

A Study of the Interaction of Ixabepilone As Anticancer Drug With Acetoxymurcuric Fluorescein Reagent by Fluorescence Quenching Approach: A Validated Method

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Abstract

A spectrofluorimetric approach has been developed and validated for determination of sulfur-containing drug; ixabepilone in raw powder, vials and human plasma. This approach studies the quenching effect of IXA on the fluorescence intensity of acetoxymymercuric fluorescein (AMF) reagent at λ_{em} of 530 nm and λ_{ex} of 500 nm. All the parameters that can affect the reaction as pH, AMF solution concentration, temperature, time and solvents were studied and optimized. The linearity range of the studied approach was 20-100 ng mL⁻¹ with correlation coefficient of ($r = 0.9998$). The proposed approach was validated and approved regarding to ICH guidelines in terms of accuracy, precision, linearity, LOD and LOQ, with mean percentage recovery of 99.79 and RSE% of 1.64. The previously obtained results were already statistically compared with that of established reported methods indicating no significant differences in accuracy and precision. Finally, the proposed approach is easy, sensitive, and inexpensive so it is suitable for routine determination of IXA in raw powder, vials and human plasma with no need for any prior separation or sample extraction.

Introduction

Ixabepilone (IXA) is (1S,3S,7S,10R,11S,12S,16R)-7,11-dihydroxy 8, 8, 10, 12, 16-pentamethyl-3-[(E)-1-(2-methyl-1,3-thiazol-4-yl)prop-1-en-2-yl]-17-oxa-4-azabicyclo [14.1.0] heptadecane-5,9-dione [1] (Figure 1) is an orally bioavailable semisynthetic analogue of epothilone B with antineoplastic activity, a natural chemical compound produced by *Sorangium cellulosum* [2]. Epothilone B itself might not be created as a pharmaceutical drug since of low metabolic stability and pharmacokinetics [3]. The epothilones parallel taxanes in that they connect to β -tubulin and trigger microtubule nucleation at numerous spots farther from the centriole. This chaotic microtubule stabilization triggers cell-cycle capture at the G2-M interface and apoptosis. Epothilones connect to a location definite from that of taxanes. In colon cancer cell lines, p53 and Bax trigger apoptosis in ixabepilone-treated cells. In vitro application, advise that ixabepilone is less inclined to P-glycoprotein-mediated multidrug resistance when compared to taxanes. Other instrument involved in epothilone resistance incorporate mutation of the β -tubulin active site of binding and upregulation of isoforms of β -tubulin [4]. Ixabepilone was constructed through medicinal chemistry advanced upon these properconnects [3]. It is very potent, able of harming cancer cells in exceptionally low concentrations, and holds action in cases where tumor cells are heartless to taxanes brand drugs [5]. As with the taxanes and other agents that target tubulin, the epothilones, counting ixabepilone, connect to the β -tubulin subunits of microtubules to initiate microtubule polymerization and stabilization, which lead to capture of cells within the G2-M stage of the cell cycle and the initiation of apoptosis (Figure 2). A lack of chemical methods deduced for determining of IXA, rather than LC [6-8], appeared in the literature as enlisted in this review.

Non-fluorescent compounds holding sulphide or sulphydryl moieties, were determined quantitatively with acetoxymymercuric fluorescein (AMF), a mercuric acetate substituted fluorescein; which consider a widely used fluorescent agent, depending on the reaction of Hg²⁺ incorporated in (AMF) with the sulfur containing groups in the analyzed compounds (Figure 3) [9], this reaction decreases the intensity of the (AMF)fluorescence that measured quantitatively with the tested compounds [10-12]. Many compounds successfully determined quantitatively using this method such as mesna, acetylcysteine, timonacac corrosive [13], penicillamine [14] and mirabegron [15]. In this study, the reaction of IXB with its sulfide group with AMF and the quenching effect on the fluorescence were measured spectrofluorimetrically at (λ_{em} 530 nm) [9]. It is worth to mention that there is no publication conducted for the IXA assay spectrofluorimetrically either in bulk, dosages forms or human biological fluids.

This work aimed to construct a spectrofluorimetric method privileged with validity, sensitivity, simplicity and reliability along with the advantages of being costly effective and rapid when compared with other widely used techniques, for the purpose of quantitative determination of IXA in bulk, pharmaceutical dosages forms or human biological fluids.

In spite of the non-existence of a procedure conducted for the assay of IXA spectrofluorimetrically until now, more improvements needed eagerly to attain more suitable conditions and better analytical performance.

Experimental

2.1 Instrumentation

All the measurements were carried out on Agilent Cary Overshadow Fluorescence Spectrofluorimeter (USA); prepared with a 150 W xenon streak light and 1 cm quartz cell were utilized. The excitation and emission opening width was 10 nm, worked with Cary overshadow check application program adaptation 1.2. pH estimations were made with HANNA pH 211 Chip pH Meter with two-fold intersection glass anode. Digital pH meter 3310 Jenway.

2.2 Materials and reagents

Ixabepilone (IXA) was gifted from Bristol-Myers Squibb (USA, Akhenaton office (Egypt)). Acetoxymymercuric fluorescein (AMF), 1×10^{-4} M solution was made by dissolving 82.3 mg of AMF raw material in 20 mL of 0.1 N NaOH, weakened with 100 mL of 0.1 M boric acid solution and the volume was completed to 1.0 L utilizing refined water [9], and the solution is suggested to be kept secured from light in fridge. Britton Robinson buffer utilized in optimization trials was made by infusing match volumes of boric acid (0.1 M), phosphoric acid (0.1 M) and acetic acid (0.1 M) in a 100 mL volumetric flask at that point the pH was adapted within the wanted area (5-9) by including acceptable volumes of sodium hydroxide (0.1 N) [16]. Methanol, ethanol, isopropanol, chloroform and dimethylformamide (DMF) solvents were acquired from El-Nasr Co. Egypt. All reagents and solvents utilized were of analytical grade. A fresh arranged bi-distilled water was utilized through all tests. Ixempra® vials 45 mg per vials (Batch no. 69019) is a brand of Bristol-Myers Squibb (USA, Akhenaton office (Egypt)). Plasma was achieved from Minia University Hospital, blood bank, Minia, Egypt and were kept solidified until utilize after delicate defrosting.

2.3 Preparation of standard stock solution

Standard stock solution of IXA (0.25 mg mL^{-1}) was prepared by dissolving 0.025 of powder in 100 mL methanol and kept in fridge.

2.4 Spectrofluorimetric procedure and construction of the calibrated curve

The proposed approach was practiced beneath the optimized conditions that will be examined afterward. Precisely measured volumes of the stock solution were relocated into a set of 10-mL volumetric flasks to achieve a IXA concentration area of 20-100 ng mL^{-1} followed by the inclusion of 1.0 mL of 1 AMF. The solutions were blended well applying a vortex and left to stand at room temperature for 10 min. Each flask was weakened quantitatively with methanol. The fluorescence intensity was detected at λ_{em} of 530 nm after excitation at λ_{ex} 500 nm. At that point the fluorescence change was determined by subtracting the fluorescence intensity of the reaction admixtures from the comparing values of so also treated blank (a solution contains 1.0 mL of AMF reagent and weakened with methanol). A calibration curve detailing the fluorescence contrasts at λ_{em} 530 nm to the comparing drug concentrations in ng mL^{-1} was developed.

2.5 Application procedures

2.5.1 Procedure for pharmaceutical preparation

Ixempra ® vials: (45 mg per vial). An aliquot of 1 mL from the blended substance of Ixempra ® vials was precisely relocated to to a 100 mL volumetric flask and broken down in methanol, then the volume was completed to the line with methanol. 0.5 mL of this solution was weakened with methanol to earn an eventual IXA working solution concentration, then the approach was completed as already explained.

2.5.2 Procedures for spiked human plasma

One-milliliter aliquots of plasma were delocated into two solution of centrifuge tubes. The plasma were spiked with 0.1, 0.2 and 0.3 mL from 12.5 mg% stock solution of IXA. The tubes were blended well by employing a vortex blender. The solutions were deproteinized twice with acetonitrile. The centrifugation was done for 15 min at 8000 rpm. The centrifugates were delocated to new and clean centrifuge tubes then vaporized. The residues were transformed in to methanol and delocated to 5 mL volumetric flasks and the volumes were adapted to the line with the same solvent. Aliquots of 2 mL from each solution were delocated to a 25 mL volumetric flask, the required volumes of buffer and AMF were taken and the volume was completed to the line with methanol. The relative fluorescence intensities were measured utilizing the previous cited fluorescence approach and subtracted from the comparing resultes of an essentially treated blank.

Results And Discussion

A reaction named the Wronski reaction [18] which describe the complexometric reaction occurred between a mercuriated compounds and the sulfur containing compounds is happened here between a mercuriated derivative of fluorescein (AMF) (a reagent with green fluorescence) [17], and mercury complexing agents such as sulfides, arising in quenching of its fluorescence. Upon the reaction is occurred, AMF is changed over to weak fluorescent ones. This is due to the alteration within the chromophore structure of the reagent particle. For encourage clarification of the reaction mechanisms, it was presumed that anions which can shape stable Hg^{2+} complexes would replace the acetoxy moiety in AMF to make a solid chelate with Hg^{2+} cation [17]. The proposed pathway is shown in scheme 1. Figure 4 appears the fluorescence quenching of the reagent within the nearness of IXA. The quenching pathway was examined by developing Stern-Volmer plot. It is a plot that appears a connection between (I_0/I) and the quencher concentration. A linear curve was achieved upon plotting (I_0/I) against concentration of the drug which demonstrates either inactive or energetic quenching happens in an inactive mechanism, as the quencher got to be a portion of the complex shaped amid the chemical reaction agreeing to (Eq. (1)) which speaks to a ground-state quenching model [19, 20]. This association constant K_a was determined and it is 0.1079. $I_0/I = 1 + K_a[Q]$ (I_0 is the fluorescence intensity of AMF in nonattendance of quencher whereas I is its fluorescence intensity in nearness of the quencher. K_a is the association constant and $[Q]$ is concentration of the quencher (drug) [19].

3.1 The stoichiometry of the reaction

The Continuous Variation Method (Job's Approach) [20] has been generally utilized with isomolar solutions to examine the complexation cases in these solutions and to decide the transcendent complexes of the reaction. It was accepted in this work to examine the reaction stoichiometry between IXA and AMF. Iso-molar concentrations of IXA and AMF (1×10^{-4} M) solutions were arranged. Precisely measured various volumes from (1×10^{-4} M) stocks of each IXA and AMF were included together into a set of test tubes in numerous proportions to get a volume of 5 mL. A connection between the achieved fluorescence difference and the proportion between the drug and the reagent was outlined in Job's plot (Figure. 5). It showed that 2.0 mol of IXA were required to full the quenching reaction of 1.0 mol of AMF, so the stoichiometric ratio between (drug: AMF) was (2:1), so it can be clarified by the trade of two acetoxy moieties in AMF by two moles of IXA [17] Scheme 1.

3.2 Optimization of the reaction parameters

Various parameters influencing the reaction were optimized to have the most sensitivity, counting concentration of AMF reagent solution, temperature, ideal pH, time and weakening solvents. The resultes of optimization of the reaction parameters are appeared in Tables 1 & 2.

3.2.1. AMF concentration

The impact of AMF solution concentration was considered utilizing various volumes (0.1–2 mL) of 1×10^{-4} M AMF to respond with a certain concentration of IXA in a solution of 10-mL volumetric flasks. The flasks' substance was blended and completed to the line with methanol and waiting for 10 min at room temperature. The fluorescence contrast was observed, at λ_{em} 530 nm, for each test solution against a fresh prepared blank solution for each estimation. The connection between AMF volume and the fluorescence contrast of the reaction blend was shown to in (Figure 6). It uncovered that; 1.0 ± 0.2 mL of 1×10^{-4} M AMF was appropriate for the proposed approach.

3.2.2. Temperature

The ideal temperature for total quenching was considered by warming the reaction blend at various temperatures (40–100 °C), and its impact on the fluorescence quenching is shown in (Figure 7). This appeared that, the greatest fluorescence quenching was achieved at room temperature, whereas it remained nearly consistent when the temperature was raised up to 60 °C, while diminished at temperatures over 60 °C and up to 100 °C. The diminish in fluorescence quenching at great temperature may be due to the separation of the shaped weak complexes that are greatly important for quenching the fluorescence [21].

3.2.3. pH

The pH plays a vital part within the sensitivity of this reaction. The impact of pH on quenching the fluorescence was examined in the pH area (5–9) utilizing the universal Britton Robinson buffer. The connection between various pH and comparing fluorescence contrast in (Figure 8) appeared that the most extreme sensitivity was achieved within the solution's pH 6.4. This data is due to the reality that at pH ranges from 6 to 7, AMF appeared exceptionally solid fluorescence. This could be due to the nearness of AMF as a doubly charged anion. It was moreover found that upon diminishing the pH underneath 6.0 or increasing it past 7.0, a drop within the fluorescence intensity of AMF happened leading to diminish within the predictable quenching by the addition of IXA.

3.2.4. The reaction time

The impact of time on the quenching of the fluorescence of AMF by IXA was considered by calculating the reactions each 5 min for 45 min, and it was shown in (Figure 9). The results shown that the overall reaction and consequently the greatest sensitivity was achieved after 10 ± 2 min, past which there were nearly slight changes within the measured fluorescence.

3.2.5. Weakening solvent

The impact of various weakening solvents was followed after the same approach. Various solvents of different polarities were attempted counting: chloroform, isopropanol, methanol, dimethylformamide (DMF) and refined water. It was found that the chief solvents to be utilized for achieving highest sensitivity at 530 nm was methanol. Typically due to the low energy gap among methanol vibrational energy levels related to water, so sensitivity in case of methanol is greater [22].

3.3 Validation of the proposed spectrofluorimetric method

The established method has been validated according to ICH guidelines [23]. All validation parameters are shown in Tables 3–5.

3.3.1. Linearity range

The linearity of the proposed approach was built up beneath the already optimized conditions employing a set of solutions of various concentrations. A calibration curve (Figure 10) was built to show the relationship of the fluorescence contrast between the signals of blank solutions of AMF and those achieved after reaction of IXA to the comparing drug concentrations in ng mL^{-1} which was found to be direct within the area of (20–100 ng mL^{-1}). Regression analysis was achieved by least squares analysis of the calibration results to determine the relation coefficient (r), slope (b), intercept (a), standard deviation of slope (S_b) and standard deviation of intercept (S_a) (Table 3), which confirmed acceptable linearity of the proposed approach as shown by the high relationship coefficient ($r > 0.9998$), % RSD of the slope ($S_b\% < 2\%$) and the small value of significance F that shown a small grade of empirical points diffusing around the regression line.

3.3.2. Limit of detection (LOD) and limit of quantitation (LOQ)

LOD is considered as the concentration which can be spoken to by $3 S/m$ and LOQ by $10 S/m$, where, S is the standard deviation and m is the slope of the calibration line. The values of LOD and LOQ displayed in (Table 3) affirmed the sensible sensitivity of the proposed approach in qualitative and quantitative analysis of IXA.

3.3.3. Accuracy and precision

To evaluate the reliability and repeatability of the proposed approach, the precision and accuracy of estimations have been assessed as beneath the main method. Three readings at each concentration level were done (Table 4). Recovery % and RSD % were determined for each level. The results were inside the satisfactory limits of 98–103% and 2% for recoveries and RSD% separately. The intra-day and inter-day precision were evaluated utilizing concentrations inside the linearity area, on the same day and on three distinctive days individually. The little RSD % shown the great precision of the proposed approach (Table 4) and affirmed the reliability of the approach for quality control tests of IXA.

3.3.4. Robustness

The already detailed approach was performed beneath little varieconnects within the optimized parameters such as volume of AMF solution (± 0.2 mL) and the reaction time (± 2 min). Low RSD% values appeared in (Table 5) affirmed that little varieties within the previously detailed had no critical impact on the analysis of IXA by the recommended approach.

3.4. Analytical applications

3.4.1. Pharmaceutical preparation

The proposed approach was practiced for the assurance of IXA in Ixempra® vials. The results achieved are appeared in (Table 6). Recovery was achieved by applying the standard addition technique where various concentrations of standard IXA solution (40-80 ng) were included to already analyzed Ixempra® vials. There was no obstructions from co-formulated excipients. Statistical analysis of the results achieved by the proposed approach and those achieved by the reported approach [6] was done utilizing the student's t-test and the variance ratio F-test (Table 7). The calculated values didn't pass the hypothetical ones showing no significant difference between the proposed approach and the reported one with respect to precision and accuracy.

3.4.2. In plasma

The sensitivity of the proposed spectrofluorimetric approach permitted the analysis of IXA drug in spiked human plasma. To defeat lattice interferences, tests were subjected to a clean-up method. In this regard, acetonitrile was utilized for protein precipitation. Three concentrations were spiked for the drug and spiked concentration was reproduced three times to affirm the accuracy and precision of the proposed approach. The recoveries were calculated and they were between 95–97% (Table 8). Appropriately, this work about spiked plasma tests propose that the proposed approach is performed for the in vivo test of the drug in real biological samples.

Conclusion

In this work an easy, reproducible, and fast spectrofluorimetric approach was created for the determination of IXA in bulk as well as in Ixempra® vials and human plasma. The approach depends on the measured fluorescence quenching of AMF due to the presence of the sulfide moiety in IXA. The approach was statistically validated with regard to precision, accuracy, linearity, LOD, LOQ and robustness. All parameters were established to be inside satisfactory limits. Linearity and area were found to be greatly specific as they gave satisfactory recoveries and the correlation coefficient (r) was 0.9998. In addition, it is sensibly delicate and reasonable for dependable investigation of low concentrations of the IXA. Both inter-day and intra-day precisions were considered. Results of this experiment were found to be inside satisfactory. Subsequently, the proposed spectrofluorimetric approach can be suggested to consider the pharmacokinetics of the drug in numerous preparations and combinations and human plasma.

Declarations

Author Declarations

- Ethics Approval and consent to participate

Collection of samples was authorized by Ethics Committee of Minia Hospital.

- Consent to Participate

Accept

- Availability of data and materials

Available upon request

- Competing interests

No conflict of interests

- Funding

No funding was received for this work from any.

- Author Contributions

All authors contributed to the study conception and design. Data collection and analysis were performed by all authors. The first draft of the manuscript was written by Hesham Salem and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

- Acknowledgements

Not applicable

- Authors information

- Compliance with Ethical Standards

- Disclosure of potential conflicts of interest

The authors declare that they have no conflict of interest.

- Research involving Human Participants and / or Animals

Not applicable

- Informed consent

Not applicable

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Tables

Table 1 Detailed data of the optimization of the reaction parameters.

AMF Con. ($\mu\text{g mL}^{-1}$)	Fluorescence Difference	pH	Fluorescence Difference	Time (min.)	Fluorescence Difference	Temp. ($^{\circ}\text{C}$)	Fluorescence Difference	Diluting solvent	Fluorescence Difference
0.1	65.689	5	18.6	0	207.987				
0.5	215.564	6	47.387	5	352.123			Water	220.699
1	240.30	6.4	366.211	10	357.254	25	270.689	Methanol	260.898
1.5	115.958	7	116.057	15	352.68	40	235.865	Chloroform	15.785
2	70.032	8	22.288	20	356.68	60	237.868	DMF	17.789
		9	27.998	25	355.964	80	188.547		
				30	351.871	100	59.98		
				35	355.329				
				40	351.985				
				45	354.259				

Mean of (n = 3) experiments for each parameter

Table 2 Assay parameters and conditions for determination of IXA by the proposed spectrofluorimetric method.

Parameters	Proposed method
AMF concentration	1x10 ⁻⁴ M
AMF volume	1.0 mL
Temperature	25 °C
Time	10 min
Diluting solvent	Methanol
pH	6.4

Table 3 Regression parameters and test results for the determination of IXA by the proposed spectrofluorimetric procedure.

Parameters	Spectral data
λ_{ex} & λ_{em} (nm)	500 & 530
Linearity range (ng mL ⁻¹)	20-100
LOD (ng mL ⁻¹)	5.145
LOQ (ng mL ⁻¹)	16.987
Slope \pm Sb	10.14 \pm 0.99
Intercept \pm Sb	-105.81 \pm 2.67
%RSD of Sb	1.39
Regression equation	Intensity 530 = 10.14 – 105.81
Significance F	5.84 x 10 ⁻⁶
Correlation coefficient (r)	0.9998

Table 4 Intra-day and inter-day accuracy and precision for the determination of IXA by the proposed spectrofluorimetric method.

IXA (ng mL ⁻¹)	Intra-day			Inter-day		
	Found \pm SD (ng mL ⁻¹)	Accuracy (%)	Precision (%RSD)	Found \pm SD (ng mL ⁻¹)	Accuracy (%)	Precision (%RSD)
40	40.58 \pm 1.06	101.45	1.045	40.82 \pm 0.75	102.05	0.735
60	59.66 \pm 0.99	99.43	0.996	59.38 \pm 0.88	98.96	0.889
80	79.23 \pm 0.89	99.04	0.899	80.63 \pm 0.69	100.79	0.685

Table 5 Robustness of the proposed method for the determination of IXA

Parameters	%RSD
AMF Volume (\pm 0.2 mL)	1.04
Reaction time (\pm 2 min)	0.84

Mean of % RSD

Table 6 Recovery of IXA by applying standard addition technique

Ixempra [®] vials (ng mL ⁻¹)	Drug added (ng mL ⁻¹)	Drug found (ng mL ⁻¹)	% Recovery
80	40	40.74	101.84
80	60	60.34	100.56
80	80	81.74	102.18
80	100	98.49	98.49
Mean			100.77
RSD %			1.85

Table 7 Statistical analysis of the results obtained by the proposed and reported procedures for the determination of IXA in Ixempra[®] vials

Parameters	Ixempra [®] vials	
	Proposed method	Reported method [6]
N ^a	5	5
Recovery %	101.62	100.94
SD	0.739	0.939
RSD%	0.716	0.937
t ^b (2.262)	1.27	
F value ^b (5.05)	1.615	

^a Number of experiments.

^b The values in parenthesis are tabulated of t and F at (P = 0.05)

Table 8 Statistical analysis of the results obtained by the proposed and reported procedures for the determination of IXA in human plasma

Parameters	IXA	
	Proposed method	Reported method [6]
N ^a	5	5
Recovery %	99.79	100.94
SD	1.597	0.939
RSD%	1.643	0.937
t ^b (2.262)	1.676	
F value ^b (5.05)	2.898	

^a Number of experiments.

^b The values in parenthesis are tabulated of t and F at (P = 0.05)

Figures

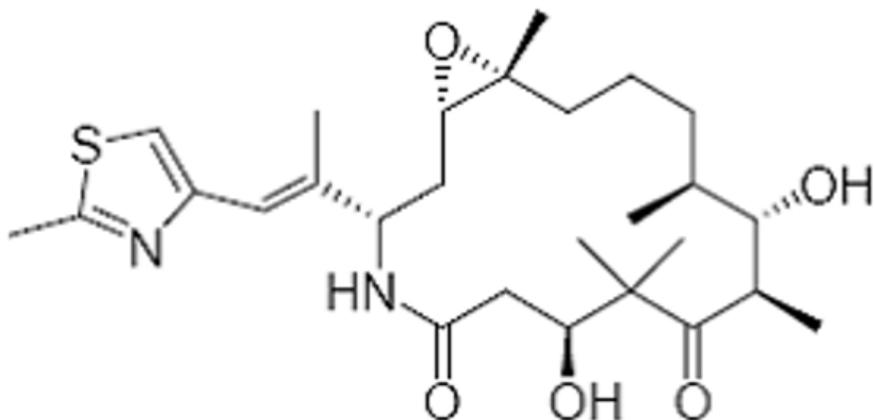


Figure 1

Chemical structure of ixabepilone

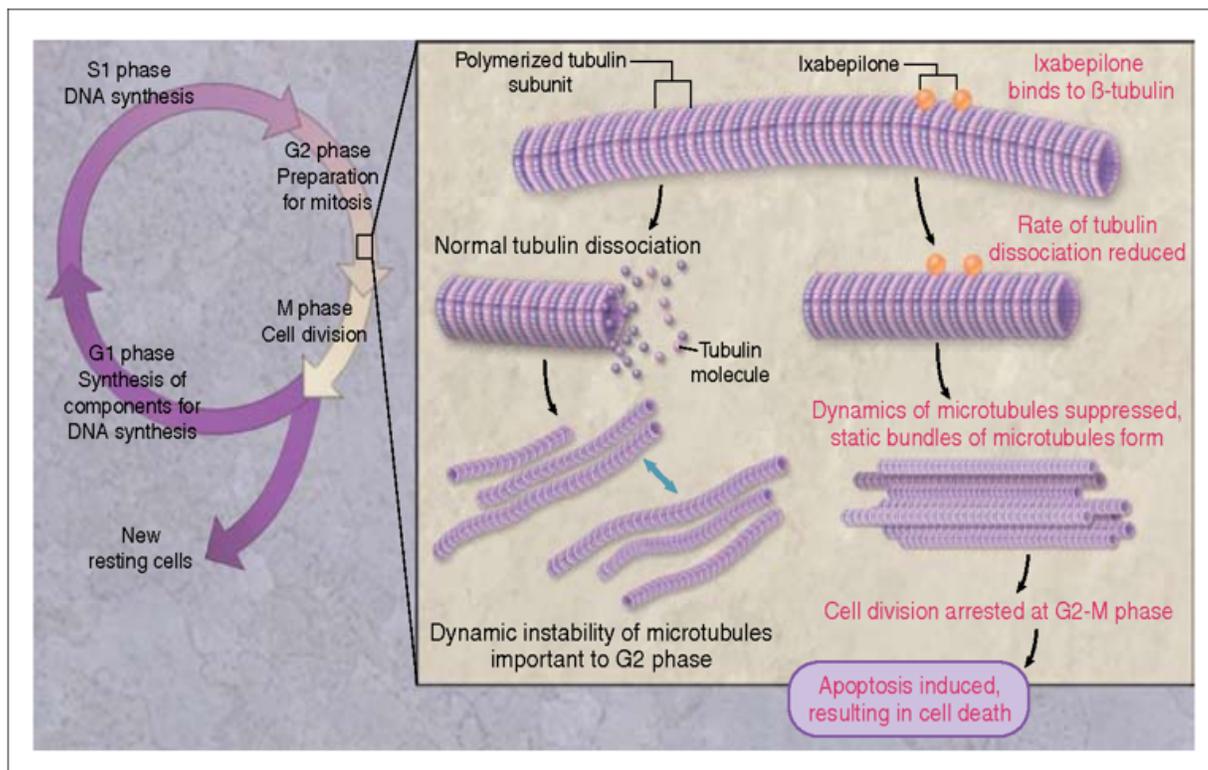


Figure 2

Mechanism of action of ixabepilone. Ixabepilone binds to the β -tubulin subunits of microtubules to induce microtubule polymerization and stabilization, which lead to G2-M arrest and the induction of apoptosis.

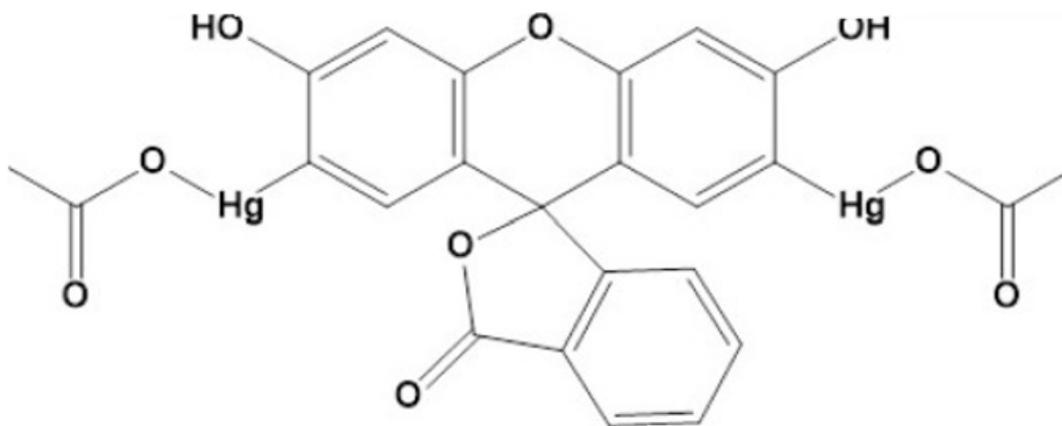


Figure 3

Chemical structure of Acetoxymercuric fluorescein reagent.

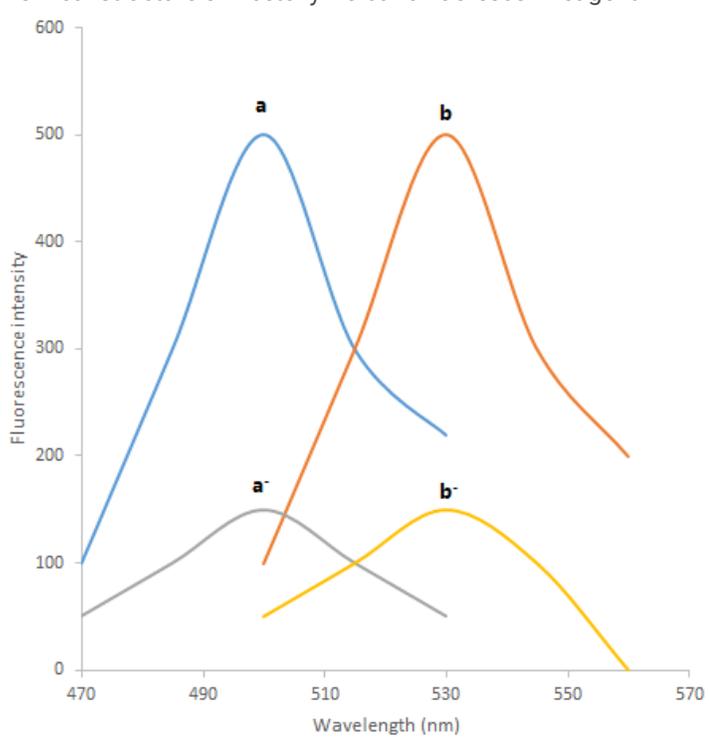


Figure 4

Excitation and emission spectra of 1.0 mL (10^{-4} M AMF solution) in the absence and presence of (120 ng mL^{-1}) IXA at 500 and 530 nm, respectively. a: Excitation blank of AMF, a-: Excitation of AMF+IXA, b: Emission blank of AMF and b-: Emission of AMF+IXA.

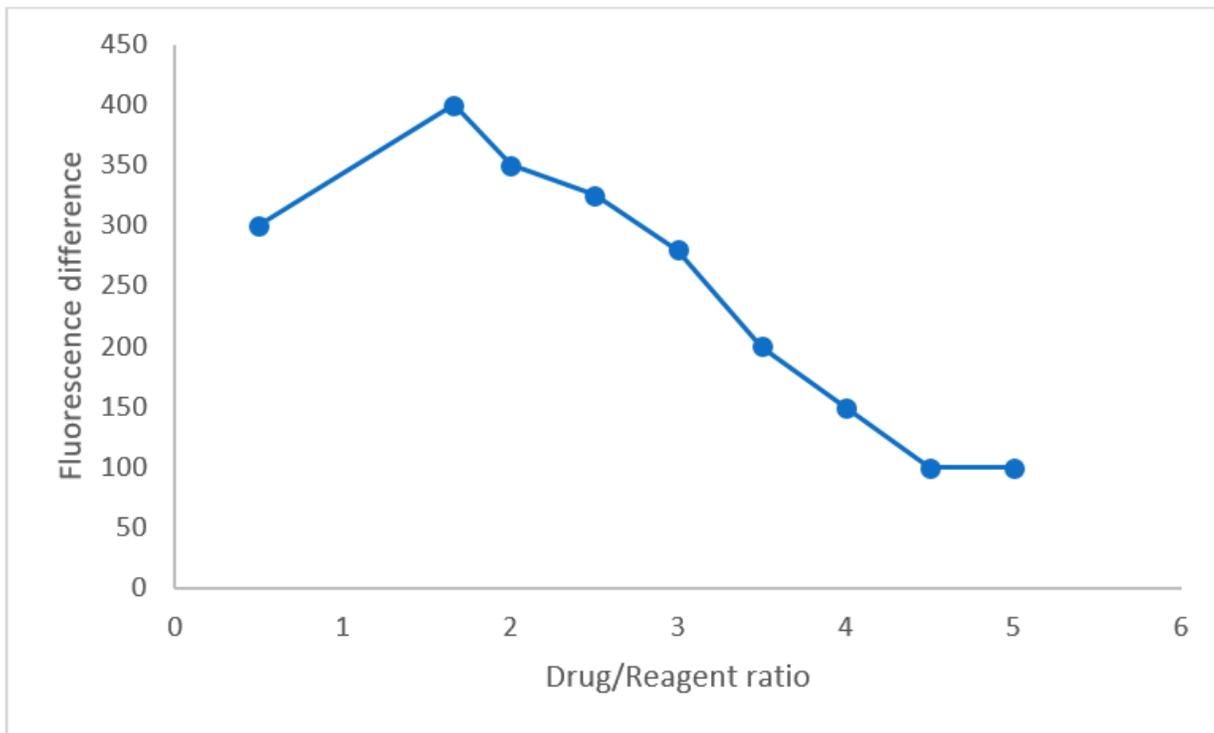


Figure 5

Stoichiometry of the reaction of IXA (1×10^{-4} M) and 1×10^{-4} M AMF by continuous variation (Job,s) method.

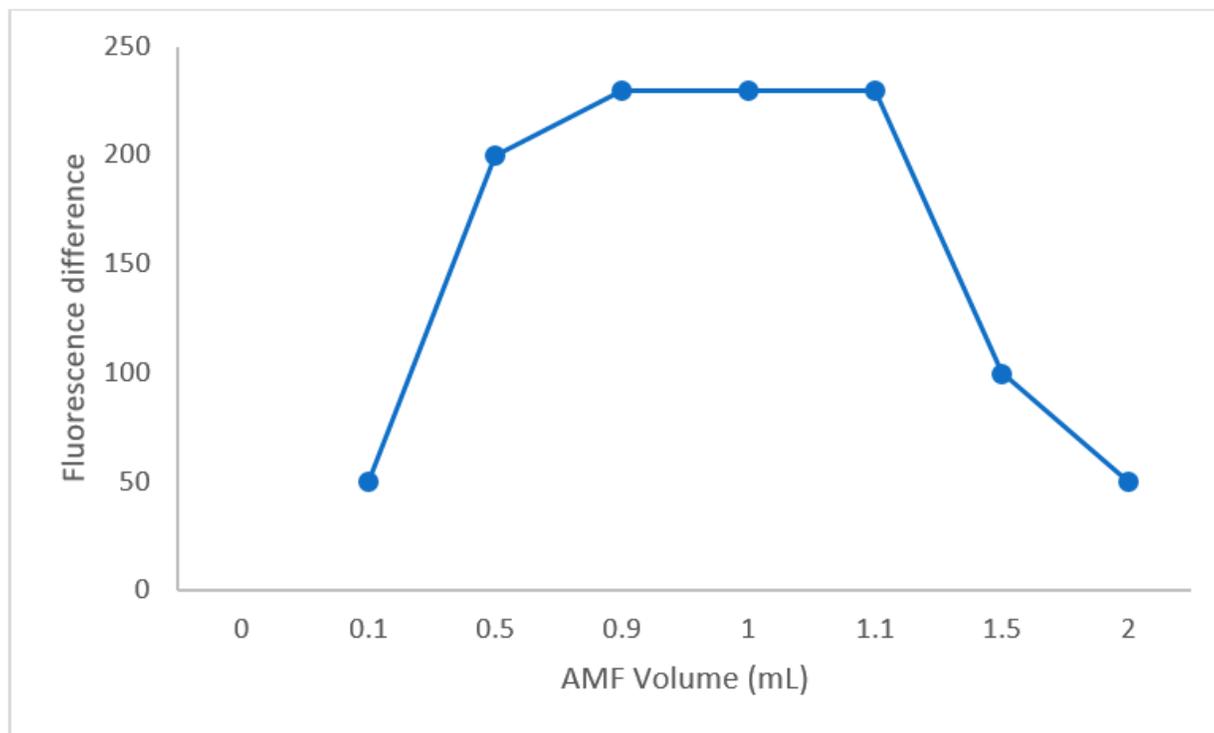


Figure 6

Effect of AMF volume on the fluorescence difference, after the reaction with 60 ng mL^{-1} IXA at 530 nm.

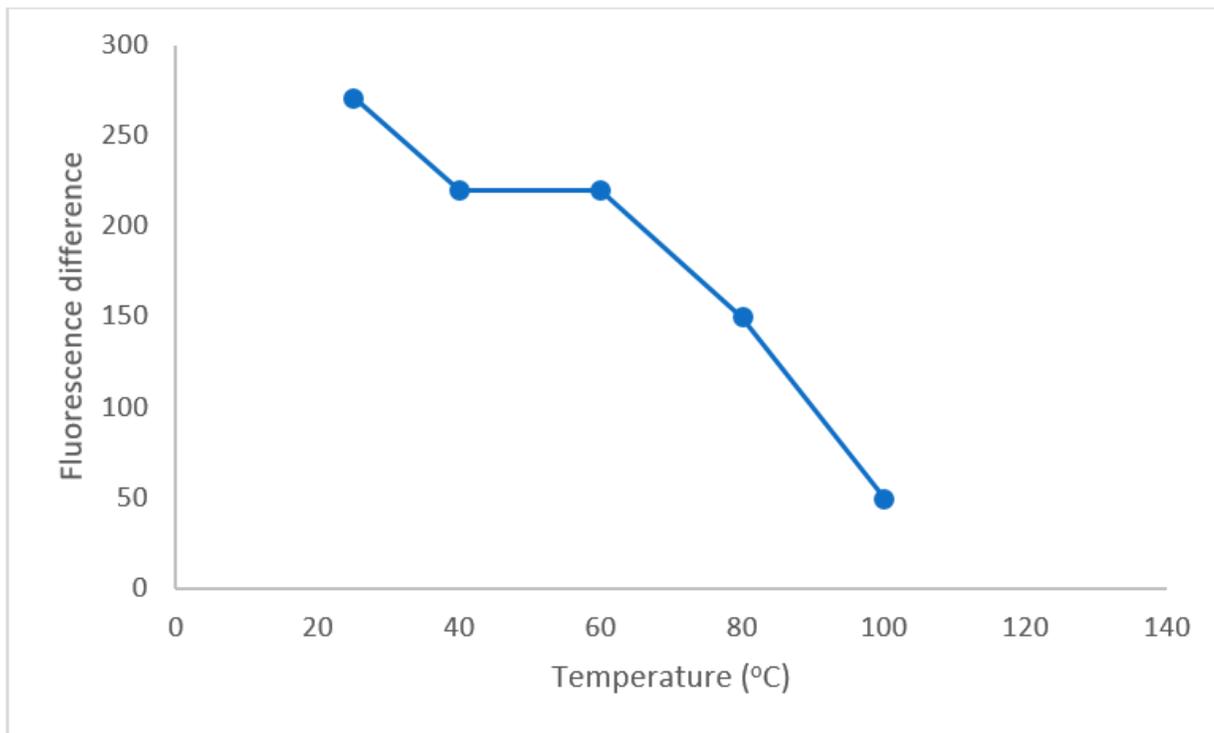


Figure 7

Effect of temperature on the fluorescence quenching 1 mL AMF after the reaction with 60 ng mL⁻¹ IXA at 530 nm.

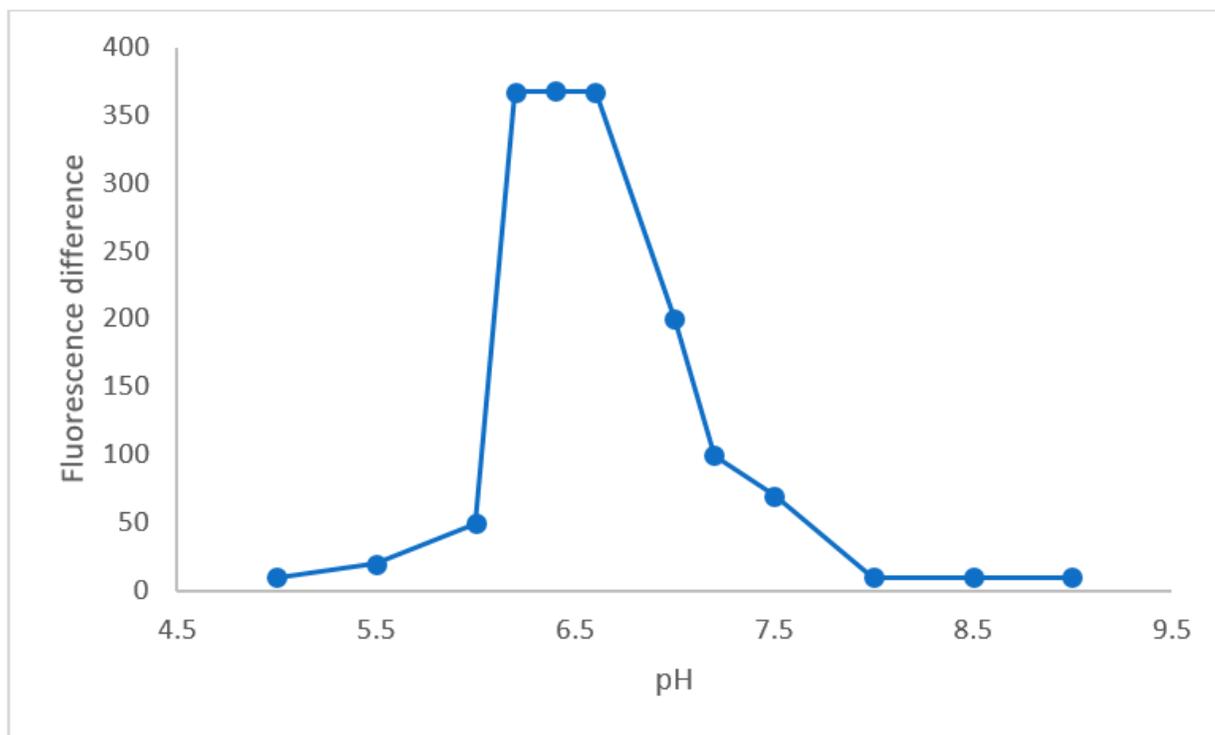


Figure 8

Effect of medium pH on the fluorescence quenching of 1 mL AMF after reaction with 60 nm mL⁻¹ IXA at 530 nm.

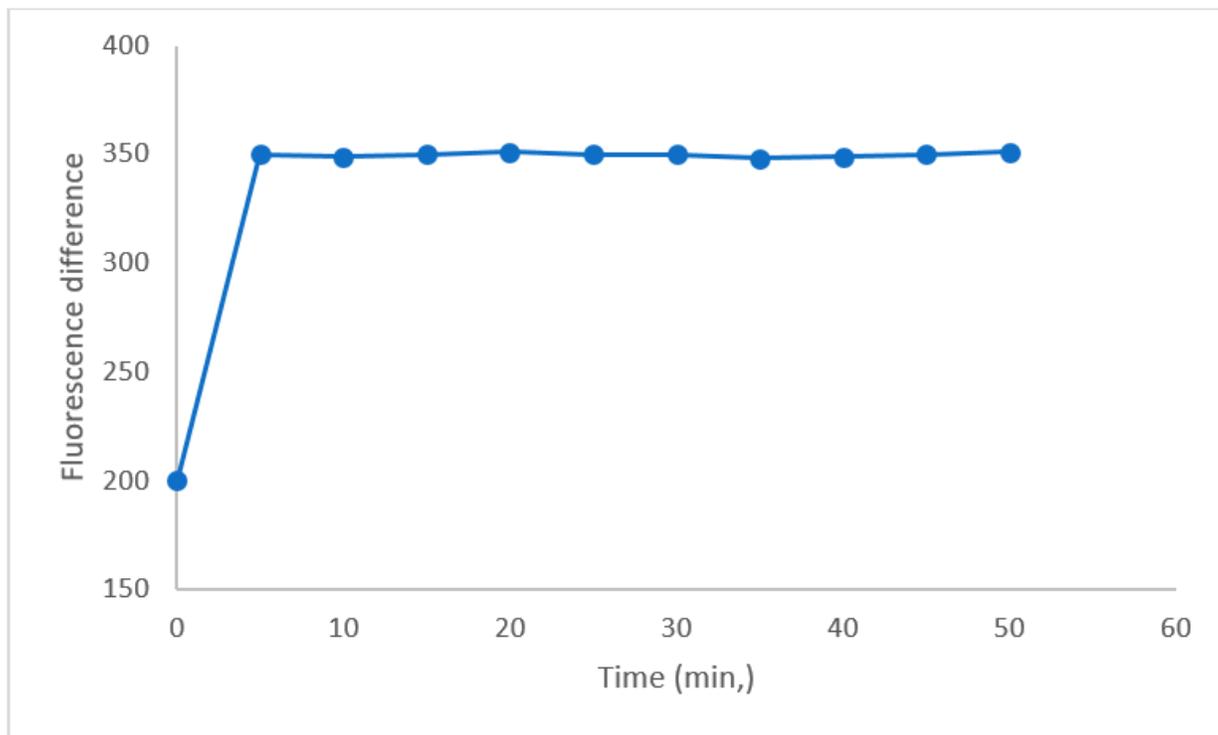


Figure 9

Effect of reaction time on the fluorescence quenching of 1 mL AMF after the reaction with 60 ng mL⁻¹ IXA at 530 nm.

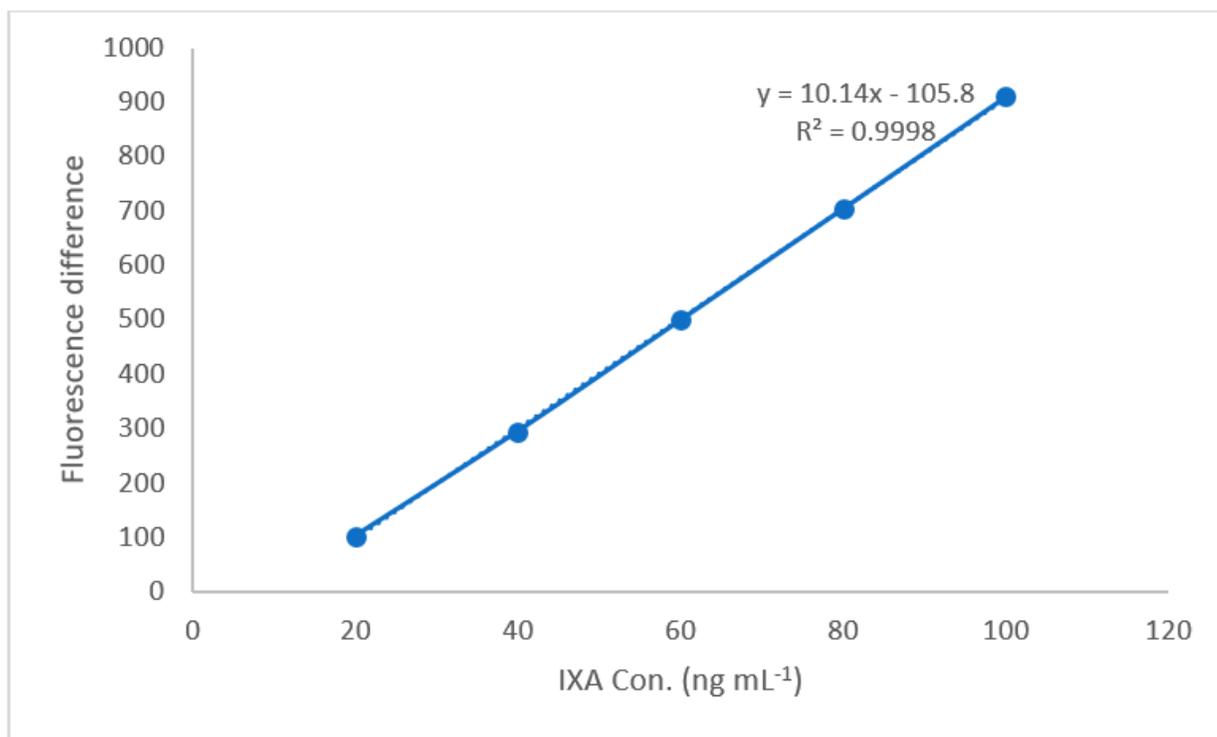


Figure 10

Calibration graph of IXA with 1x10⁻⁴ M AMF at λ_{em} 530 nm.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [Scheme1.png](#)