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A Fast and Sensitive Method for the Detection of Leuprolide Acetate: A High-Throughput Approach for the *In Vitro* Evaluation of Liquid Crystal Formulations

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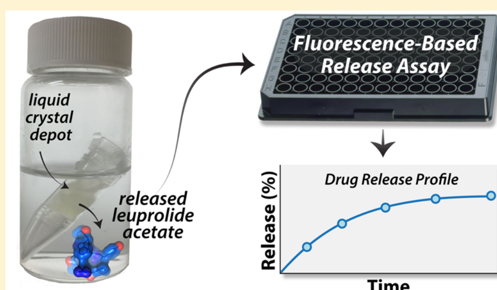
ABSTRACT: The suitability of using fluorescence spectroscopy to rapidly assay drug release by quantifying the time-dependent increase in total intrinsic protein fluorescence was assessed. Leuprolide acetate, a synthetic nonapeptide analogue of gonadotropin-releasing hormone (GnRH or LHRH), is the active pharmaceutical ingredient used to treat a wide range of sex hormone-related disorders, including advanced prostatic cancer, endometriosis, and precocious puberty. During the *in vitro* evaluation of drug delivery technologies for leuprolide acetate, one of the most time-consuming steps is the detection and accurate quantification of leuprolide release from formulation candidates. Thus far, the dominant means for leuprolide detection involves conventional multistep high-performance liquid chromatography (HPLC) methods, requiring sampling, dilutions, sample filtration, and chromatography, which can take up to 40 min for each sample. With the increasing demand for assay adaptation to high-throughput format, here we sought to exploit fluorescence spectroscopy as a tool to develop a novel method to rapidly assay the *in vitro* release of leuprolide acetate. By utilizing the intrinsic fluorescence of the tryptophan (Trp) and tyrosine (Tyr) amino acid residues present in the leuprolide nonapeptide, the *in vitro* release from liquid crystal formulations was accurately quantified as a function of fluorescence intensity. Here, we demonstrate that assaying leuprolide release using intrinsic protein fluorescence in a 96-well format requiring volumes as low as 100 μL is a cost-effective, rapid, and highly sensitive alternative to conventional HPLC methods. Furthermore, the high signal-to-noise ratios and robust Z′-factors of >0.8 indicate high sensitivity, precision, and feasibility for miniaturization, high-throughput format adaptation, and automation.



Detection and quantification of total drug release are usually the most pivotal steps in formulation development. Traditionally, high-performance liquid chromatography (HPLC) is the dominant analytical technique for assaying the *in vitro* release of leuprolide acetate, a synthetic nonapeptide analogue of gonadotropin-releasing hormone (GnRH) (also known as luteinizing hormone-releasing hormone (LHRH)). Leuprolide acetate is the active pharmaceutical ingredient used for treatment in a wide range of sex hormone-related disorders, including advanced prostatic cancer, endometriosis, and precocious puberty.[1] Several HPLC methods are now available for the analysis of leuprolide acetate.[2−5] Each method is sensitive and extremely precise, especially when combined with mass spectrometry (MS), where the limit of quantitation can be as low as 0.018 ng/mL.[5] However, despite all the well-known benefits of traditional HPLC methods, the use of HPLC is often associated with high cost, time-consuming sample preparation, and chromatography, which can require large volumes of expensive organic solvents. These drawbacks associated with analytical HPLC methods may render them inadequate for characterization of drug release kinetics for a large number of formulation candidates.

In protein biochemistry, the use of intrinsic protein fluorescence is a ubiquitous practice applied to the study of folding/unfolding, protein−protein interactions, substrate binding, and external quencher accessibility, among others.[6] An emerging but less commonly used application of intrinsic protein fluorescence is protein quantification.[7] In principle, intrinsic protein fluorescence originates from the aromatic amino acids residues tryptophan (Trp), tyrosine (Tyr), and phenylalanine (Phe), with Phe being the weakest fluorophore.[8] While Tyr and Trp have similar quantum yields, the indole group of Trp is considered the dominant source of UV absorbance and emission in proteins.[8,9] Leuprolide acetate is a synthetic Trp/Tyr-containing nonapeptide drug with the primary sequence of pGlu-His-Trp-Ser-Tyr-D-Leu-Leu-Arg-Pro-NHEt. Here, we utilized the intrinsic fluorescence of Trp and Tyr amino acid residues as a fit-for-purpose approach to the detection and quantification of leuprolide release, with the intended purpose of fast and sensitive routine drug release screening of liquid crystal formulations *in vitro*. For two liquid crystal formulations, a weeklong drug release kinetic study was assayed in a 96-well format, requiring volumes as low as 100 μL. The release patterns obtained from the quantitative fluorescence-based method

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allowed for the rapid assessment of drug burst release, and the data are in agreement with parallel studies conducted using traditional HPLC methodologies. In conclusion, the fluorescence-based quantitative 96-well assay demonstrated good signal strength, wide dynamic range, and high precision; key parameters in assay adaptation for high-throughput format and automation.

■ EXPERIMENTAL SECTION

Materials. Leuprolide acetate was purchased from Polypeptide Group (San Diego, CA, USA). Soy phosphatidylcholine (PC) was purchased from Lipoid GmBH (Ludwigshafen, Germany). Sorbitan monooleate (SMO) was purchased from Seppic (Puteaux, France). Tocopherol acetate (TA) was purchased from DSM Nutritional Products Limited (Sisseln, Switzerland). The 96-microwell plates (Corning Costar 96-well black flat-bottomed (No. 3915) and UV transparent flat-bottomed (No. 3635)) were purchased from Fisher Scientific. Pur-A-Lyzer Mini Dialysis Kit (PURN12100) was purchased from Sigma–Aldrich. Captiva syringe filters and Microsorb-MV 100-5 C18, 150 mm × 4.6 mm column were purchased from Agilent Technologies. All other chemical and reagents were of analytical grade and were purchased from Sigma–Aldrich or Fisher Scientific.

Fluorescence Spectroscopy. The absorption, excitation, and emission spectra of leuprolide acetate were recorded at room temperature, using the Synergy H1 multimode reader from BioTek. Leuprolide acetate was dissolved in simulated body fluid (SBF) buffer[10] to a final concentration of 1 mg/mL. Standard samples of leuprolide acetate were prepared in serial dilutions, and 100 μL were transferred to clear (for absorbance) and black (for fluorescence) 96-well microplates. The absorption spectra were recorded from 230 to 700 nm. The fluorescence excitation spectra were recorded from 250 to 310 nm at fixed emission wavelengths (λ_{em}) of 330, 340, and 350 nm (top read at fixed 16 nm monochromator bandwidth). To achieve maximum fluorescence, the excitation wavelength (λ_{ex}) was established at 280 nm, also corresponding to the wavelength of maximum absorbance (λ_{max}). The fluorescence emission spectra were recorded from 310 to 500 nm, resulting in an emission peak at 340 nm. Fluorescence interference by assay components was determined by evaluating the spectral properties of leuprolide acetate in release media containing excess amounts of liquid crystal (LC) excipients that could emerge from the disintegration of the LC during the *in vitro* experiments. For this, to promote and accelerate the disintegration of the LC, a sample was prepared by injecting 100 μL of LC into 10 mL of SBF followed by probe sonication at room temperature for 5 min at 40% amplitude.

Method Validation: Analysis of Dynamic Range, Sensitivity, Precision, and Assay Quality. Standard solutions of leuprolide acetate dissolved in SBF at concentrations ranging from 1 mg/mL to 0.001 mg/mL were used to determine the optimal region for the calibration curve and the dynamic range of the assay, where the arbitrary fluorescence units (AFU) has a linear response to increasing the concentration of leuprolide acetate. Assay quality, sensitivity, and precision were evaluated in a 96-well format and 100 μL assay volume using the equations described below. In these equations, μ_S and μ_B are the mean AFU values from the sample set (leuprolide acetate positive controls, 125 μg/mL) and background set (release media SBF, negative controls), respectively. The standard deviation of the AFU from the sample set and background set are expressed as σ_S and σ_B, respectively. First, the dynamic range and sensitivity were evaluated by calculating the signal-to-background ratio (S/B) using eq 1.

$$S/B = \frac{\mu_S}{\mu_B} \quad (1)$$

Because the S/B does not contain any information regarding data variability, the signal-to-noise ratio (S/N) was calculated using eq 2. The S/N ratio measures the degree of confidence with which a signal can be regarded as real and can capture the variability in the SBF background controls.

$$S/N = \frac{\mu_S - \mu_B}{\sigma_B} \quad (2)$$

Data variability in the sample set was evaluated by calculating the percent coefficient of variation (%CV), using eq 3, which divides the standard deviation of leuprolide acetate samples by the mean. A low %CV value indicates high precision, whereas a high %CV value indicates poor precision.

$$\%CV = \frac{\sigma_S}{\mu_S} \times 100 \quad (3)$$

Assay quality was evaluated using the statistical parameter Z'-factor described by Zhang et al.[11] (see eq 4), which reflects both the assay signal dynamic range and data variation associated with the signal measurements in the sample set and background control set. In practice, as the standard deviations approaches zero (small data variation), or as the dynamic range increases (the average of the sample signal minus the averages of the background signal approaches infinity), the Z'-factor approaches unity with a coefficient of 1 being an ideal assay, $0.5 \leq Z' < 1$ representing an excellent assay, $0 < Z' \leq 0.5$ denoting a marginal assay, and $Z' < 0$ being a nonuseful assay, since there is too much overlap between positive and negative controls.

$$Z' = 1 - \frac{(3\sigma_S + 3\sigma_B)}{|\mu_S - \mu_B|} \quad (4)$$

Preparation of Liquid Crystal (LC) Systems. The LC forming material was prepared by mixing PC, SMO, and TA in ethanol (90:10, % w/w) in a 20 mL scintillation vial and allowed to mix overnight by continuous vortex agitation at room temperature. In a second 20 mL scintillation vial, a total of 75 mg of lyophilized leuprolide acetate was dissolved in 100 mg of dimethyl sulfoxide (DMSO). A total of 1825 mg of LC-forming material was then added to the leuprolide acetate solution and was mixed overnight by continuous vortex agitation at room temperature. The final dose contained 3.75 mg of leuprolide acetate in a total of 100 μL of the LC forming material designed as formulation 1 (PC, SMO, and TA, 60:30:10, % w/w) and formulation 2 (PC and Span 80, 60:40, % w/w). Formulations 1 and 2 were chosen because their highly divergent release kinetics allowed for method development and evaluation at the lower and upper detection limits.

Development of a 96-Well Fluorescence-Based Assay for the *In Vitro* Evaluation of Liquid Crystal Formulations. To evaluate the *in vitro* release of leuprolide acetate from the LC formulations, 100 μL of LC-forming material loaded with 3.75 mg of leuprolide acetate was injected into a Pur-A-Lyzer tube and transferred to a 20 mL scintillation vial containing 10 mL of release media SBF and 0.01% sodium azide. The *in vitro* release of formulations 1 and 2 was evaluated in triplicate at 37 °C under constant shaking at 100 rpm. Aliquots of 100 μL and 200 μL of release media were taken at predetermined time points, followed

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by volume replacement with 100 μL or 200 μL of fresh SBF, respectively. Time point samples were placed in 96-well black microplates, and the concentration of leuprolide acetate in the release media was quantified by fluorescence spectroscopy using the Synergy H1 multimode microplate reader. The 96-well assay plate was designed to include leuprolide acetate standards in duplicate (24 wells), positive and negative controls (12 wells), and 60 sample wells, allowing for the analysis of the first week of drug release from two formulations in 100 μL and 200 μL assay formats. Positive and negative controls correspond to the maximum detection limit (125 μg/mL leuprolide acetate in SBF) and SBF, respectively. The relative fluorescence intensities from released leuprolide acetate were measured using a gain of 78, a monochromator bandwidth fixed at 16 nm, λ_{ex} = 280 nm, and λ_{em} = 340 nm. All measurements were taken within the assay's dynamic range. Formulations with fast release resulting in high concentrations of released leuprolide acetate were diluted to a 1:1 ratio with SBF and the resulting AFU were corrected for the dilution factor. Results were reported as the mean ± standard deviation of triplicate measurements.

Analysis of Drug Release by High-Performance Liquid Chromatography (HPLC). HPLC analysis of drug release on day 7 was performed with an Agilent 1260 Infinity system (Agilent Technologies) equipped with a quaternary pump and degasser, diode-array detector, autosampler, and thermostated autosampler, and column compartment. The mobile phase was composed of solution A (15.2 mg/mL of triethylamine in water) at pH 3.0 adjusted with phosphoric acid, mixed to a 3:1 ratio with solution B (acetonitrile and 2-propanol, 3:2). Samples of 1 mL taken at day 7 were filtered using a 0.45 μm Captiva syringe filter. A 50 μL injection volume was used for chromatographic separation using a Microsorb-MV 100-5 C18 column, 150 mm × 4.6 mm. The isocratic separation was performed at a flow rate of 1 mL/min with UV detection at 220 nm.

RESULTS AND DISCUSSION

Optical Properties of Leuprolide Acetate by Absorption and Fluorescence Spectroscopy. Fluorescence methods have evolved into powerful tools for polymer research[12−14] and the study of biological molecules.[15,16] High-throughput assays used in drug discovery frequently utilize fluorescent reporters to enable large numbers of experiments to be performed in miniaturized and automated fashion.[17] The use of intrinsic protein fluorescence, using Trp as a reporter, has provided a sensitive measurement of protein structure, function, and dynamics.[6,15,16] More recently, Trp fluorescence has been applied to the determination of total protein concentration under denaturing conditions.[7] Here, we explore the use of intrinsic protein fluorescence to develop a quantitative methodology for the rapid and accurate quantification of the Trp/Tyr-containing synthetic nonapeptide drug, leuprolide acetate. As expected for peptides containing aromatic amino acid residues, the absorbance spectral scan of leuprolide acetate resulted in a λ_{max} at 280 nm (Figure 1A). While the excitation spectra scan at a fixed λ_{em} of 340 nm had a peak maxima closer to ~270 nm (data not shown), the resulting fluorescence intensities were found optimal at a λ_{ex} value of 280 nm (Figure 1A). Therefore, at fixed λ_{ex} of 280 nm, the resulting spectral emission peak of 340 nm (Figure 1A) indicates that both chromophores (Trp indole ring and Tyr phenolic hydroxyl group) can contribute to the intrinsic fluorescence of leuprolide acetate.[9]

The study of protein biochemistry using Trp as a fluorescent reporter is possible, because of the high sensitivity of Trp

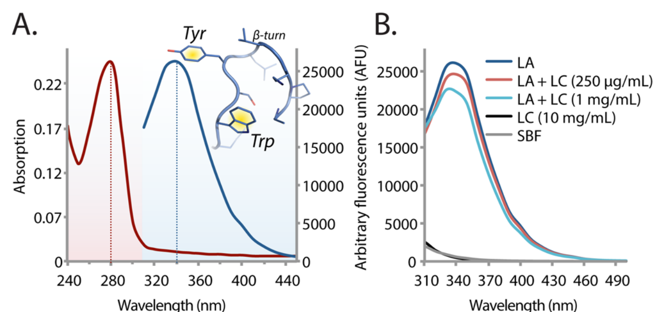

Figure 1. Absorbance spectra and fluorescence emission spectra of leuprolide acetate. (A) Absorption (red line, first *y*-axis) and fluorescence emission (blue line, second *y*-axis) spectral scan of leuprolide acetate (125 μg/mL in SBF) under excitation at 280 nm. The inset shows a cartoon representation of the NMR solution structure of leuprolide acetate (PDB: 1YY2)[19] depicting the location of Tyr and Trp amino acids (highlighted). (B) Effect of liquid crystal (LC) excipients on the fluorescence emission spectra of leuprolide acetate (LA) under excitation at 280 nm.

fluorescence to its local chemical microenvironment.[16] As a result, in nondenaturing conditions, Trp fluorescence can have a nonlinear relationship with changes in protein conformation, limiting its usefulness in protein quantification. Under denaturing conditions or for peptides having limited secondary structure, however, Trp fluorescence has been shown to be suitable for total protein determination of whole tissue lysates and peptide quantification in a 96-well format.[7] In the case of leuprolide acetate (and synthetic peptide analogues), the solution NMR structure indicates that the putative bioactive conformations contain a type II β-turn in the Ser-Tyr-D-Leu-Leu segment (Figure 1A),[18,19] potentially leaving the chromophore-bearing fragment Trp-Ser-Tyr solvent-accessible. Because solvent accessibility can also lead to fluorescence interference by assay components, the effect of the formulation excipients on the fluorescent properties of leuprolide acetate was evaluated. In the presence of excess amounts of disintegrated LC, the emission maximum shifted slightly from 340 to 330 nm, causing <5% signal quenching at λ_{em} = 340 nm (Figure 1B). However, because there is no detectable disintegration of the LC in our *in vitro* assay, we concluded there is no significant fluorescence interference or Trp quenching under these conditions. Nevertheless, it is worth noting that for release studies containing Tween 80 in the release media, which promotes the erosion of the LC depot, further analysis would be required to determine the most appropriate λ_{ex} and λ_{em} to minimize any potential fluorescence interference or quenching effect. As a result, the versatility of the method is limited to samples lacking other components that are optically active near these excitation and emission wavelengths, especially release media containing Tween 80, TA, or other Trp/Tyr-bearing proteins commonly present in fetal bovine serum (FBS), fetal calf serum (FCS), and other biological fluids.

Development of a 96-Well Fluorescence-Based Assay for the Detection and Quantification of Leuprolide Acetate Release. Key differences from orthodox HPLC approaches permit the direct application of existing high-throughput method validation paradigms for the development of a new fluorescence-based quantitative method for leuprolide acetate. In assay miniaturization for high-throughput screening (HTS) applications, several parameters are obtained during assay development to gauge the precision and reliability of the assay

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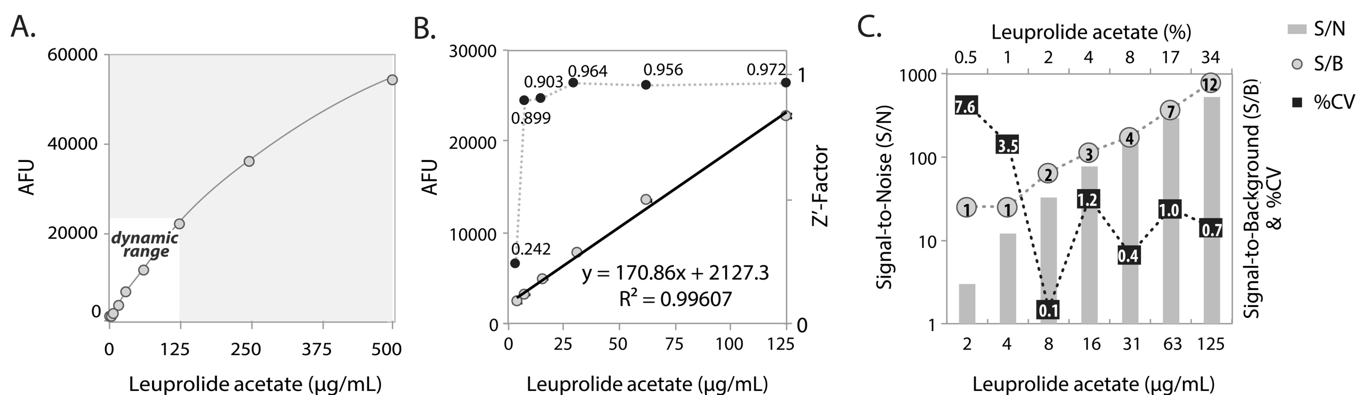

Figure 2. Dynamic range, assay quality, sensitivity, and precision. (A) Dynamic range and optimal region of calibration curve (highlighted). (B) Standard calibration curve of AFU (first y-axis) versus amount of leuprolide acetate displays linearity at concentrations ranging from 4 μg/mL to 125 μg/mL. Robust Z'-factor (black, second y-axis) values of >0.899 were achieved at concentrations above 8 μg/mL. (C) High sensitivity and precision are detected at leuprolide acetate concentrations ranging from 8 μg/mL to 125 μg/mL with both the signal strength (S/N) and dynamic range (S/B) ratios increasing, while the %CV values decrease.

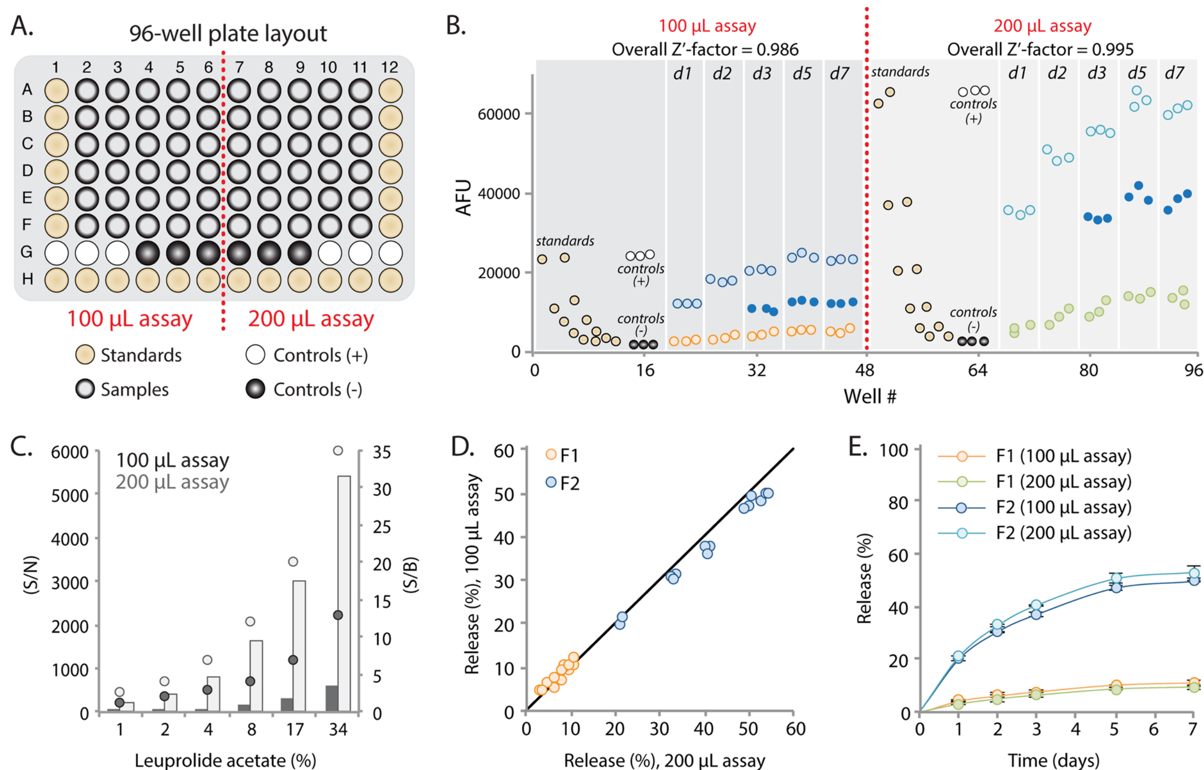

Figure 3. *In vitro* release analysis of leuprolide acetate in a 96-well-based assay. (A) 96-well-based assay layout for 100 μL and 200 μL assay volumes, depicting the well location of standard samples, *in vitro* release samples, positive and negative control samples. (B) Scatter plot of raw AFU values from the 96-well-based 100 μL and 200 μL assays, showing the fluorescence intensities of the standard samples (beige), positive and negative controls (white and black, respectively), and leuprolide acetate released from formulation 1 (orange and green) and formulation 2 (blue and teal) at day (d) 1, 2, 3, 5, and 7 from triplicate *in vitro* release experiments. Dark blue dots represent the 1:1 dilution of samples from formulation 2 with AFU values close to the positive controls. (C) Comparison of the S/N (bars, first y-axis) and S/B (dots, second y-axis) from the 100 μL (dark gray) versus 200 μL (light gray) assays. (D) Replicate plot of the cumulative percentage (%) of leuprolide release in triplicates, from formulation 1 (F1) and formulation 2 (F2) in 100 μL (y-axis) versus 200 μL (x-axis) assays. (E) A weeklong release profile of F1 and F2 expressed as mean cumulative percentage (%) in 100 μL and 200 μL assay formats.

and HTS data.[20] Among these are the statistical parameter Z'-factor coefficient, signal-to-noise ratio (S/N), signal-to-background ratio (S/B), and percent coefficient of variation (%CV) (*vide infra*). Initially, to assess the feasibility of the assay for the intended purpose, it is imperative to determine the assay's dynamic range. The dynamic range is the range over which the AFU are proportional to the amount of leuprolide acetate in solution under predetermined assay conditions. The dynamic range of the assay is then further evaluated to determine accuracy, precision, and robustness for adaptation to a microwell assay format.

Here, the dynamic range of the assay was evaluated in a 96-well format containing 100 μL assay volumes, and the AFU of the leuprolide acetate standard samples and background controls

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Table 1. Comparison between Fluorescence- and HPLC-Based Methods

method	Release (%) on Day 7 formulation 1, F1	Release (%) on Day 7 formulation 2, F2	sample preparation	analysis format	analysis time per sample	detection limit, DL
fluorescence-based						
100 μL assay	11 ± 1	50 ± 0.2	no	96-well throughput	<1 s	5 μg/mL
200 μL assay	10 ± 1	53 ± 2	no	96-well throughput	<1 s	5 μg/mL
HPLC-based	7 ± 1	51 ± 1	yes	single injection	45 min	1.5 μg/mL

(release media SBF) were recorded at a fixed λ_{ex} of 280 nm and λ_{em} of 340 nm. The linear response of AFU to leuprolide acetate at concentrations ranging from 125 μg/mL to 4 μg/mL indicates the optimal concentration range for calibration curve analysis (see Figures 2A and 2B). The resulting dynamic range, as shown by the S/B ratio, corresponds to AFU outputs increasing the background signal of the release media from 2-fold to 12-fold (see Figure 2C). The parameter S/B gauges the strength of the AFU signal from the standard samples, in comparison with the SBF background measurements, and is often evaluated in combination with the S/N parameter. The S/N parameter evaluates the AFU signal strength, in relation to the noise (signal scatter), capturing the variability of SBF background control samples. In other words, the S/N measure the degree of confidence with which a signal can be regarded as real, and when the S/B is low, an S/N of >10 is desired. Although our assay yields S/B < 10, high precision and accuracy at concentrations above 4 μg/mL are indicated by the high S/N ratios, with increasing values from 12 to 500, in relation to increasing leuprolide amount.

The assay's dynamic range was further evaluated by calculating the statistical parameter Z'-factor. The Z'-factor measures assay quality by incorporating the assay signal dynamic range, data variation associated with signal measurements, and the data variation associated with the reference control measurements.[11] At 8 μg/mL leuprolide acetate, the Z'-factor of >0.899 indicates small data variation and a broad dynamic range, suitable for high-throughput applications (Figure 2B). When the concentration of leuprolide acetate exceeds 8 μg/mL, the Z'-factor approaches 1, which is indicative of an excellent assay. Similar trends associated with leuprolide acetate concentration were observed in the S/N and S/B ratios, and %CV. The assay demonstrated good signal strength, large dynamic range, and high precision, as determined by the increasing ratio values, while the %CV decrease with increasing leuprolide acetate concentration (see Figure 2C). Finally, the detection limit (DL), the concentration of analyte that can be readily detected, was determined per the International Conference on Harmonization (ICH) Q2(R1) guidelines[21] and was found to be 5 μg/mL. Therefore, under these assay conditions, the dynamic range was established at leuprolide acetate concentrations ranging from 8 μg/mL to 125 μg/mL, and all further measurements were taken within this concentration range.

One-Week *In Vitro* Release of Leuprolide Acetate for Assessment of Burst Release. *In vivo*, the 1-month release of leuprolide acetate from lyotropic liquid crystal systems composed of PC:SMO:TA is characterized by an initial burst release effect that progresses over time to a reduced release rate.[22] In the development of sustained-release drug delivery technologies, limiting the burst effect can prolong the sustained release ability of the formulation and potentially reduce any adverse side effects.[23,24] A limiting factor in developing liquid crystal formulations with optimal drug delivery characteristics is the requirement for screening a large number of formulation candidates by means of long HPLC methods. Therefore, to develop formulations with reduced burst release, it is imperative to achieve the rapid evaluation of the burst release in a sensitive and accurate manner.

In an attempt to develop a rapid means for evaluating the burst release, here we developed 100 μL and 200 μL 96-well-based assays to evaluate the first 7 days of *in vitro* release from formulation 1 and formulation 2 (Figure 3A), previously characterized in our laboratory using traditional HPLC methods. The 96-well plate layout was designed to accommodate the first 7 days of drug release from the two formulation candidates, leuprolide acetate standard samples, and internal positive (leuprolide acetate 125 μg/mL in SBF) and negative (release media SBF) controls. The *in vitro* release of leuprolide acetate from LC formulations (3.75 mg of leuprolide acetate per 100 μL of LC) was performed in 20 mL scintillation vials containing 10 mL of SBF, shaking at 100 rpm and incubated at 37 °C. Under these conditions, at 100% release, a total of 3750 μg (or 375 μg/mL) of leuprolide acetate is present in the release media. Hence, in our *in vitro* assay containing 10 mL of release media, the DL corresponds to <1.5% released leuprolide acetate and is well below the assay's dynamic range, equivalent to 2%−34% release (Figure 2C). Importantly, the dynamic range of the release study (2%−34% release) can be adjusted as needed by simply increasing or reducing the volume of the release media in the *in vitro* assay for formulations with fast or slow release, respectively.

The raw AFU data from the 96-well-based assays described in Figure 3A is presented in Figure 3B. Large signal separation is observed between the controls and the *in vitro* release samples, with increasing dynamic range relative to increasing the sample volume from 100 μL to 200 μL. When the sample's fluorescence intensity approached the intensity of the positive controls, they were diluted 1:1 and reanalyzed (Figure 3B). Both assays displayed robust overall Z'-factors above 0.9 (Figure 3B); however, an additional ~10-fold increase in assay sensitivity was achieved by increasing the sample volume from 100 μL to 200 μL (Figure 3C).

Repeatability, robustness, and feasibility for assay adaptation from 100 μL to 200 μL were evaluated by comparing the intra-assay precision between the two independent 96-well-based assays. The replicate plot displays the first 7 days of cumulative percent release of leuprolide acetate from triplicate *in vitro* release vials (Figure 3D). The intra-assay precision between the triplicate samples, and between the 100 μL- versus 200 μL-based assays is revealed by the data points aligning within the replicate line, indicative of high precision and reproducibility. The cumulative percent release of leuprolide acetate from formulation 2 displayed increased drug burst release relative to formulation 1, with almost 6-fold higher percent released at day 7 (Figure 3E). Although further analyses are required to fully characterize the role of each excipient, the significantly reduced release rate of formulation 1 could indicate a potential role for TA in limiting the burst release. More importantly, the quantities

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presented herein are in good agreement with results obtained from side-by-side HPLC analysis (Table 1). Thus, even with a higher DL (5 μg/mL), compared to HPLC-based methods, which can detect as little as <1 ng/mL,[5,25] the *in vitro* drug release from slow- or fast-releasing formulations can be rapidly and accurately assayed in a cost-effective manner.

Because of its ease, low cost, lack of sample preparation, and high-throughput data acquisition and analysis, the miniaturized fluorescence-based method provides a practically feasible, rapid, and highly accurate alternative method for *in vitro* applications. Furthermore, to our knowledge, this is the first study that employs intrinsic protein fluorescence for detection and quantification of drug release *in vitro*. More importantly, as a subsidiary method for formulation assessments prior to *in vivo* analysis, the method can be applied to release studies of other Trp/Tyr-containing peptide drugs; a proof-of-concept of how intrinsic protein fluorescence, a simple and ubiquitous approach in protein science, have significant applications in the field of drug delivery.

■ CONCLUSIONS

In drug release studies, most methodologies for the detection and accurate quantification of peptide release rely on long, multistep, and tedious high-performance liquid chromatography (HPLC) methods. Currently, analytical HPLC methods are the benchmark for detection and quantification of the synthetic nonapeptide drug, leuprolide acetate. However, incompatibility with high-throughput applications and high associated costs are limiting factors when screening a large number of formulations *in vitro*. The use of intrinsic protein fluorescence as a method to study protein−protein interactions, ligand binding, or changes in molecular conformation has been widely established in the field of protein biochemistry. An emerging but less commonly used approach for protein quantification also uses tryptophan (Trp) fluorescence. A fit-for-purpose approach to detection and quantification of leuprolide acetate is proposed, keeping in mind the intended use for fast and sensitive routine *in vitro* screening of liquid crystal formulations. By using leuprolide intrinsic fluorescence, detection and quantification of leuprolide release from formulation candidates were achieved with accuracy in a 96-well format. The straightforward data analysis within this context of a fit-for-purpose method is well-suited for successful implementation as a routine screening *in vitro* assay. Quantification of leuprolide acetate using the fluorescence-based method allows for an increased number of release kinetic analyzes, faster formulation development, and evaluation of large numbers of formulation candidates before advancing to *in vivo* studies.

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Notes

The authors declare no competing financial interest.

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