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Review Article

A simple and cost effective method for preparing FL and LG solutions

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ABSTRACT

Purpose: The purpose of this study was to develop a clinically feasible method for obtaining dye concentrations of 2% fluorescein (FL) and 1% lissamine green (LG) by soaking commercially available dye impregnated strips in saline.

Methods: Calibration curves were established to related known concentrations of dye to prepared FL fluorescence and LG absorbance. To determine the optimum number of dye strips and soaking times (preliminary testing), 1, 2, 3 FL or LG strips were soaked in 200 μl commercially available saline for 0.5, 1, 2, 3, 4 and 5 min, using calibration curves to determine FL and LG concentrations. The best combination of number of dye strips and soaking time was soaking 3FL and 3LG strips for 5 min and these were finally tested in 2 ml centrifuge tubes, selected for ease of use in a clinical setting.

Results: Preliminary testing indicated that soaking 3 FL or 3 LG strips for 5 min in saline yielded an average (±standard deviation) of 2.0 ± 0.000% FL and 0.93 ± 0.010% LG. Final testing of FL in centrifuge tubes (strips soaked for 3–15 min) yielded an average of 1.99 ± 0.040% FL, with no significant difference among time periods or dye lots tested. However, LG showed more variable results with an average of 0.80 ± 0.160% LG (5–15 min), with significant differences among dye lots and times (2-way ANOVA, $p < 0.05$).

Conclusions: This simple, reliable and relatively inexpensive method involves soaking 3 FL or LG strips in saline solution, yielding concentrations close to the 2%FL and 1%LG recommended for clinical trials, although LG showed more variability.

1. Introduction

Vital dyes, which are used to stain living tissues, have long been used clinically to identify defects in the ocular surface and highlight the tear film [1–3]. Three dyes are typically used, sodium fluorescein (FL), lissamine green (LG), and rose bengal (RB). However, because of toxicity [4,5] and patient discomfort [6,7] associated with RB solutions, RB has been largely replaced in clinical use by LG [8].

FL ($C_{20}H_{10}Na_2O_5$) is a fluorescent dye with a peak excitation occurring at wavelengths of 465–490 nm (blue) and emission at 520–530 nm (green). LG dye ($C_{27}H_{25}N_2NaO_7S_2$) is a non-fluorescent dye with a maximum absorbance at 550–670 nm, resulting in the dye's blue-green color [9]. FL is typically used to highlight corneal staining and the tear film, while LG is most often used to stain the conjunctiva, for example, in dry eye [8]. Clinically, both dyes are most often instilled into the eye using dye impregnated strips wetted with saline. However, for more precise applications and for clinical trials, instillation of known concentration and volume of dye is often desirable, as the amount of dye instilled can affect the intensity of fluorescence (FL) [10] and absorbance (LG) [11] when viewed on the ocular surface. To address this concern for more precise applications and in clinical trials, instillation of known amounts of FL (1 or 2 μl of 2%FL) [9,12] and LG (2–10 μl of 1%LG) [13,14] are often recommended.

Until recently, 2%FL and 1%LG solutions designed for this purpose have been readily available from several vendors in the U.S. However, changing regulations for compounding pharmacies have rendered these solutions more expensive and more difficult to obtain. The purpose of this study was to determine whether FL and LG dye impregnated strips could be used to consistently generate solutions of 2%FL and 1%LG that could be used in clinic and in clinical trials.

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2. Methods

2.1. Dye calibration

Before testing the concentration of FL and LG dyes obtained from strips, it was necessary to establish calibration curves for the dyes. Compounded solutions of 2% FL (FL powder, Sodium Chloride [usp], unpreserved sterile water) and 1% LG (LG powder, Sodium Chloride [usp], sterile unpreserved water) solutions were obtained (O'Brien pharmacy, Mission, Kansas) and diluted using a commercially available sterile solution (OcuFresh®, purified water 98%, sodium chloride, sodium borate, boric acid and benzalkonium chloride). FL was diluted in 0.1% steps from 0.4 to 2% and LG was diluted in 0.1% steps from 0.1 to 1%. All dilutions were performed under low light conditions (75.6 Lux) due to the potential photosensitivity of FL dyes [15]. Intensities of known dye concentrations were measured using a plate reader (Fluostar Galaxy, Mount Holly, NJ) at an excitation and emission wavelength of 485 nm and 520 nm, respectively, for FL and an absorbance wavelength of 595 nm for LG. Calibration experiments were repeated three times on three different days and read off immediately (<5 min after preparation) in the plate reader.

FL calibration data was fitted with a curve based on the known relationship between fluorescein concentration and intensity [16]. For LG calibration data, the MATLAB curve fitting tool (MATLAB®, The MathWorks, Inc.) was used to find the best fit curve between LG absorbance and concentration. These calibration curves were used to calculate dye concentrations from the measured FL intensities [16] (obtained using a lookup table) or LG absorbance of test solutions. In the following experiments, dye concentrations were calculated using equations derived from the best curve fit of the FL and LG calibration curves.

2.2. Test solutions: preliminary testing

Preliminary testing was done to determine the number of FL and LG strips and soaking times required to achieve concentrations of 1% LG and 2% FL solutions. According to Stock et al. [17], using 200 μl of artificial tears (preservative-free artificial tears (PFAT; Alcon, Ft Worth, TX), balanced salt solution (BSS; Alcon), and Proparacaine hydrochloride ophthalmic solution 0.5% (Akorn, Lake Forest, IL)] to soak either 4 LG strips for 1 min or 2 LG strips in 1.5-mL microcentrifuge tubes for 5 min produced concentrations of 1% LG. We could not find similar published data for soaking FL strips.

In our study, plastic 24-well plates (Corning ™ Costar ™ flat bottom cell culture plates with 1.9 cm^2 culture area, diameter of 1.6 cm and well volume of 0.5 ml) were used, as the width and depth of the wells allowed the dye-impregnated portion of the strips to lie flat on the bottom of the well when bent at a 90° angle. As was done for the calibration experiments, each dilution was prepared fresh on different days and disposed of after intensities were read immediately. A minimum of 100 μl was required for reading. After placing 1, 2, 3, or 4 strips (stacked) in each well, the dye-impregnated portions of FL (GloStrips, AmCon Laboratories, Inc. St. Louis, Missouri) and LG (GreenGLO, Hub Pharmaceuticals LLC Sigma, St Louis, MO) strips were covered with 200 μl of solution and soaked for 30 s, 1 min, 2 min, 3 min, 4 min, and 5 min. After soaking each 1, 2, 3, 4 for the above stated times, 100 μl of the resultant solution was pipetted into a sterile 96-well plate and covered with aluminum foil (to protect from light) until the dye intensities were measured. FL dye intensities were read at an excitation wavelength of 485 nm and an emission wavelength of 520 nm, and LG was read at an absorbance wavelength of 595 nm.

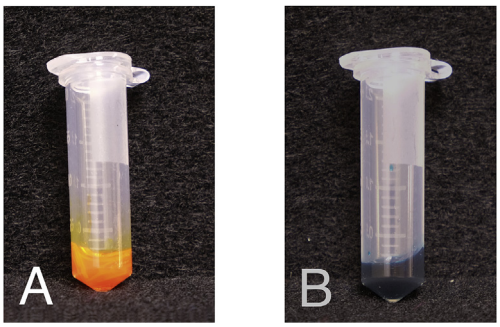

Fig. 1. Examples of three dye impregnated FL (A) and LG (B) strips soaking in 2.0 ml centrifuge tubes.

2.3. Test solutions: final testing

Because the use of 24-well plates is not practical in the clinical setting, further experiments were conducted using sterile plastic 2.0 ml centrifuge tubes (2.0 ml centrifuge tubes, VWR®, Radnor, PA) [16]. In this final phase of the experiment, we used data from preliminary testing to determine that 3 FL and 3 LG strips would be adequate to obtain approximate concentrations of 2%FL and 1%LG. The main purpose of the final testing was (1) to retest the time of soaking using tubes rather than 24-well plates, and (2) to determine the ability to obtain consistent dye concentrations.

FL and LG strips were cut at the junction where the dye-impregnated portion meets the non-impregnated portion of the strips. Sterile scissors and gloves were used during all procedures. Dye strips were then cut into 3 roughly equal-sized pieces to allow the strips to fit in the centrifuge tubes and be covered completely with the 200 μl of solution (Fig. 1A and B). Immediately after adding the eyewash, centrifuge tubes were shaken up and down twice to further ensure complete coverage of the strip pieces by the solution.

After each time period to be tested, FL and LG dye concentrations were determined from the respective calibration curves. Each experiment was repeated three times on three different days, and the mean and standard deviation of the resultant dye concentrations were determined. A two-way ANOVA with Bonferroni post hoc testing was used to compare FL and LG final results in vials across time and between dye lots.

3. Results

3.1. FL and LG dye calibration

Calibration results for FL and LG are shown in Fig. 2. FL data (Fig. 2A) was nonlinear, and fluorescence intensity decreased with increasing concentration. This was presumably due to the phenomenon of quenching at higher FL dye concentrations [16,18,19]. The negative slope of the line for our FL calibration suggests that all of our measurements were higher concentrations, indicating that increasing dye concentrations result in decreasing fluorescence intensity due to the phenomenon of concentration quenching [10,16,18]. A previously published mathematical model [16,19] was used to relate fluorescence intensity to concentration.[1] As Fig. 2A shows, the overall variability of FL calibration measures was low, although it increased with more dilute solutions. LG calibration data (Fig. 2B) was based on absorbance of the dye and was best fitted with a linear function ($y = 2.166x+0.669$; $r = 0.996$, $p < 0.001$,

[1] $I = I_0(1\text{-}e^{-\varphi f_0})/1+(f_0/f)^2)$, where I = FL intensity, f = FL concentration, f_0 = critical FL concentration, I_0 = scalar constant, e = Exponential, φ = molar extinction coefficient.

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