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Methods of Inner Filter Effect Correction in Fluorescence Measurements of Protein–Ligand Association: A Re-Examination of the Association of Anti-HIV Drugs to Serum Albumin

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ABSTRACT

Fluorescence-based methods are widely used to assess protein–ligand and protein–protein interactions. Thanks to their high sensitivity, these techniques allow very low dissociation constants to be determined. However, in some cases, results must be critically revised because some artifacts can occur, one of the most common being the inner filter effect (IFE). In this article, we show how multiple strategies should be used to correct data obtained from fluorescence quenching experiments to avoid overestimation of binding affinities. The extent of the proposed corrections will depend on the spectroscopic properties of the protein and the ligand, i.e., the fluorophore involved in the experiments, as well as on the specific experimental conditions in each case.

1 | Introduction

1.1 | Fluorescence Studies of Protein/Ligand Association

Spectroscopic techniques are powerful tools to study protein–ligand interactions, to determine association or dissociation constants, and to investigate competition for the same binding site of different molecules [1]. The main advantage of fluorescence spectroscopy, as well as fluorescence polarization-based techniques, is the possibility to obtain data from a direct measurement, even in very low concentrated samples (i.e., nanomolar). For instance, under the proper experimental and theoretical conditions, fluorescence intensity can be immediately correlated to the binding constant [2–4]. Generally, in protein–ligand binding, the intrinsic protein fluorescence can be exploited to estimate the dissociation constant. By titrating the protein of interest with increasing ligand concentration, changes in the emission intensity of the fluorophores are observed and can be exploited to quantify the association phenomenon. The use of fluorescence or fluorescence-based spectroscopic techniques in the field of protein science is widely documented in decades of scientific literature [5–8], providing for countless association data and significantly contributing to the progress of protein science and to the development of new drugs [9]. According to the methodologies applied, we can cite different fluorescence-based experimental techniques that allow to quantify the association process: fluorescence emission spectra, that permit us to directly correlate the fluorescence intensity of the fluorophore with the different species within the sample (i.e., bound and free molecules) [3]; fluorescence anisotropy and other polarized techniques, from

This work is dedicated to the memory of Prof. Lorenzo Stella, our mentor and friend. He prematurely passed away on August 24th, 2025. His life was always driven by a deep love for science, a passion he knew how to communicate to all his students and collaborators. A man of the highest moral stature, anyone who met him could immediately sense it. We will miss him every day, both as a man and as a scientist, and we will always follow his teaching.

which the dissociation constant can be determined avoiding at the same time interfering phenomena that can occur during a classic fluorescence experiment such as fluorophore bleaching or fluctuations in the lamp intensity [1]; Förster resonance energy transfer (FRET) experiments, that allow the association processes to be followed by measuring the efficiency of the FRET phenomenon, a nonradiative energy transfer that take place from an excited specie (the donor) to another molecule (the acceptor) when they are in close proximity [10]. However, fluorescence quenching remains the most widely used method to measure protein–ligand interactions, encompassing the previously discussed FRET phenomenon. A Scopus database inspection, based on the keywords Fluorescence + protein + binding returns 41,517 results only in the 2020–2025 time range. If we refine the research as "Fluorescence + protein + binding + quenching" gives back 9879 documents. These numbers clearly show how widespread the use of techniques based on fluorescence quenching is. In the past years, other studies have underlined possible pitfalls within the data analysis of fluorescence quenching experiments [2, 11]. However, many fluorescence studies of protein–ligand binding still do not consider one of the major sources of artifacts that can lead to an overestimate of the binding affinity between the protein and the ligand, the inner filter effect (IFE).

1.2 | Excitation Inner Filter Effect

The simplest form of IFE is manifested by the fact that solutions at increasing concentrations of a fluorophore do not exhibit a linear dependency of the emission intensity on the concentration of the fluorescent molecule [12, 13]. The fluorescence intensity can be defined according to Equation (1)

$$F = K\phi I_0 10^{-a_F^{exc} L_{exc}} \left(1 - 10^{-a_F^{exc} \Delta x_{exc}}\right) \quad (1)$$

where K is the detection efficiency of the instrument, ϕ the quantum yield of the fluorophore, I_0 the intensity of the exciting light, a_F^{exc} the sample absorbance per cm of pathlength, and L_{exc} and Δx_{exc} are the region of the sample from which the fluorescence is collected (thickness and distance from the front face of the cuvette, respectively).

Only in the limit of low absorbance values, the fluorescence becomes linear with a_F^{exc} Equation (2)

$$\lim_{a_F \to 0} F = K\phi I_0 \ln 10 \Delta x_{exc} a_F^{exc} \quad (2)$$

The excitation inner filter is always potentially present, since even the solution of a single fluorophore necessarily absorbs at the selected wavelength for the excitation.

In binding studies, the fluorophore concentration is essentially constant, and relatively low (typically in the low μM range), and therefore the effect described above can be neglected. However, the ligand involved in the titration experiment has a significant absorbance at the wavelength used for sample excitation that cannot be neglected. When a chromophore (C) absorbing at the excitation wavelength is present in addition to the fluorophore (F), the following Equation (3) holds, that considers the total sample absorbance

$$F = K\phi I_0 10^{-\left(a_F^{exc} + a_C^{exc}\right) L_{exc}} \left[1 - 10^{-\left(a_F^{exc} + a_C^{exc}\right) \Delta x_{exc}}\right] \frac{a_F^{exc}}{a_F^{exc} + a_C^{exc}} \quad (3)$$

1.3 | Emission Inner Filter Effect

The emitted light must pass through a thickness of sample too, but in the case of a single fluorophore (F), absorbance at the emission wavelengths is often negligible, thanks to the Stokes shift. However, a partial overlap of the absorbance and emission spectra is possible, and inner filtering of the emitted light (emission IFE) takes place also when an additional chromophore (C) is present, which absorbs at the emission wavelengths of the fluorophore [14]. Considering this phenomenon, the total intensity reaching the detector is given by Equation (4)

$$F_{meas.} = \frac{F_{em}}{\Delta x_{em}} \frac{10^{-\left(a_F^{em} + a_C^{em}\right) L_{em}}}{\ln 10 \left(a_F^{em} + a_C^{em}\right)} \left[1 - 10^{-\left(a_F^{em} + a_C^{em}\right) \Delta x_{em}}\right] \quad (4)$$

In any case, when IFE (either in excitation or emission) affects the association measurements, a correction factor must be determined to adjust the measured fluorescence and restore an ideal behavior, in which fluorescence intensity increases linearly with absorbance. The "corrected" fluorescence value can be obtained by multiplying the measured fluorescence for the correction factor, that can be determined accordingly to the experimental condition (see Supporting Information for an accurate description of the correction factor).

In this study, we demonstrate that fluorescence quenching, when used to determine dissociation constant, must be critically reassessed and properly corrected. We compare different methods of inner filter correction by exploiting the well characterized interaction between HSA and five commonly used anti-HIV drugs: didanosine, lamivudine, nevirapine, stavudine, and zidovudine [15]. Since these molecules significantly absorb at the wavelengths used in affinity binding experiments to estimate the K_d, it is highly probable that IFE will play a role. We therefore estimated the constant of the association process by applying different IFE correction methods and compare them with the values reported in literature.

2 | Experimental Section

2.1 | Materials

L-tryptophan, N-acetyl-tryptophan-amide (NATA), and human serum albumin (HSA) were obtained from Sigma Aldrich (St. Louis, MO). The nucleoside reverse transcriptase inhibitors didanosine, lamivudine, stavudine, zidovudine, and non-nucleoside reverse transcriptase inhibitor nevirapine were provided by the Italian National Institute of Health (ISS). Spectroscopic-grade acetonitrile (Carlo Erba, Milano, Italy) was used.

2.2 | Instrumentation

Absorbance measurements were performed with a Cary 4000 spectrophotometer (Agilent, Santa Clara, CA). Steady-state fluorescence measurements were performed with a HORIBA

FluoroMax–4 fluorimeter (Edison, NJ, USA). Time-resolved experiments employed an Edinburgh Instruments LifeSpec-ps apparatus (Edinburgh, UK). For all experiments, the temperature was controlled with a thermostatted cuvette holder set at 25°C.

2.3 | Titrations with Anti-HIV Drugs

A fixed concentration of HSA (0.5 μM) was titrated with different concentrations of anti-HIV drugs (7.6×10^{-2}, 0.15, 0.23, 0.30 0.37, 0.44, 0.51, 0.58, 0.65, and 0.71 mM). After each addition, the fluorescence spectra were recorded repeatedly, until stability was achieved. Experiments were performed with integration time 0.4 s, and bandwidths of 6 nm in excitation and emission, except where stated otherwise. Excitation and emission wavelength, as well as the cuvette path length, are specified for each individual experiment in the Result and Discussion. The ratio between the fluorescence of HSA in the presence of increasing concentrations of the different drugs (F) and in the absence of ligand (F_0) at $\lambda_{em} = 350$ nm (except where stated otherwise), as a function of the concentration of drugs ([C]), was then fitted with the following equation, to obtain the dissociation constant (K_d)

$$\frac{F}{F_0} = \frac{1}{1 + \frac{[C]}{K_d}} \quad (5)$$

This equation approximates the free ligand concentration with the total concentration in the sample, an assumption fully justified under the condition [HSA]<<[C]. In addition, it assumes a 1:1 stoichiometry and a totally quenched complex [2]. We could not assess the reliability of these assumptions because the curves were far away from saturation; however, the fits were used solely to obtain an order-of-magnitude estimate of the dissociation constant.

2.4 | Correction with the Method Based on the Experimental Determination of the Instrumental Parameters

For excitation IFE correction, the fluorescence and the absorbance of a solution of increasing concentrations of tryptophan in phosphate buffer 10 mM, pH 7.4, were measured in a 0.4 cm × 1 cm cuvette (in excitation and emission, respectively). The excitation correction factor was then determined as reported in Supporting Information. For emission IFE correction, the fluorescence and the absorbance of a fixed concentration of 1.5-dimethoxynapthalene (DMN) in acetonitrile, titrated with increasing concentrations of 1,6-diphenyl-1,3,5-hexatriene (DPH), were measured in a 0.4 cm × 1 cm cuvette in excitation and emission, respectively. Fluorescence was recorded with $\lambda_{exc} = 272$ nm and $\lambda_{em} = 350$ nm, integration time 1 s. The emission correction factor was then determined as reported in Supporting Information.

2.5 | Correction Based on a Reference N-Acetyl-L-Tryptophanamide Sample

A fixed concentration of NATA (0.5 μM) was titrated with increasing concentrations of drugs (7.6×10^{-2}, 0.15, 0.23, 0.30 0.37, 0.44, 0.51, 0.58, 0.65, and 0.71 mM). After each addition, the fluorescence spectra were recorded. Experiments were performed with $\lambda_{exc} = 295$ nm, integration time 0.4 s, and bandwidth of 6 nm in excitation and emission, in a 0.4 cm × 1 cm cuvette (in excitation and emission, respectively). The ratio of NATA fluorescence intensities at $\lambda_{em} = 350$ nm in the absence and in the presence of the different concentrations of drugs were used to calculate the correction factor (see Results and Discussion).

2.6 | Time-Resolved Experiments

Time-resolved fluorescence experiments were performed with the time-correlated single photon counting technique in a LifeSpec-ps fluorometer (Edinburgh Instruments, UK). Lifetime measurements of NATA were performed in the absence and in the presence of stavudine 8×10^{-4} M in a fluorescence black-bottom cuvette, 10 mm × 4 mm, exciting along the shorter path length, using a magnetic stirrer, and the following experimental conditions: excitation with a pulsed light-emitting diode (Horiba NanoLED-280) with emission peaked at $\lambda_{exc} = 282$ nm, $\lambda_{em} = 350$ nm, cut-off filter in the emission channel at 305 nm, emission monochromator bandwidth = 8 nm, pulse repetition frequency = 1 MHz, acquisition time range = 50 ns, 1024 channels, 10.000 peak counts. The temperature was controlled with a thermostatted cuvette holder set to 25°C for all experiments. Fluorescence decays were fitted using a triple exponential and a scattering component, and the values of the average lifetime were calculated from the two lifetimes and pre-exponential factors.

3 | Results and Discussion

3.1 | Selection of the Compounds

In the following sections, we present and discuss the correction of the IFE for a selected set of compounds taken from previous study [15]. This selection was made with the aim of removing, as far as possible, sources of error that could obscure the correction results. For instance, the protease inhibitors atazanavir, nelfinavir, saquinavir, and the nucleoside reverse transcriptase inhibitor abacavir were discarded as they are strongly fluorescent: their emission spectrum overlapped with that of HSA, and the emission intensity was considerably higher than intrinsic protein fluorescence at the concentrations needed to observe significant quenching with a sufficient signal-to-noise ratio (data not shown). Finally, the water-solubility of the protease inhibitor ritonavir was very limited. Its solutions were visibly turbid, perturbing the spectroscopic measurements and presumably the binding equilibrium, too, due to likely aggregation processes. Based on these considerations, we decided to concentrate the present study on the five reverse transcriptase inhibitors reported in Figure 1: the nucleoside inhibitors didanosine, lamivudine, stavudine, and zidovudine, and the non-nucleoside inhibitor nevirapine.

3.2 | Absorption Spectra

The extinction coefficients of the investigated compounds are reported in Figure 2. All drugs show significant absorption at 280 nm, the excitation wavelength of the tryptophan maximal absorbance which was used in the reference paper [15]. In addition, nevirapine also absorbs in the spectral region of the HSA

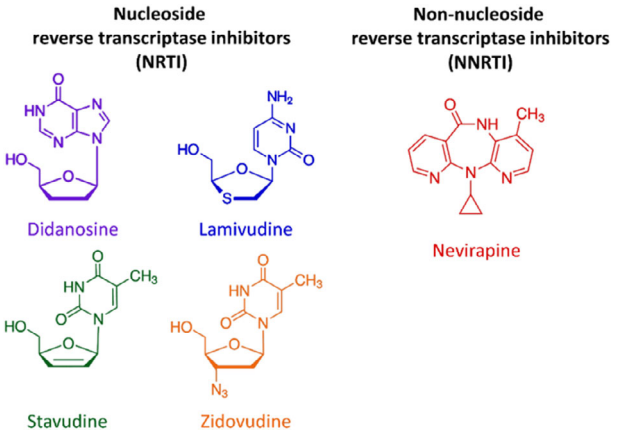

FIGURE 1 | Chemical structures of the investigated compounds. Compounds are reported in the color used for their respective data in the following figures.

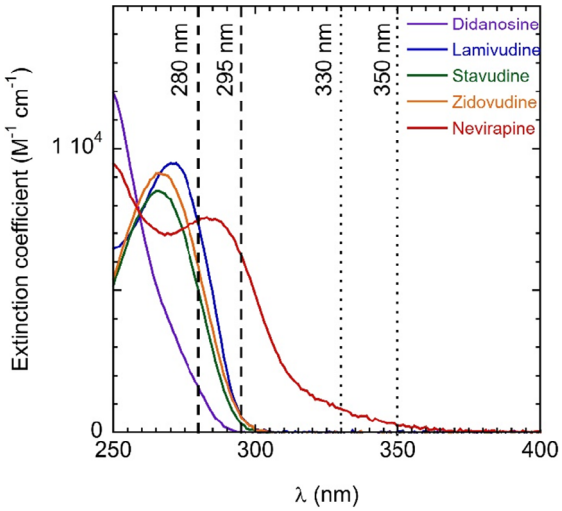

FIGURE 2 | Absorption spectra of the investigated compounds. Vertical lines indicate the excitation (dashed lines) and emission (dotted lines) wavelengths employed in different measurements.

emission spectrum. Therefore, excitation IFE is possible for all drugs, and emission IFE could be present in the case of nevirapine.

In the following sections, we present various correction methods that can be applied to measure affinity constants without artifacts arising from IFE. These range from a simple fluorescence correction based on total sample absorbance at the center of the cuvette, to more advanced methods using experimental parameters to calculate the correction factor, and titrations with non-interacting molecules, where fluorescence changes reflect only IFE.

3.3 | Effect of Experimental Parameters on Apparent Ligand-Induced Fluorescence Quenching

The simplest way to check for the presence of IFE artifacts is to change experimental parameters that influence sample absorbance, i.e., the optical path or the excitation and emission wavelengths. Obviously, these parameters should not affect the binding equilibrium. Therefore, if quenching was caused by complex formation, the quenching curves obtained under different experimental conditions should be superimposable. Figure 3A shows the decrease in fluorescence intensity observed in a titration of HSA with lamivudine, using an excitation wavelength of 280 nm, emission at 330 nm and a 1 cm × 1 cm pathlength cuvette (as in [15]) or with an excitation wavelength of 295 nm, where lamivudine and all other compounds absorption (except nevirapine) is several folds lower. With $\lambda_{exc} = 295$ nm, the apparent quenching efficiency was significantly reduced compared to experiments with $\lambda_{exc} = 280$ nm. This finding is a clear indication of the presence of IFE artifacts, as the change in the excitation wavelength is not expected to have effects on the F/F_0 ratio. This interpretation was also supported by experiments performed in cuvettes with different excitation optical paths (5, 4, and 3 mm) (Figure 3B). A shorter optical path reduced the quenching efficiency, a finding that can only be explained as due to IFE. Based on these data, it is possible to conclude that the quenching effects, and thus the binding affinities reported [15], were overestimated.

3.4 | Titrations Performed Under Optimized Conditions

To minimize IFE, the association experiments between HSA and the five different anti-HIV drugs were then repeated using optimized instrumental parameters: a 295 nm excitation wavelength (rather than 280 nm, as in the original article), 350 nm emission wavelength (rather than 330 nm), 4 mm excitation pathlength (rather than 10 mm), while emission pathlength was kept at 10 mm (we observed that smaller optical paths cause a reduction in the reproducibility of the measurements). The data obtained in experiments performed under the optimized conditions are shown in Figure 4A, and the dissociation constants determined by a simple Langmuir isotherm fit, assuming total quenching in the complex, are reported in Table 1. In our experimental conditions, we are far from saturation, limiting an exact determination of the dissociation constant. Nevertheless, a clear and robust estimation of the K_d is possible and allow us to compare the different IFE correction methods. With the single exception of nevirapine, all values of the derived dissociation constant are approximately one order of magnitude larger than those previously reported, further confirming that the original data were affected by IFE. Unfortunately, significant sample absorbance was still present even using the optimized wavelengths and optical path. For this reason, different IFE correction methods described in each section were applied and compared. Since nevirapine also absorbs at the emission wavelength selected for the data analysis, in this case, correction for emission IFE was required.

3.5 | Correction with Total Absorbance at the Center of the Cuvette

A first-level straightforward correction can be performed assuming that the geometry of the instrument is such that the collected intensity comes exactly from the center of the cuvette so that $\Delta x \to 0$ and $L = l/2$, where l is the optical path of the cuvette [16]. In this case the correction factor that account for both excitation and emission inner filter becomes Equation (6)

$$C_{tot} = 10^{\left(a_F^{exc} + a_C^{exc}\right)\frac{l}{2}} \cdot 10^{\left(a_F^{em} + a_C^{em}\right)\frac{l}{2}} \quad (6)$$

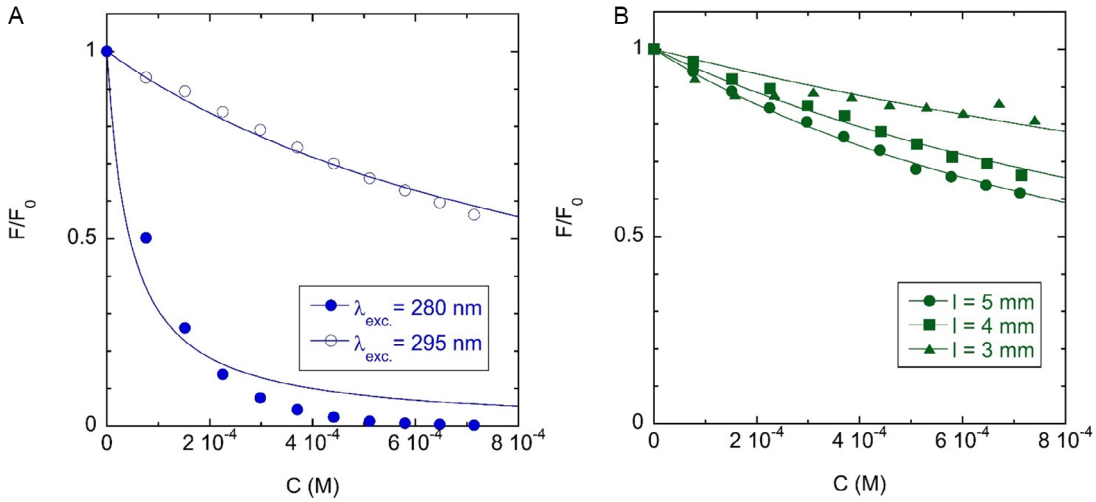

FIGURE 3 | Effect of experimental parameters on apparent ligand-induced fluorescence quenching. Panel A: Effect of the excitation wavelength on the apparent quenching of HSA fluorescence by lamivudine (optical path 10 × 10 mm). Panel B: Effect of the excitation path length on the apparent quenching of HSA fluorescence by stavudine (excitation wavelength 295 nm). The ratio of the fluorescence intensity F to the fluorescence intensity in the absence of ligand F_0 is reported as a function of ligand concentration C.

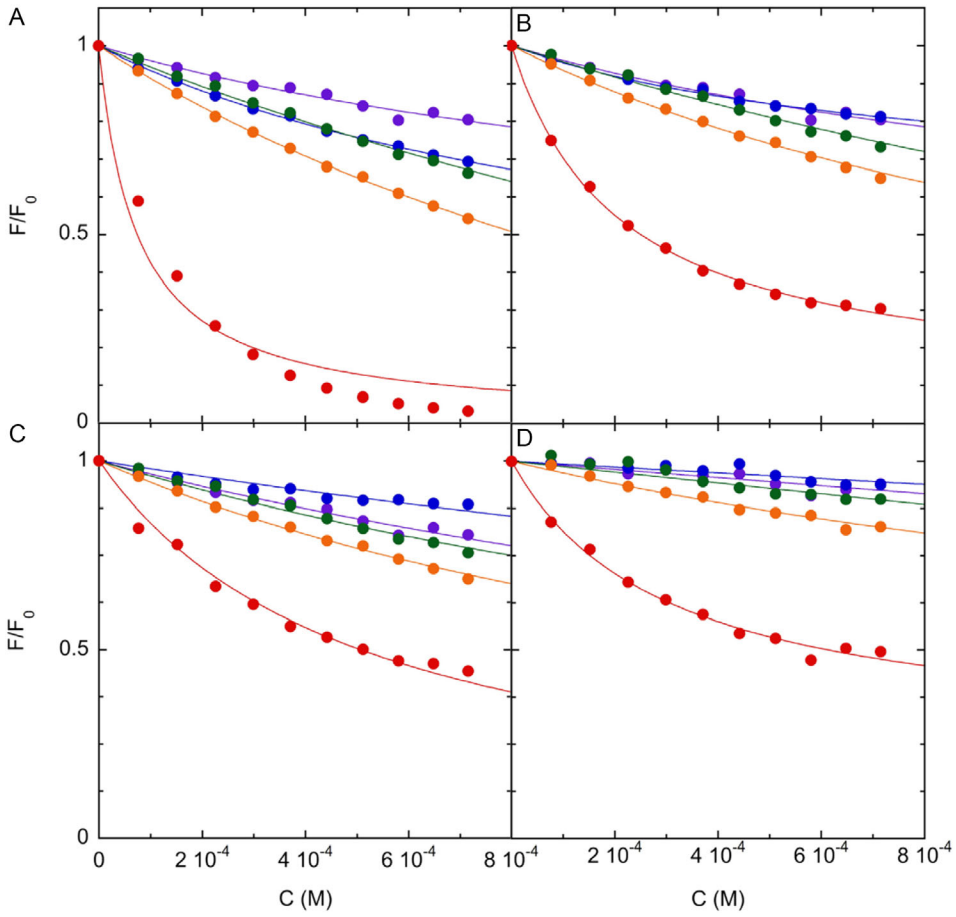

FIGURE 4 | Uncorrected and corrected quenching curves, measured with optimized instrumental parameters (295 nm excitation and 350 nm emission 4 mm × 10 mm cuvette). Panel A: uncorrected quenching curves. Panel B: quenching curves corrected with the approximate equation (see Equation (5)). Panel C: quenching curves obtained based on the derivation of the instrumental parameters $L_{exc.}$, $\Delta x_{exc.}$, $L_{em.}$, $\Delta x_{em.}$. Panel D: quenching curves corrected with NATA measurements. The quenching curves obtained with the different compounds are colored according to Figure 1.

TABLE 1 | Estimated dissociation constants (K_d) obtained with the different used IFE correction methods, compared to the ones described in [15].

	K_d (mM)				
Compound	**Ref. [15]**	**Optimized experimental parameters**	**$10^{A/2}$ correction**	**Correction with instrumental parameters**	**NATA correction**
Didanosine	0.10 ± 0.01	2.77 ± 0.01	2.77 ± 0.01	2.77 ± 0.01	8.7 ± 0.1
Lamivudine	0.10 ± 0.01	1.56 ± 0.02	2.79 ± 0.01	3.4 ± 0.1	13 ± 1
Stavudine	0.09 ± 0.03	1.53 ± 0.04	2.17 ± 0.01	2.54 ± 0.03	6.5 ± 0.6
Zidovudine	0.16 ± 0.01	0.93 ± 0.02	1.41 ± 0.02	1.62 ± 0.02	3.3 ± 0.1
Nevirapine	0.12 ± 0.01	0.07 ± 0.01	0.26 ± 0.01	0.41 ± 0.02	0.56 ± 0.02

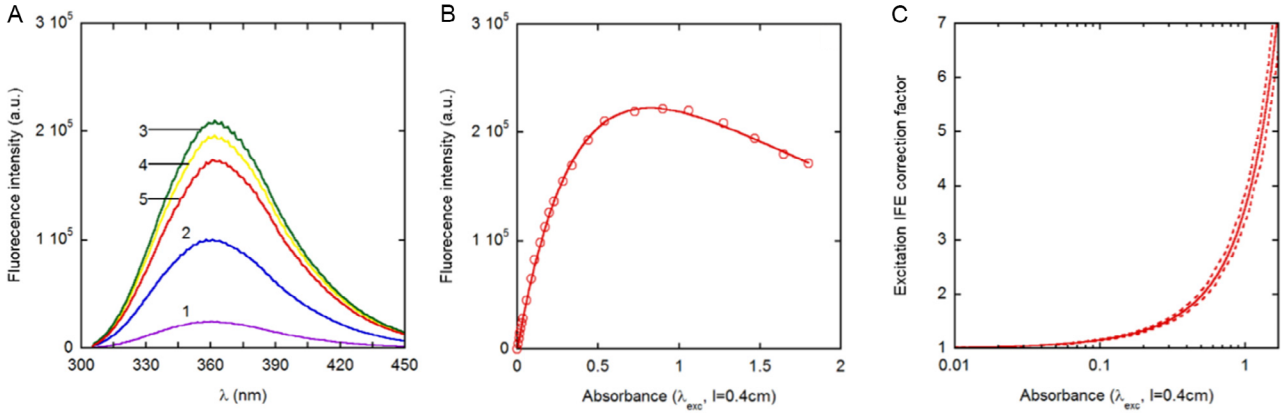

FIGURE 5 | Determination of the correction factor for IFE in the excitation path. Panel A: emission spectra of five representative additions of a tryptophan solution. For each spectrum, the absorbance values at 295 nm are: 0.03 (spectrum 1, purple), 0.14 (spectrum 2, blue), 1.28 (spectrum 3, green), 1.47 (spectrum 4, yellow), and 1.80 (spectrum 5, red). For each spectrum, the $\lambda_{exc} = 295$ nm. Absorbance values were measured in a 0.4 cm optical path cuvette. Panel B: fluorescence intensity ($\lambda_{exc} = 295$ nm, $\lambda_{em} = 450$ nm, bandpass 6 nm in both excitation and emission, 4 mm × 10 mm cuvette), emitted from a tryptophan solution of increasing concentration, reported as a function of sample absorbance at the excitation wavelength, in a 0.4 cm optical path. Panel C: correction factor determined from the data in Panel B reported as a function of sample absorbance at the excitation wavelength in a 0.4 cm optical path. Dotted lines correspond the error in the parameter, determined from repeated experiments.

Figure 4B and Table 1 report the results of correction performed with this simplified method. Although this correction is not extremely sophisticated, it can give reasonable results at low absorbance values. A further reduction in the apparent affinity was observed for all compounds, except for didanosine, which does not absorb at the wavelengths used in these experiments. These results confirm that IFE is still at play even under these conditions.

3.6 | Correction with the Method Based on the Experimental Determination of the Instrumental Parameters

Another possible method is based on the derivation of the instrumental parameters. This method is limited by the preliminary construction of a standard curve in both fluorescence and absorbance spectroscopy to define the optical parameters of the specific instruments used in the binding experiment to calculate the correction factors.

Equation (1) can be used to fit a series of measurements performed on a reference fluorophore solution, as a function of sample concentration [14]. These parameters can then be used in Supporting Equations (1)–(4) in order to obtain a correction factor. To apply this IFE correction method in excitation based on the experimental determination of the specific instrumental geometry, the fluorescence intensity at $\lambda_{em} = 365$ nm of a solution of increasing concentrations of tryptophan, and therefore at increasing values of its absorbance, was recorded (Figure 5A). Figure 5B shows representative data for the measured fluorescence intensity as a function of sample absorbance, fitted with Equation (1). From the fit, the following values were obtained for the instrumental parameters, and used in the calculation of the correction factor according to supporting Equation (3): $L_{exc.} = 0.073$ cm and $\Delta x_{exc.} = 0.392$ cm. The correction factor determined from these experiments is reported in Figure 5C. It is worth mentioning that in the binding studies, the HSA concentration was 0.5 μM, and therefore, a_F^{exc} was negligible.

The same approach can be applied for the correction of emission IFE. In this case, DMN was titrated with increasing concentrations of the chromophore DPH, which absorbs in the spectral region where the fluorophore emits (Figure 6A). After each addition of DPH the absorption spectrum of the solution was recorded (Figure 6B), and the emission intensity was measured at $\lambda_{em} = 350$ nm with a $\lambda_{exc} = 272$ nm. The measured intensity was corrected for excitation IFE using the previously obtained correction factor (Supporting Equation (3)). Even so, the fluorescence

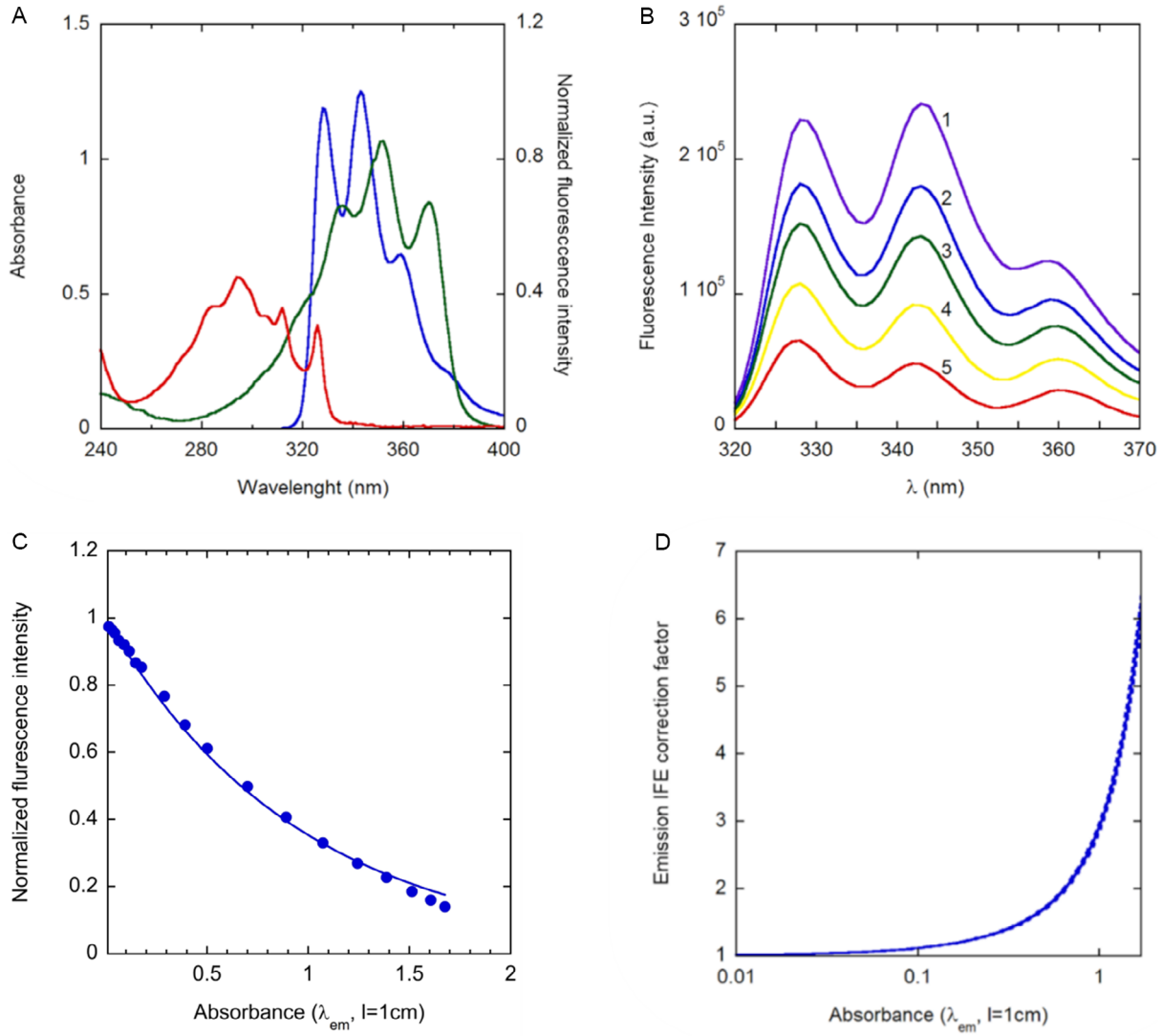

FIGURE 6 | Determination of the correction factor for IFE in the emission path. Panel A: absorption spectrum of DPH (green), compared with the absorption (red) and normalized emission (blue) spectra of DMN. Panel B: Emission spectra of DMN solution (in acetonitrile) in the presence of five representative additions of DPH. For each spectrum, the absorbance values at 350 nm are: 1.43 (spectrum 1, purple), 0.93 (spectrum 2, blue), 0.54 (spectrum 3, green), 0.33 (spectrum 4, yellow), and 0.04 (spectrum 5, red). For each spectrum, the following conditions were used: $\lambda_{exc} = 272$ nm, bandpass 6 nm in both excitation and emission, 4 × 10 mm cuvette). Panel C: normalized fluorescence intensity ($\lambda_{exc} = 272$ nm, $\lambda_{em} = 350$ nm, bandpass 6 nm in both excitation and emission, 4 × 10 mm cuvette), emitted from a DMN solution (in acetonitrile) in the presence of increasing concentrations of DPH, reported as a function of sample absorbance at the emission wavelength, in a 1 cm optical path, after correction for excitation IFE. Panel D: correction factor for IFE in the emission path determined from the data in Panel C, reported as a function of sample absorbance at the emission wavelength, in a 1 cm optical path. Dotted lines indicate the error determined from repeated experiments.

intensity of DMN decreased, due to the partial absorption of the emitted light by the added DPH (Figure 6C). By fitting these data with Equation (4), the following values were obtained for the instrumental parameters: $L_{em.} = 0.416$ cm and $\Delta x_{em.} = 0.073$ cm, that were then used to calculate the emission correction factor (Supporting Equation (4)). Figure 6D reports the emission correction factor. Figure 4C and Table 1 report the data obtained with the correction factors of Figures 5 and 6. A further decrease in apparent affinity was observed with respect to the simple correction that take into account the absorption of incident light at the centre of the cuvette (again, except for didanosine, since this molecule has no absorption at the wavelengths used in these experiments). This finding indicates that the simplest correction may not be fully justified, at least for the instrumentation used in this work.

3.7 | Correction Based on a Reference NATA Sample

An alternative correction method is based on the hypothesis that no direct interaction takes place between the drugs and a reference fluorescent compound (NATA, in our case) [17, 18]. If this is true, any reduction in fluorescence intensity observed by titrating the fluorophore with the drugs is due to IFE alone. In contrast to the other methods presented in this study, this correction requires neither assumptions about nor determination of the instrumental geometric parameters and it avoids the problems of the previous approaches. In addition, it corrects for excitation and emission IFE at the same time. Since the spectral properties of the protein and NATA samples are the same at the excitation wavelengths used in this study, and measurements are performed

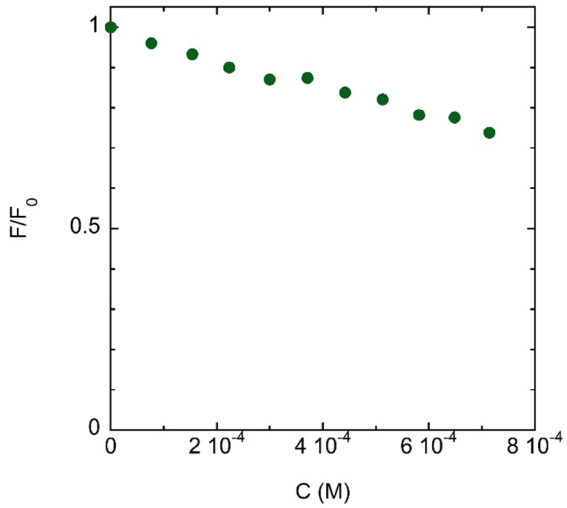

FIGURE 7 | NATA titrations. Decrease in the normalized fluorescence intensity caused by titration of stavudine. The experimental conditions are the same as the HSA titrations (Figure 4).

in the same fluorimeter, both set of measurements will be affected by IFE in the same extent. In the case of the reference molecule, no additional variations in the fluorescence are expected to occur, and therefore the correction factor can be calculated according to Equation (7)

$$C([L]) = \frac{F_{ref}([L]=0)}{F_{ref}([L])} \tag{7}$$

Figure 7 reports the results of the experiments of NATA titration with stavudine (see S1 for the titration of all the compounds tested). A decrease in the fluorescence intensity was observed for these drugs. Figure 4D and Table 1 report the data corrected with this approach. The apparent affinities in this case were even lower than those determined with the other correction methods. A direct interaction between fluorophore and drug would invalidate the hypotheses on which this correction method is based.

To further investigate this possibility, we compared fluorescence lifetime measurements of NATA performed in the absence and in the presence of stavudine at a concentration of $8{\cdot}10^{-4}$ M (Figure 8). Of course, fluorescence lifetimes are not affected by the IFE and can be used to test for the presence of real quenching, without the need for any corrections. We obtained an average lifetime of 2.8 ns, for both NATA alone, and in the presence of stavudine. This result confirmed that no interaction between NATA and stavudine takes place. By contrast, fluorescence lifetime measurements of HSA alone and in the presence of stavudine at a concentration of $8{\cdot}10^{-4}$ M yielded an average fluorescence lifetime of 5.4 and 5.2 ns, respectively (Figure 8), confirming a slight binding-induced quenching.

4 | Conclusion

Fluorescence spectroscopy is one of the most sensitive techniques for studying ligand binding to a wide range of biological molecules. In a typical experiment, a ligand is titrated into a solution containing a fluorescent macromolecule. In the case of proteins, the intrinsic fluorescence of aromatic residues can be exploited. Upon ligand addition, changes in the emission intensity of the fluorophores are observed and, if quenching arises from intermolecular association, the binding constant can be determined. However, if appropriate experimental conditions or correction factors are not applied, the affinity constant may be significantly overestimated. In the present work, we evaluated different methods for correcting the IFE to obtain more accurate affinity constants. The method based on the $10^{A/2}$ factor is extremely simple but, due to the stringent assumptions on which it is based, provides reliable results only at low absorbance values. The second method of correction, based on experimental determination of the instrumental parameters, requires the construction of a standard curve by measuring both fluorescence and absorbance. Since these measurements are typically performed using different instruments with distinct optical properties, this approach may introduce inaccuracies in the derivation of the correction factor.

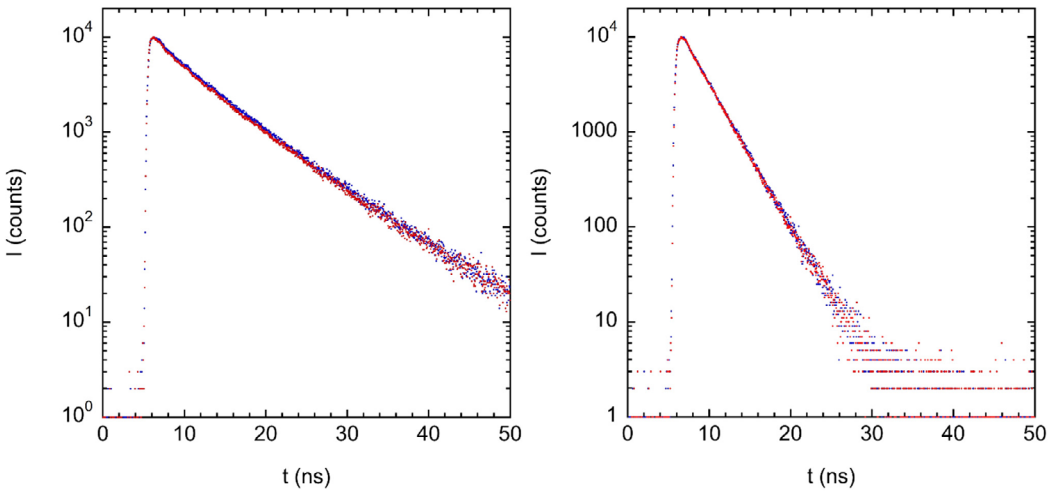

FIGURE 8 | Lifetimes. Left panel: fluorescence intensity decay of NATA in the presence of stavudine (red dots) (8×10^{-4} M) and NATA alone (blue dots). Right panel: fluorescence intensity decay of HSA in the presence of stavudine (red dots) and HSA alone (blue dots). Average lifetimes: HSA = 5.4 ns HSA + stavudine = 5.2 ns; NATA = 2.8 ns; NATA + stavudine = 2.8 ns, using a triple exponential and a scattering component. NATA 10 μM, stavudine 0.77 mM, HSA 0.5 μM, λ_{exc} = 298 nm, λ_{em} = 360 nm, cutoff filter 320 nm, slits 2 mm.

Finally, the method based on a reference sample overcomes the limitations of the previous approaches, as it does not rely on assumptions regarding instrument geometry and simultaneously corrects for both excitation and emission IFE. Importantly, the single assumption underlying the last method, the absence of direct ligand–HSA quenching, was experimentally validated in our measurements. Moreover, this method yielded a more substantial IFE correction compared to alternative experimental procedures, indicating its higher reliability. The efficacy of this method is reflected in a better estimation of the dissociation constants, resulting quite different compared to the $10^{A/2}$ and instrumental parameters methods. This experimental excursus suggests that reference sample correction may be considered the most promising approach when IFE occurs. Overall, our results demonstrate that fluorescence spectroscopy is a powerful technique for quantifying association processes; however, IFE artifacts can lead to severely misleading conclusions if not properly addressed. Therefore, appropriate correction methods must be carefully considered in fluorescence-based binding studies.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.