

Fluorescence Lifetime Imaging of Propranolol Uptake in Living Glial C6 Cells

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Abstract. Uptake of the β -blocker drug propranolol by living glial C6 cells has been observed using fluorescence lifetime imaging with two-photon excitation at 630 nm. Both uptake and release of propranolol occur within minutes and are temperature dependent, being about 5 times faster at 37°C than at 20°C. The intracellular fluorescence lifetime of propranolol is generally shorter than the value of 9.8 ns determined in dilute neutral aqueous solution, and the difference is ascribed to concentration quenching. Within the cells, propranolol is accumulated within intracellular acidic vesicles and the cytoplasm but is excluded from the cell nucleus. On incubation of cells in medium containing 100 μ M propranolol, the drug is accumulated to reach intracellular concentrations up to 10 mM in a process that is believed to be driven by protonation within acidic cellular compartments.

Keywords: Propranolol, fluorescence, lifetime, imaging, multiphoton, living cell

1. Introduction

Propranolol is a nonselective β -blocker drug which prevents binding of epinephrine and noradrenaline to β 1- and β 2-adrenergic receptors [1] as well as inhibiting cellular uptake of both serotonin and dopamine [2]. Propranolol is a weakly basic lipophilic compound known to interact and modify the behaviour of bilayer lipid membranes [3–5]. Studies have shown propranolol to be taken up into a range of cells including epithelial cells [6] and hepatocytes [7]. In the latter case, it has recently been reported that there are two intracellular sites, one of high affinity and low capacity, and the other a low affinity and nonsaturable site proposed to be the cellular membranes [7].

It is known that propranolol exhibits intrinsic fluorescence in the ultraviolet region with a solvent-dependent maximum between 320 and 360 nm [8–11]. This has been used for a number of purposes, including quantification of propranolol in drug assays, detection during microchip electrophoresis, and monitoring binding to imprinted polymers. However, the wavelengths required for one-photon fluorescence excitation in the ultraviolet (260–320 nm) are damaging to cellular systems, producing potentially toxic intermediates [12], and are poorly transmitted by normal microscope optics [13]. These constraints therefore make difficult direct excitation and imaging of propranolol fluorescence

in cellular systems with UV excitation. In contrast, two-photon microscopy using red and near infrared wavelengths with the benefit of confining the region of excitation to the focal volume of a femtosecond laser beam focussed to the diffraction limit, which further allows pseudoconfocal imaging [14, 15] with reduced overall phototoxicity. Multiphoton microscopy has applications in pharmaceutical sciences [16] effectively permitting imaging of UV intrinsic fluorescence from biochemical chromophores such as serotonin [17, 18] and dehydroergosterol [19].

2. Materials and Methods

Reagents

All chemicals were obtained from Sigma-Aldrich and used as received. The rat glioma line, designated as C6 glial cells, was purchased from LGC Promochem (ATCC number CCL-107). They were grown as monolayer cultures in F-12 K medium supplemented with 2.5% (v/v) FCS at 37°C with humidified 5% CO₂. For imaging, cells were treated with 0.25% trypsin and seeded into dishes with a number 1 coverslip base (MaTek Corporation) at a concentration of $\sim 2.5 \times 10^5$ cells mL⁻¹ and grown for over 50 hours.

The microscope system for fluorescence lifetime imaging has been previously described [18]. Briefly, it is based on a Ti: sapphire laser (Coherent Mira) pumping an optical parametric oscillator (APE) coupled to an inverted microscope (Nikon TE2000U) with a water-immersion ultraviolet corrected objective (Nikon VC x60, NA 1.2). For cell work, power at the sample was limited to <1 mW to minimize localised photodamage. Fluorescence was detected through a combination of a saturated copper sulphate solution and 340 nm (U340, Comar) interference filters using time-correlated single photon counting with a Hamamatsu R3809U photomultiplier linked to a PC module SPC830 (Becker and Hickl, Germany).

3. Results and Discussion

In aqueous solution at pH 7.3, propranolol and tryptophan have similar absorption maxima at about 280–290 nm with comparable extinction coefficients (see Table 1). However, excitation on the red edge at 310–320 nm allows selective stimulation of propranolol fluorescence in the presence of tryptophan-containing proteins in a manner analogous to that previously described for observation of serotonin and 5-hydroxytryptophan fluorescence [18, 23]. For propranolol, the long wavelength absorption peak is at 289 nm ($\epsilon = 5,900 \text{ M}^{-1} \text{ cm}^{-1}$) whilst the extinction coefficient at 315 nm is $1,730 \text{ dm}^3 \text{ mol}^{-1} \text{ cm}^{-1}$. In neutral aqueous solution, the fluorescence spectrum of propranolol has a peak at 353 nm and overlaps substantially with that of tryptophan. The fluorescence quantum yield (ϕ_f) of 0.50 ± 0.03 for propranolol at pH 7.3 was measured on excitation of 315 nm by comparison of the integrated fluorescence intensities in solutions of matched absorbance values when using 5-hydroxytryptophan as a standard ($\phi_f = 0.27$ [21]).

For imaging studies of cells, 2-photon excitation (2PE) at around 630 nm was achieved using laser pulses of 180 fs duration at a repetition frequency of 76 MHz. Under these conditions, propranolol fluorescence intensity exhibits the anticipated quadratic dependence on laser power. two-photon cross-sections (σ_2) for propranolol as the sample (*s*) at 590 nm and 630 nm were obtained by the method

Table 1: Spectroscopic properties of tryptophan, serotonin, and propranolol including fluorescence quantum yields (ϕ_f), fluorescence lifetimes (τ_f), and 2-photon excitation cross sections (σ_2).

	Propranolol	Serotonin	Tryptophan
$\varepsilon(295 \text{ nm})^a$	5310	4280	1490
$\varepsilon(315 \text{ nm})^a$	1730	1140	20
ϕ_f	0.50 ± 0.03	0.28 [20]	0.27 [21]
$\tau_f(\text{ns})$	9.8	3.9 [20]	2.79, 0.26 [21]
$\sigma_2(590 \text{ nm})^b$	0.40	0.94	0.21 [22]
$\sigma_2(630 \text{ nm})^b$	$0.17^c, 0.22^d$	0.35	—

^a Units $\text{M}^{-1} \text{cm}^{-1}$, ^bGM units ($10^{-50} \text{cm}^4 \text{s photon}^{-1}$), ^cdetermined using tryptophan as standard, ^ddetermined with 9-chloroanthracene as standard.

outlined by Mathai et al. (2007) [24] using a reference fluorophore (r) with a known value of σ_{2r} using (3.1), in which I represents the measured fluorescence intensity in the two-photon experiment, c the concentration, ϕ the fluorescence quantum yield, and FI^λ the fluorescence intensity of the fluorophore in the one-photon excited spectrum at the wavelength λ used in the two-photon experiment relative to the integrated spectral intensity ΣFI :

$$\sigma_{2s} = \frac{I_s \cdot c_r \cdot (\text{FI}_r^\lambda / \Sigma\text{FI}_r) \cdot \phi_r}{I_r \cdot c_s \cdot (\text{FI}_s^\lambda / \Sigma\text{FI}_s) \cdot \phi_s} \cdot \sigma_{2r}. \quad (3.1)$$

Using this method for determination of cross-sections at one excitation wavelength (here 590 nm) effectively calibrates the sensitivity of the emission/detection section of the apparatus and allows determination of cross-sections at other excitation wavelengths. Only corrections for the transmission of the microscope objective at the excitation wavelength and laser power at each excitation wavelength are required. A two-photon excitation spectrum for tryptophan has been reported by Rehms and Callis [22] and σ_2 of 1.9 GM units ($10^{-50} \text{cm}^4 \text{s photon}^{-1}$) calculated for 3-methyl indole at 552 nm. Their data suggests σ_{2r} of 0.21 GM for tryptophan two-photon excitation at 590 nm, and this was used as one of the standard values. The results summarised in Table 1 indicate values of σ_2 of 0.40 and 0.17 GM units for propranolol at 590 and 630 nm, respectively. The corresponding cross-sections for serotonin are shown for comparison and are approximately twofold higher than for propranolol despite the lower extinction coefficients for the former at 295 and 315 nm. For comparison, a second determination of σ_2 for propranolol was made with 2PE at 628 nm using 9-chloroanthracene as a standard with $\sigma_{2r} = 0.20$ GM at 630 nm [25]. Using again the method outlined by Mathai et al. [24], a value of σ_2 for propranolol of 0.22 GM at 628 nm was obtained which is in excellent agreement with the value of 0.17 GM units obtained with tryptophan as the standard.

Fluorescence lifetimes of propranolol under various conditions were measured using two-photon excitation at 630 nm with the microscope system and placing a drop of solution on a coverslip mounted on the thermostatically controlled microscope stage. At 20°C in dilute neutral aqueous solution, a good single exponential lifetime of 10.1 ns ($\chi^2 = 1.03$) was measured. Buffering the solution with phosphate resulted in a slight decrease in lifetime to 9.8 ns in 0.1 mol dm³ phosphate. As shown in Figure 1(a), the lifetime remained constant over the range pH 5 to 8.5 but decreased in alkaline solutions in a manner

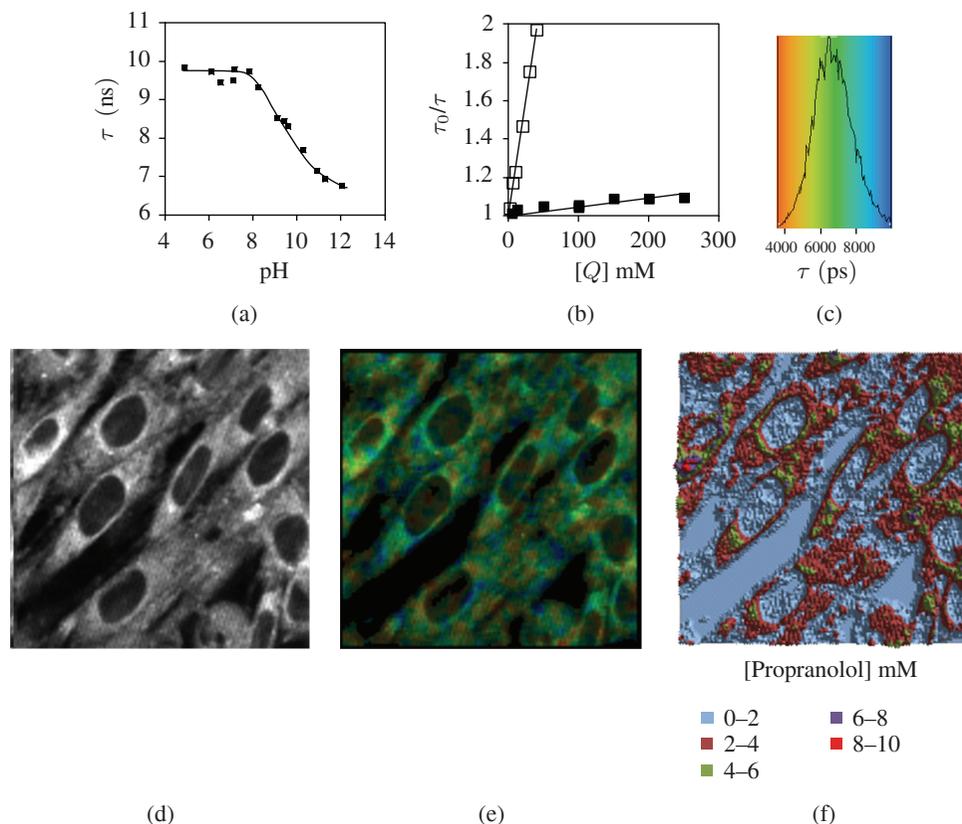


Figure 1: Fluorescence of propranolol in solution and after uptake into C6 glioblastoma cells. (a) Effect of pH on the fluorescence lifetime of propranolol in aqueous solution; (b) Stern-Volmer plots at pH 7 for the self-quenching of the fluorescence lifetime of propranolol (\square) and quenching by phosphate (\blacksquare). Images (c) through (f) show results obtained on incubation of C6 glioblastoma cells with SR-propranolol ($100 \mu\text{M}$) at 37°C for 10 minutes. Fluorescence at 340 nm was observed following 2PE at 630 nm. The results show the fluorescence intensity (d) and lifetime (e) images (field $76 \mu\text{m}$ square) together with the lifetime distribution within the image (c). Image (f) shows the intracellular propranolol concentration profile calculated from (d) and (e) as described in the text.

consistent with the reported pK_a of 9.53 [26]. At 37°C in neutral aqueous solution, the fluorescence lifetime also decreased to 9.4 ns. Self-quenching of the fluorescence lifetime at concentrations above 1 mM was measured and followed Stern-Volmer kinetics for kinetic quenching, plotted according to (3.2) in Figure 1(b) giving a second-order rate constant, k_q , of $(2.5 \pm 0.1) \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$:

$$\frac{\tau_0}{\tau} = 1 + k_q \tau_0 [Q], \quad (3.2)$$

which is close to the diffusion-controlled limit. Weaker kinetic quenching of the propranolol fluorescence lifetime by phosphate at pH 7 was also observed with k_q $(3.2 \pm 0.5) \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$. The lifetimes measured for propranolol in dilute solutions are longer than those of tryptophan (major long

lifetime component 2.88 ns [21]) enabling good discrimination against intrinsic tryptophan fluorescence in biochemical systems as demonstrated below.

Intracellular Imaging of Propranolol

C6 cells are capable of protecting neuronal cells against excessive levels of monoamines through sodium- and chloride-dependent monoamine transporters [27]. Using the fluorescence properties of propranolol described above, it proved possible to image intracellular accumulation of propranolol in cells using two-photon fluorescence lifetime imaging with excitation at 630 nm and detection of ultraviolet (340 nm) fluorescence. Uptake was initiated by addition of propranolol (100 μ M final concentration) to the culture medium above a confluent monolayer of cells attached to a cover-slip forming part of a thermostatically controlled chamber. A typical image obtained after incubation of C6 cells with propranolol at 37°C is shown in Figure 1. The intensity image (Figure 1(d)) shows propranolol mainly distributed within the cell cytoplasm and excluded from the cell nucleus. The fluorescence lifetime image (Figure 1(e)) indicates a range of lifetimes from the solution value of 9.4 ns at 37°C to quenched values of less than 5 ns, with an average value of approximately 6.5 ns (Figure 1(c)). Calibration of the microscope system with propranolol solutions of known concentration allows an estimate of intracellular propranolol concentrations after correcting for Stern-Volmer quenching using the lifetime data. Plots of fluorescence intensities of the calibration solutions versus concentration themselves required correction because of the effects of self-quenching according to (3.2). Applying a similar lifetime adjustment to the cell intensity data provides a concentration map of propranolol concentration within the cells (Figure 1(f)). It can be seen that substantial regions of the cell cytoplasm contain around 5 mM propranolol with peak concentrations reaching up to 10 mM. These values are up to two orders of magnitude higher than the concentration of propranolol (100 μ M) added to the external medium.

The rates of accumulation of propranolol within C6 glial cells were evaluated by measuring the total fluorescence intensity within the microscope field. Figure 2(a) shows that at 37°C uptake was rapid and occurred with a half-life of less than 2 minutes. Although S-propranolol is about 100 times more effective as a drug than R-propranolol, no significant difference in the rate of uptake between the 2 enantiomers and a racemic mixture was observed. At 22°C, the rate of uptake was much slower with a half-life of about 15 minutes. The loss of propranolol from previously loaded cells could also be measured on replacement of the external medium containing propranolol with phosphate-buffered saline. The results in Figure 2(b) show half-lives of <2 and 8 minutes at 37 and 20°C respectively.

Propranolol is a weak base ($pK_a = 9.53$) and is known to accumulate within acidic vesicles through protonation and trapping of the acidic form [28]. Our previous imaging experiments [29] with rat smooth muscle aorta cells show colocalisation of propranolol with LysoTracker Green DND-26 at 20°C, but more extensive distribution through the cell cytoplasm at 37°C. Further imaging experiments in C6 glial cells (not shown) provide further support for this and suggest that initial accumulation occurs in lysosomes and mitochondria. At higher temperatures, the incorporation of propranolol into the membranes of these organelles, due to the lipophilicity of the drug, may lead to leakage into more widespread regions of the cell cytoplasm. The rate of propranolol uptake observed directly in these fluorescence lifetime imaging experiments is in accordance with previous nonimaging measurements using radiochemical analysis [7]. The fluorescence method here, however, not only gives details of uptake but also enables direct imaging of the intracellular location and concentration of the accumulated drug.

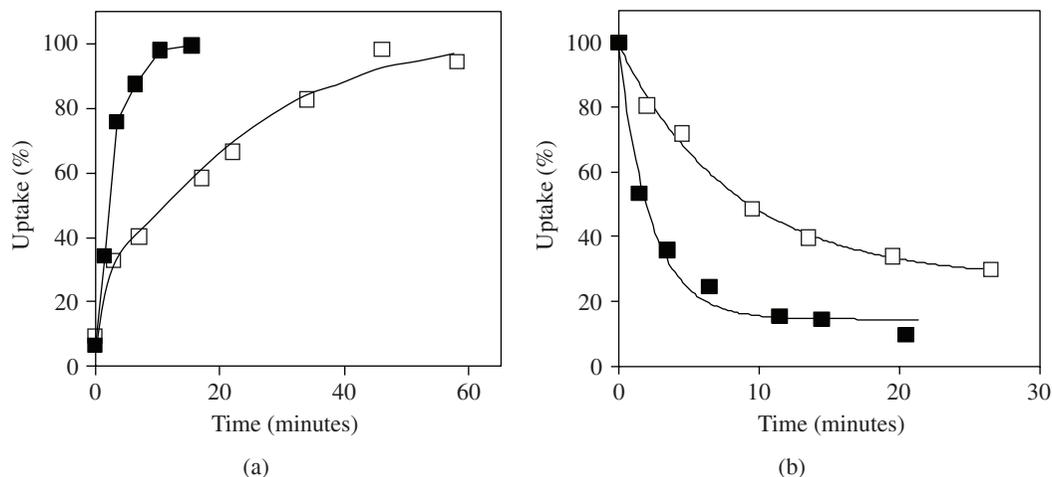


Figure 2: Time courses for uptake and release of propranolol from rat C6 glial cells. Uptake (a) was measured at 37°C from a solution containing SR-propranolol (100 μ M, ■) and at 22°C with S-propranolol (100 μ M, □). Cells previously loaded with propranolol (100 μ M) were washed with phosphate-buffered saline to observe release (b) of SR-propranolol at 37°C (■) and 22°C (□).

Acknowledgments

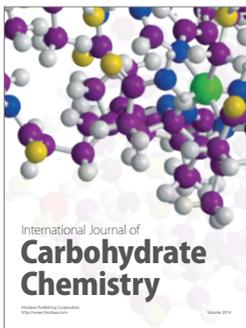
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