10-ml volumetric flasks. To each flask, 0.5 ml of 0.2 M acetate buffer (pH 5.4) was added then completed to marked volume with ethanol. SFS measurements were acted upon scanning both excitation and emission monochromators, concurrently. Each measurement was performed with a constant wavelength difference (Δλ) of 60 nm. A blank experiment was carried out at the same time and the results were utilized to determine the relative synchronous fluorescence intensity (RSFI) for each drug. To create the

TABLE 1 Analytical performance data for the proposed method

Validation parameter	FPV	HCQ
Wavelength difference (Δλ)	60 nm	
Linearity range (ng/ml)	1.0–18.0	10.0–120.0
Intercept (a)	−10.995	8.447
Slope (b)	52.196	7.625
Correlation coefficient (r)	0.9999	0.9999
S.D. of residuals ($S_{y/x}$)	6.21	4.90
S.D. of intercept (S_a)	4.01	3.52
S.D. of slope (S_b)	0.37	0.05
Limit of detection, LOD (ng/ml)	0.25	1.52
Limit of quantitation, LOQ (ng/ml)	0.77	4.62

calibration plots, the correlative regression equations were calculated by plotting RSFI against the ultimate drug concentration in ng/ml.

2.5.2 | Analysis of FPV/HCQ in laboratory prepared mixtures

To produce four synthetic combinations within each drug linear range, various aliquots of each FPV and HCQ working standard solution were transferred into a set of 10.0 ml volumetric flasks. The flasks were completed to volume with ethanol after adding 0.5 ml of acetate buffer (0.2 M, pH 5.4). The procedure was then followed as instructed in Section 2.5.1.

2.5.3 | Analysis of FPV in Avipiravir® tablets

Ten tablets of Avipiravir® were weighed, finely ground, and thoroughly mixed. An accurately weighed quantity of the powdered tablets equivalent to 10.0 mg of FPV was transferred into a 100-ml volumetric flask and then 70 ml of methanol was added and sonicated for 30 min. The resulting solution was filtered into another 100-ml volumetric flask and completed to the mark with methanol to produce solution of final concentration of 100 μg/ml, then serially diluted to reach the working concentration range. The procedure was processed as described under Section 2.5.1.

2.5.4 | Analysis of HCQ in their tablets

Ten tablets of each of Plaquenil®, Futarhomal®, Hydroxytoid® and Hydroquin® containing (200 mg HCQ/tablet) were separately weighed, and finely crushed. A quantity of finely pulverized tablets powder equivalent to 10.0 mg of HCQ was transferred separately into a 100-ml volumetric flask, then dissolved in 60 ml of methanol and sonicated for 30 min. The resulting solution was filtered into a 100-ml volumetric flask and completed to the mark with methanol to produce solution of final concentration of 100.0 μg/ml, then serially diluted to reach the working concentration range. The procedure was completed as described under Section 2.5.1.

The nominal concentrations of FPV and HCQ in their medical dosage forms were then calculated using the correlative regression equation derivatization method.

2.5.5 | Assessment of FPV and HCQ in spiked human plasma samples

The concurrent detection of FPV and HCQ in spiked human plasma was conducted to estimate their curative amounts. A 1000 μl aliquot of plasma was transferred independently into a set of 15.0 ml screw-capped plastic centrifugation tubes. Aliquots from FPV and HCQ stock solutions were added to each tube to reach final concentrations of 2.0–10.0 and 20.0–100.0 ng/ml for both drugs, respectively. To allow entire separation of the medications from the plasma material, acetonitrile was added to each tube up to 5 ml. Then each tube was vortex blended for 1 min and centrifuged for another 30 min at 3600 rpm. The upper clear layer was filtered using a 0.45-μm syringe filter. Quantitatively, 1000 μl aliquots of the filtrate were transferred into a set of 10 ml volumetric flasks, 0.5 ml of acetate buffer (0.2 M, pH 5.4) was added and diluted to the volume with ethanol. A blank experiment was performed at the same time. After then, the process for plotting the calibration graph was used. The % recoveries of each drug were calculated using the corresponding regression equation.

3 | RESULTS AND DISCUSSION

A detailed review of the literature revealed that no methods for determining FPV and HCQ in pharmaceutical preparations were yet reported. As a result, the demonstration of an environmentally friendly, simple, and sensitive fluorimetric technique was critical for

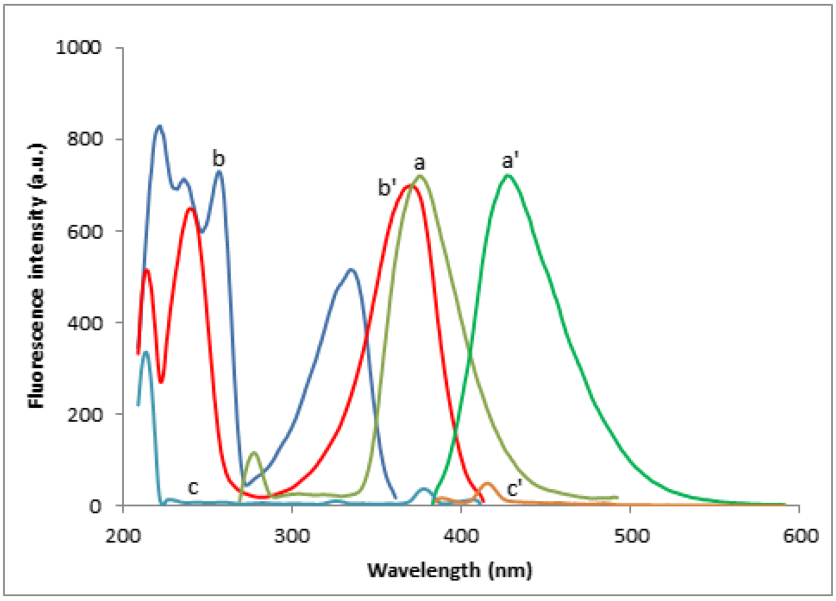


FIGURE 2 Excitation and emission fluorescence spectra of FPV (20.0 ng/ml) (a, a′), HCQ (100.0 ng/ml) (b, b′) in ethanol (c, c′)

the determination of the drugs studied in pharmaceuticals and spiked human blood plasma. As shown in Figure 2, the spectra of FPV and HCQ shows maximum emission in ethanol at 427 or 375 nm following excitation at 370 or 256 nm, respectively. Therefore, the SFS method was an excellent option for analyzing this binary combination in a single iteration with minimal interference. This technique had major aspects, including superior selectivity and sensitivity, diminished light scattering, and spectral simplification.[37,42] The SFS findings revealed a high degree of tolerance for exogenous compounds, especially when estimating studied components in complex biological matrices and pharmaceuticals. As a result, using constant wavelength synchronous spectrofluorimetry (Δλ), an ecofriendly and easy SFS approach for concomitant assessment of FPV and HCQ was established for the first time in a single run without any preprocessing procedures. To establish the best Δλ for resolution of such a mixture, a wide variety of Δλ in the range (20–200 nm) was investigated. It was found that 60 nm was the best one to achieve the resolved spectra for each drug without interference from the other as shown in Figure 3.

Figure 4 depicts the SF spectra of varying concentrations of FPV at 372 nm in the presence of a given concentration of HCQ (60.0 ng/ml), whereas (Figure 5) depicts the SF spectra of varying concentrations of HCQ at 323 nm in the presence of a given concentration of FVP (10.0 ng/ml).

3.1 | Investigation and optimization of process variables

Various factors influencing the fluorescence intensity of the two drugs, were properly examined:

3.1.1 | Impact of diluent

The influence of diluent on fluorescence value of the studied medications was tested using distilled water, acetone, acetonitrile, methanol, and ethanol.

For FPV, distilled water produced the highest fluorescence value followed by ethanol and methanol, respectively, whereas acetonitrile resulted in very low fluorescence intensity compared with other studied diluents. For HCQ, distilled water produced very low fluorescence readings compared with ethanol and methanol as the optimum organic solvents. As a consequence, ethanol was identified as the primary diluent for this approach in terms of sensitivity and environmental aspects.

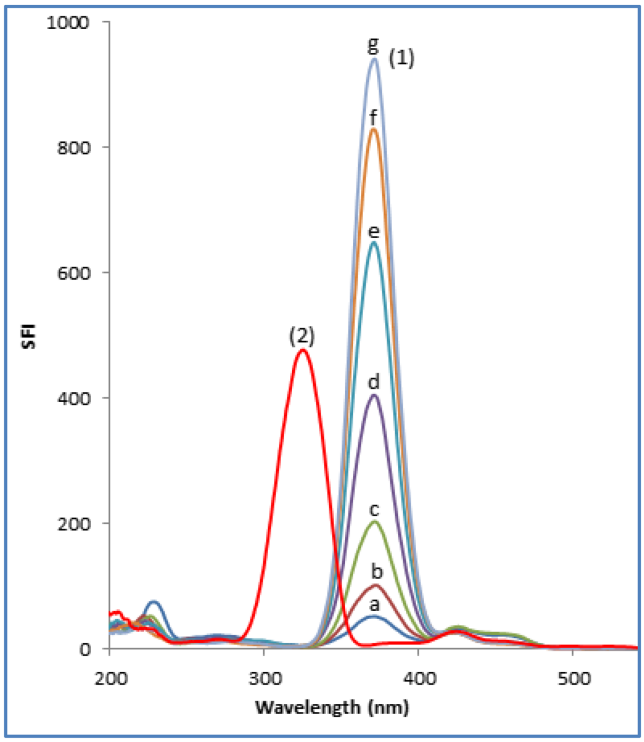


FIGURE 4 Synchronous fluorescence spectra at Δλ = 60 nm of (1) FPV (a–g; 1.0, 2.0, 4.0, 8.0, 12.0, 16.0 and 18.0 ng/ml) in the presence of (2) HCQ (60.0 ng/ml)

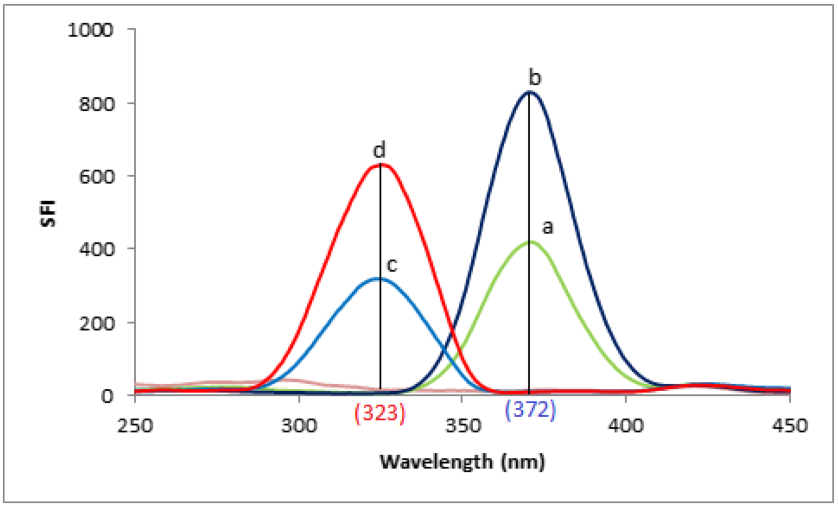


FIGURE 3 Synchronous fluorescence spectra of the studied drugs at Δλ = 60 nm. Where (a, b): 8.0 and 16.0 ng/ml of FPV, (c, d): 40.0 and 80.0 ng/ml of HCQ

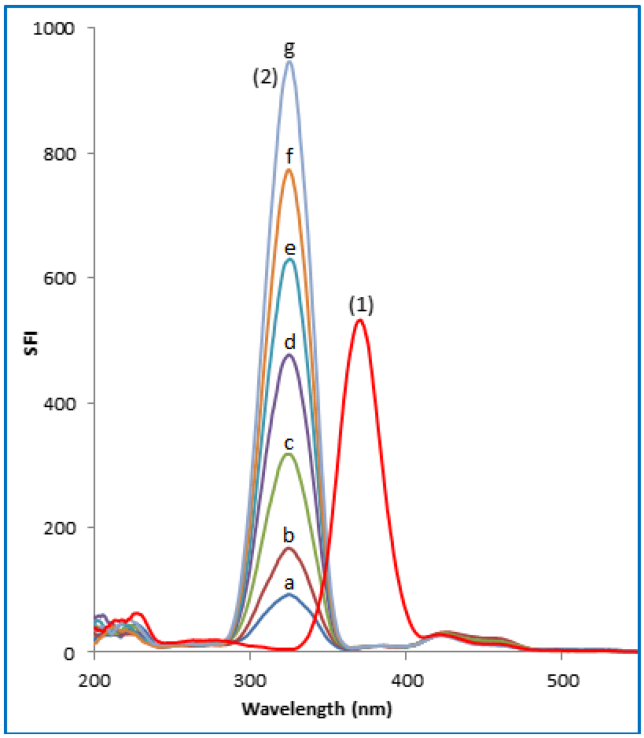


FIGURE 5 Synchronous fluorescence spectra at $\Delta\lambda = 60$ nm of (1) FPV (10.0 ng/ml) and (2) HCQ (a-g; 10.0, 20.0, 40.0, 60.0, 80.0, 100.0 and 120.0 ng/ml)

3.1.2 | pH of different buffer solutions and its influence on the fluorescence value of the drugs being studied

The buffer solutions were prepared according to USP,[43] in which acetate and borate buffers of concentration of 0.2 M were tested with pH ranges 3.6–5.6 and 6.0–10.0, respectively. Following multiple runs, it was ascertained that the maximum fluorescence value for each of FPV and HCQ was achieved at pH 5.4 and with 0.2 M acetate buffer. The pH values that were either extremely acidic or basic did not lead to considerable enhancement in fluorescence signals.

As a consequence, several volumes of acetate buffer (0.2–2.5 ml) were investigated. It was found that the highest sensitivity was obtained upon using 0.5–1.5 ml of 0.2 M acetate buffer. But unfortunately, increasing volumes of buffer results in increasing overlapping between the two spectra and affects resolution. Therefore, 0.5 ml of acetate buffer was utilized throughout the investigation to compensate sensitivity and resolution.

3.1.3 | The effect of surfactants and macromolecules

Several surfactants at concentrations higher than respective critical micelle concentrations were investigated for their potential improvement of the fluorescence values of the mentioned drugs.[44] Surfactants such as cetrimide, sodium dodecyl sulphate, and Tween-80, as well as macromolecules such as carboxy methyl cellulose and β-cyclodextrin, are among the organized media examined. None of the specified surfactants and macromolecules resulted in a substantial improvement in the fluorescence signal of the cited medications in studied approach.

3.1.4 | Study of $\Delta\lambda$ and their effect on resolution of FPV and HCQ spectra

In the interval between 20 and 200 nm, several $\Delta\lambda$ settings were thoroughly examined. FPV seemed to have the highest intensity at $\Delta\lambda = 60$ nm, which is equivalent to the difference between its excitation and emission wavelength,[45] whereas HCQ had highest fluorescence intensity at $\Delta\lambda = 40$ nm. However, SF spectra for the two studied medications were obtained in a single iteration with adequate sensitivity using $\Delta\lambda$ value of 60 nm, which was determined to be suitable and provided a legitimate response for both compounds. At 372 nm for FPV and 323 nm for HCQ, the two drugs could be determined without interference in the presence of each other because of the well resolved spectra produced (Figure 3).

3.2 | Method validation

The rules of the International Conference on Harmonization (ICH) Q2 (R1) were used to determine whether or not the suggested strategy was validated and approved.[46]

A linear correlation between RSFI and concentration was recorded between (1.0–18.0 ng/ml) for FVP and (10.0–120.0 ng/ml) for HCQ at 372.0 and 323.0 nm, respectively. The characteristics listed below are required for a straightforward relationship:

$$\mathrm{RSFI}_{(\mathrm{FPV})} = 52.196\,\mathrm{C} - 10.995\,(r = 0.9999)\ \text{at}\ 372.0\,\mathrm{nm}$$

$$\mathrm{RSFI}_{(\mathrm{HCQ})} = 7.625\,\mathrm{C} + 8.447\,(r = 0.9999)\ \text{at}\ 323.0\,\mathrm{nm}$$

where RSFI stands for relative synchronous fluorescence intensity, C stands for concentration of the drug in ng/ml and r stands for correlation coefficient.

Conferring to statistical analysis data,[47] the calibration graphs are suitably linear as demonstrated in Table 1. For both FPV and HCQ, the corresponding limit of detection (LOD) and limit of quantitation (LOQ) values were calculated and summarized in Table 1. In terms of sensitivity, the LOD and LOQ values revealed that the proposed methodologies can analyze the aforementioned pharmaceuticals with great sensitivity down to nanogram levels, and therefore may be effectively used for their biological applications. To evaluate whether the suggested method was accurate or not, the results obtained of proposed approach were compared with those obtained by another published methods.[3,25] Statistical analysis of data[47] revealed no significant differences between both techniques as demonstrated in Table 2. The present work also has been evaluated in terms of

TABLE 2 Application of the proposed method for the determination of the studied drugs in their raw materials

Parameters	FPV			HCQ		
	Amount taken (ng/ml)	Amount found (ng/ml)	% found[a]	Amount taken (ng/ml)	Amount found (ng/ml)	% found[a]
	1.0	1.019	101.90	10.0	10.130	101.30
	2.0	1.970	98.50	20.0	19.883	99.42
	4.0	3.923	98.08	40.0	39.944	99.86
	8.0	8.008	100.10	60.0	60.266	100.44
	12.0	12.227	101.89	80.0	80.203	100.25
	16.0	15.881	99.26	100.0	98.842	98.84
	18.0	17.970	99.83	120.0	120.725	100.60
Mean ± SD			99.94 ± 1.51			100.10 ± 0.81
	Comparison method ($n = 3$) [3]			Comparison method (n = 3) [25]		
Mean ± SD	100.28 ± 1.34			99.48 ± 1.15		
t[b]	0.34 (2.31)			0.93 (2.31)		
F[b]	1.27 (19.30)			2.02 (5.79)		

[a]Average of three replicate determinations.
[b]The values between parentheses are the tabulated values of t and F at $P = 0.05$.[47]

TABLE 3 Interday and intraday precision data for the studied drugs using the proposed method

Concentration (ng/ml)		Intraday precision			Interday precision		
		Mean ± SD	% RSD	% error	Mean ± SD	% RSD	% error
FPV	5.0	99.92 ± 0.38	0.38	0.22	100.95 ± 1.18	1.17	0.68
	10.0	99.40 ± 0.68	0.68	0.39	100.93 ± 1.01	1.00	0.58
	15.0	99.93 ± 1.24	1.24	0.72	100.29 ± 1.68	1.68	0.97
HCQ	20.0	100.63 ± 1.54	1.53	0.88	101.40 ± 0.37	0.36	0.21
	40.0	101.81 ± 0.45	0.44	0.25	101.19 ± 1.21	1.20	0.69
	80.0	101.02 ± 0.91	0.90	0.52	100.67 ± 1.65	1.64	0.95

intraday and interday precision (Table 3). The % RSD of three concentrations inside the calibration curve for each sample were found to be small, confirming the repeatability and intermediate exactness of the suggested technique. The robustness of the suggested technique was verified by assessing the effect of small deliberate changes in variable parameters involved, such as buffer pH (5.4 ± 0.1) and buffer volume (0.5 ± 0.1) on the obtained RSFI. It was found that, RSFI of FPV and HCQ were not affected by slight intended changes in pH and volume (% RSD < 2%). Furthermore, the selectivity of the novel method was demonstrated by estimating the FPV and HCQ in pharmaceutical formulation as well as in complex matrices of human plasma samples. The suggested approach was shown to have sufficient selectivity to analyze the stated medications with good percentage recoveries and percentage RSD (2%), indicating and confirming the absence of interference from common excipients in pharmaceutical formulation. As a result, their findings supported strong selectivity of contemporaneous assessment in human plasma without any intervention from endogenous matrices.

3.3 | Applications

3.3.1 | Analysis of FPV/HCQ in laboratory prepared mixtures

The anticipated approach was used to estimate prepared mixtures with various FPV and HCQ ratios as shown in Figure 6. The concentrations of the specified drugs in their combinations were estimated using the associated regression equations. As shown in Table 4, the acquired results demonstrated the method's accuracy.

3.3.2 | Analysis of FPV and HCQ in their pharmaceutical preparations

The designated approach was successfully used to estimate FPV in Avipiravir® and HCQ in Plaquenil®, Futarhomal®, Hydroxytoid® and Hydroquin® tablets without any intrusion from co-formulated

excipients. The variance ratio *F*-test and Student's *t*-test[47] were used to compare the proposed and comparison methods,[3,25] and no significant differences in precision and accuracy were found, as shown in Table 5.

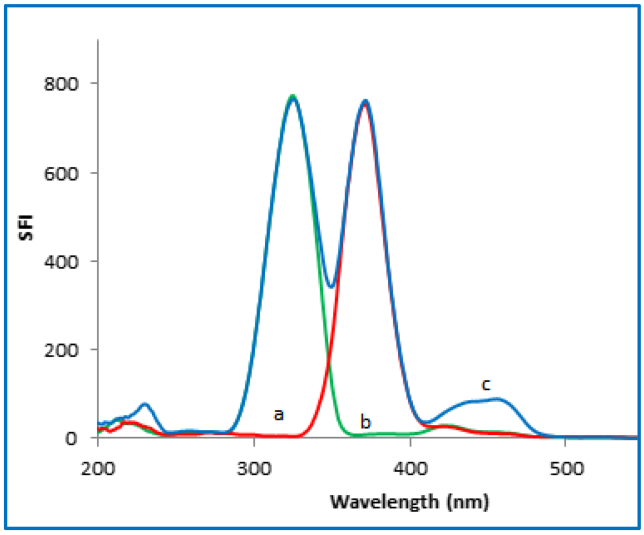


FIGURE 6 Synchronous fluorescence spectra of: (a) 14.0 ng/ml FPV, (b) 100.0 ng/ml HCQ, and (c) synthetic mixture of both

3.3.3 | Analysis of FPV and HCQ in spiked human plasma

FPV is given in the form of a prodrug. It has a high bioavailability (94%) and protein binding (54%) as well as a limited volume of distribution (10–20 L).[10] In most regions, including India, the recommended dose is 1800 mg bid on day 1, continued by 800 mg bid on days 2–14.[10] Peak plasma concentration was 4.43 μg/ml.[48] HCQ has a bioavailability of 67–74% and there was no substantial difference in bioavailability between two enantiometric form (R and S).[49] Peak plasma concentration of HCQ was 129.6 ng/ml in the blood and 50.3 ng/ml in the plasma after a 200 mg oral dosage.[50] In COVID 19, HCQ was administered at a dose of 400 mg twice daily.[51] Therefore, it was possible to estimate FPV and HCQ in biological fluids due to the great sensitivity of designed approach as demonstrated in Figure 7. The lower detection limit of the designated approached down to 0.25 ng/ml and 1.52 ng/ml for FPV and HCQ, respectively, allowed for biological detection of both drugs. A linear correlation was established when the RSFI value was plotted versus ultimate drug concentrations (ng/ml) in spiked samples (Table 6). The developed methods were assumed to have high percentage recoveries and low % RSD values, indicating that they might be utilized to accurately quantify the previously mentioned drugs in spiked plasma samples.

TABLE 4 Assay results for the determination of FPV and HCQ in synthetic mixtures using the proposed method

Mix no.	Amount taken (ng/ml) FPV	Amount taken (ng/ml) HCQ	Amount found (ng/ml) FPV	Amount found (ng/ml) HCQ	% found[a] FPV	% found[a] HCQ
1	10.0	10.0	9.890	9.825	98.90	98.25
2	5.0	10.0	5.016	9.975	100.32	99.75
3	8.0	40.0	8.052	39.211	100.65	98.03
4	14.0	100.0	14.136	98.612	100.97	98.61
Mean					100.21	98.66
± SD					0.91	0.76
% RSD					0.91	0.77
% Error					0.46	0.39

[a]Mean of three determinations.

TABLE 5 Determination of FPV and HCQ in pharmaceutical preparations using the proposed and comparison methods

Pharmaceutical formulations	Parameters				
	Mean±%RSD	N	Comparison methods[3,25]	*t*-test (2.78)*	*F*-value (19.00)*
Avipiravir® tablets (200 mg FPV/tablet)	100.84 ± 0.63	3.00	99.84 ± 1.20	1.20	3.63
Plaquenil® tablets (200 mg HCQ/tablet)	98.36 ± 0.53		99.31 ± 0.97	1.36	3.35
Futarhomal® tablets (200 mg HCQ/tab.)	99.01 ± 1.10		99.77 ± 1.32	0.78	1.44
Hydroxytoid® tablets (200 mg HCQ/tab.)	99.29 ± 1.55		99.68 ± 0.63	0.39	6.05
Hydroquin® tablets (200 mg HCQ/tab.)	97.81 ± 0.54		99.23 ± 0.9	2.12	2.72

*The values between parentheses are the tabulated values of *t* and *F* at $P = 0.05$.[47]

3.3.4 | Assessment of greenness of the proposed method

In recent years, determining whether or not a certain analytical approach is environmentally friendly has become a worldwide issue. Different metrics were included, namely the National Environmental Method Index (NEMI), which is regarded as the earliest and most easily decipherable approach in this discipline, but it has some drawbacks, such as being labour intensive, not representative for quantitation, and requiring a considerable amount of time and effort to prepare its circular pictogram.[52] Simply, this pictogram is divided into four portions, each of which is tinted white or green relying on whether the requirement is accomplished or not. The following is a list of the four criteria: the pH range utilized ought to be between 2 and 12; the utilized reagent should not be included in the list of persisting, bioaccumulative, and deleterious[53] or toxic waste[54]; and ultimately, the cumulative waste volume should not exceed 50 g or L. Our methods meet all of the requirements for being designated as a green method by referring to the aforementioned criteria (Table 7). Another novel technique known as the Green Analytical Procedure Index (GAPI) has successfully been applied.[55] This technique is recognized as one of the most recent and excellent recommendations for evaluating greenness, as it overcomes the problems encountered by the previous techniques. The GAPI method utilizes a pictorial graph to categorize the greenness of every step of an analytical technique, using a chromaticity scale: red, yellow, and green. When GAPI was applied to the suggested spectrofluorimetric techniques, it was observed that the majority of GAPI's requirements were met. Fields 1 and 15 are both red, indicating that they pertain to off-line sampling and trash that has not been handled, respectively. Field 10 is yellow coloured related to the National Fire Protection Association (NFPA) and hazard of solvent. Field 14 is similarly yellow due to the 10 ml trash created for each sample. Additionally, GAPI has been applied to the method used for the estimation of the drugs in spiked plasma samples. It was found that micro-extraction with simple treatments were followed (yellow colour) but using acetonitrile as nongreen solvent (red colour). The ultimate point drawn from this graphic depiction is that the techniques, to a large extent, conformed to GAPI's greenness criteria, due to their low burden on human and environmental health (Table 7). Lately, the AGREE evaluation method was developed.[56] Built on the 12 principles of Green Analytical Chemistry,[57] AGREE generates a clock-shaped chart with a circumference segmented into 12 sections. Each section is represented by a single criterion on a colour scale (red-yellow-green) to assess the analytical procedure's compliance with the Green Analytical Chemistry standards. The AGREE chart's heart has an overall evaluation colour as well as an overall assessment score on a scale from 0 to 1 (Table 7). As a result, a sustainable and ecofriendly SFS method for investigating FPV and HCQ using ethanol as a green solvent was developed.[58–60] The method was applied for determination of the studied drugs in commercially available tablets and human plasma samples with minimum laboratory needs.

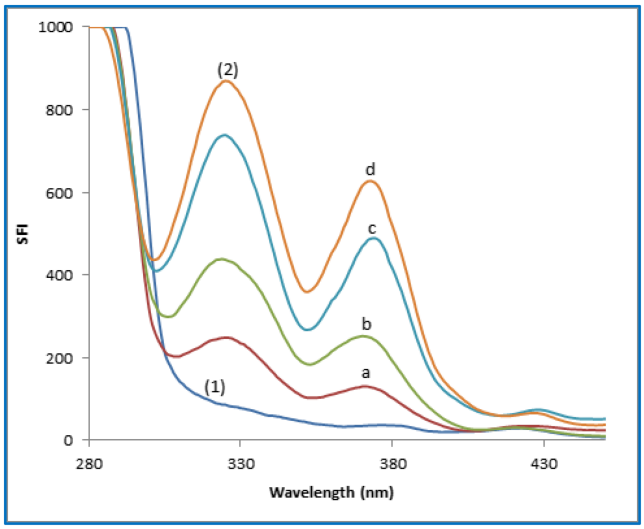


FIGURE 7 Application of drugs in spiked human plasma where: (1) blank plasma; (2) (a–d) concentration of drugs spiked in plasma samples (2.0 + 20.0 ng/ml), (4.0 + 40.0 ng/ml), (8.0 + 80.0 ng/ml) and (10.0 + 100.0 ng/ml) for FPV and HCQ, respectively

TABLE 6 Application of the proposed spectrofluorimetric method to the determination of the studied drugs in spiked human plasma

Parameters	FPV			HCQ		
	Amount taken (ng/ml)	Amount found (ng/ml)	% recovery	Amount taken (ng/ml)	Amount found (ng/ml)	% recovery
	2.0	2.036	101.80	20.0	19.087	95.44
	4.0	4.011	100.28	40.0	40.852	102.13
	8.0	7.851	98.14	80.0	81.273	101.59
	10.0	10.108	101.08	100.0	98.820	98.82
Mean ± SD			100.33 ± 1.58			99.50 ± 3.07
r	0.9996			0.9994		
Regression equation	y = 61.55 x – 29.80			y = 7.77 x + 13.30		

TABLE 7 Results for evaluation of the greenness of the developed method by different green analytical chemistry metric tools

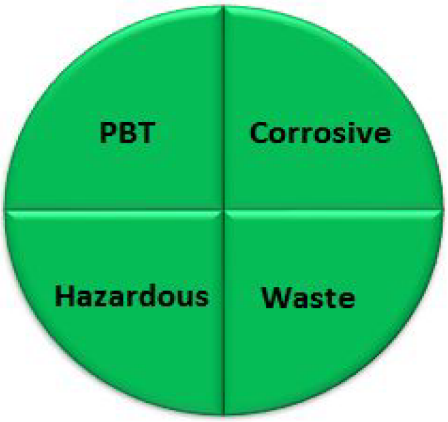
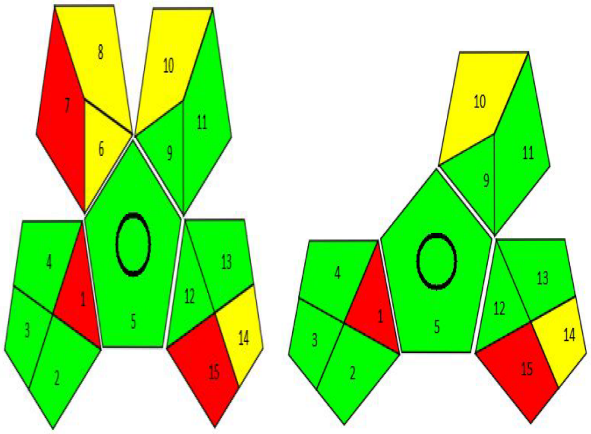
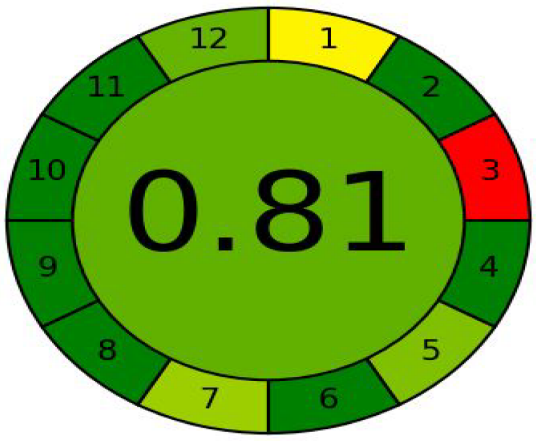
(1) NEMI pictogram	(2) Green Analytical Procedure Index (GAPI)
	
(3) AGREE Assessment.	
	

TABLE 8 Superiority of the proposed method over the other reported methods

Method	FAV		
	Linearity	Calculated LOD	Ref
Proposed method	1.0–18.0 ng/ml	0.25 ng/ml	
Spectrophotometry	2–10 μg/ml	0.095 μg/ml	[11]
Spectrofluorimetry	0.02–0.35 μg/ml	0.004 μg/ml	[3]
	40–280 ng/ml	9.44 ng/ml	[12]
HPLC-UV	10–100 μg/ml	0.985 μg/ml	[3]
	10–100 μg/ml	1.20 μg/ml	[13]
LC/MS	0.048–50 μg/ml	0.059 μg/ml and 0.045 μg/ml for positive and negative modes, respectively	[16]
Electrochemical methods	–	0.0028 μg/ml and 0.023 μg/ml	[17]
	9.0×10^{-9}	1.0×10^{-8}_5.5×10^{-5} M	[18]

TABLE 8 (Continued)

Method	HCQ		
	Linearity	Calculated LOD	Ref
Proposed method	10.0–120.0 ng/ml	1.52 ng/ml	
Spectrophotometry	1–20 μg/ml	–	[25]
Spectrofluorimetry			
HPLC-UV	1–300 μg L^{-1}	0.2 μg L^{-1}	[26]
	50–4000 ng/ml	–	[27]
LC/MS	5–2000 ng/ml	2 ng/ml	[28]
	8.3–6075 ng/ml	–	[29]
Electrochemical methods	–	5.0×10^{-6} mol L^{-1}	[31]
	2×10^{-5}_ 5×10^{-4} mol L^{-1}	11.2 ± 2.6 μg/ml	[32]

3.3.5 | Superiority of the proposed method over the other reported methods

Despite the fact that spectrophotometric methods are often used in routine work analysis because they are quick, simple, and inexpensive, they have the limitation of low sensitivity. However the chromatographic techniques are more frequent in pharmaceutical quality control and research laboratories, they are considered as a sophisticated technique due to the need for expensive column and detector, as well as the use of toxic organic solvent which has a negative effect on the environment. Therefore, the primary benefit of the developed spectrofluorimetric approach is energy saving, as the spectrofluorometer uses the least amount of energy (less than 0.1 kWh) compared with HPLC (~1.5 kWh).[61] Moreover, the method has high sensitivity (nanoscale), which is similar to ultra HPLC (UHPLC) techniques combined with MS/MS detection, but it is considerably simpler, greener (as assessed by AGREE and GAPI), cheaper, and time-saving as demonstrated in (Table 8).

4 | CONCLUSION

Within the most protocol, favipiravir and hydroxychloroquine are the regularly prescribed medications used to treat infection caused by COVID-19. The quantitative evaluation of a very small amount of FPV and HCQ in their pharmaceutical formulations was carried out using synchronous spectrofluorimetric approach. The sensitivity of the suggested procedure permits the synchronized estimation of both drugs in spiked human plasma with high percentage recovery (95.44–102.13%). Furthermore, the existing method was an environmentally friendly, easy, and cost-effective procedure that does not require sophisticated tools. Furthermore, the established methodology allows the analysis of the mentioned medications in a short period of time, with minimal sample processing, and with a wide linearity ranges 1.0–18.0 ng/ml and 10.0–120.0 ng/ml for FPV and HCQ, respectively. Using NEMI, GAPI, and AGREE principles, the approaches' greenness was successfully assessed. The new procedure was fully evaluated in conformity with ICH standards.

ACKNOWLEDGEMENTS

The authors would like to thank the Alexander von Humboldt foundation, Bonn, Germany for donating the instrument used in this study (spectrofluorometer) to one of the authors (FB).

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

ETHICS STATEMENT

This article does not include any studies on human participants or animals carried out by any of the authors.

INFORMED CONSENT

Informed consent is not applicable in this study.

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How to cite this article: M. E. El Sharkasy, M. M. Tolba, F. Belal, M. Walash, R. Aboshabana, *Luminescence* **2022**, *37*(6), 953. https://doi.org/10.1002/bio.4240