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Short communication

Simple liquid-chromatographic method for Nile Red quantification in cell culture in spite of photobleaching

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Abstract

Nile Red fluorescent marker is widely-used for different purposes, such as staining cell structures and for the visualization and localization of colloidal drug carriers. However, when fluorescence-dependent imaging or quantification is performed, the risk of inexact results is increased due to photobleaching. The proposed, simple quantification method of using an HPLC–UV–Vis system allows the determination of Nile Red even in photobleached samples. The intra- and inter-assay accuracies for all analytes were found to be within 94.9 and 100.8%, respectively, of target values. When samples underwent photobleaching by laser, UV–Vis detection varied at around 99±5%, whereas fluorescence decreased down to 86%. Such results show this method to be interesting for approaches where quantification should be performed after analysis such as fluorescent imaging.

Keywords: Photobleached samples; Nile Red

1. Introduction

Nowadays, nano-size drug delivery systems are thought to be one of the future prospects in medical and pharmaceutical development, allowing drugs to be targeted to diseased tissues or organs resulting in a distinct accumulation at the designated site of action [1]. During the development of such colloidal formulations, a major interest consists of the localization and quantification of the drug carriers, e.g. liposomes and nanoparticles, in order to determine their biodistributive behavior. For mechanistic purposes the colloidal carriers are usually tested in cell culture to follow their cell uptake pathways and to analyze the biological activity in-vitro. Since their major constituents, such as phospholipids or polymers, are not easily visualized by ordinary microscopy, markers are required in order to improve their detection by microscope.

Nile Red is known for its use as a lipid dye for different purposes [2–6]. It has a wide range of applications due to its fluorescent properties which allow low detection limits. Nile Red is in current use for such localization approaches for nanoparticles [7,8], liposomes [9], and other lipid dispersed carrier systems [10]. The fluorescent properties of Nile Red have especially identified it as a dye for imaging processes such as fluorescence microscopy, where it is used in the structural analysis of samples [11,12].

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However, quantification can still be difficult in cases where prior photobleaching produces inexact results. Until now it has been impossible to use the same sample for a fluorescent imaging procedure, such as confocal laser scanning microscopy, and a later quantitative determination of the marker molecule. Inverse microscopes provide access to observe living cell culture systems. It could therefore be important to quantitatively analyze carrier uptake that has previously been observed under fluorescence microscopy.

In this work, the quantification of Nile Red is proposed by using a simple and fast high-performance liquid chromatography (HPLC) method using a UV–Vis detection system. Such a method could permit the exact determination of Nile Red in the sample even after an intensive investigation of the sample under fluorescent microscopy. The influence of bleaching on the stability of UV–Vis detection has also been analyzed.

2. Materials and methods

2.1. Materials

Nile Red was purchased from Sigma (Steinheim, Germany). HPLC grade methanol was obtained from Fluka (Deisenhofen, Germany). A Milli-Q Plus 185 water-purification system (Millipore Corp., Molsheim Cedex, France) was used to obtain purified water for the HPLC solvent. A C_6 glioma cell line was obtained from ATCC (Manassas, VA, USA). Approximately 10 000 cells per well were seeded in 24 cavity well-plates and grown for about 96 h under standard cell culture conditions in Dulbecco's Modified Eagle Medium (DMEM) before use [13].

2.2. HPLC method

The HPLC system consisted of the Waters 717plus Autosampler (Waters Chromatography Division, Milfold, MA, USA), a Solvent Delivery Module Waters 600 Controller, a Waters in-line vacuum degasser, and a Waters 996 Photodiode Array Detector. Separation was carried out on a reversed-phase RP-18 column (LiChrospher® 100, Merck, Darmstadt, Germany). The mobile phase was kept as simple as possible which consisted of an isocratic eluent of methanol–water 93:7. The flow-rate was maintained at 1.0 ml/min. The effluents were monitored for their UV–Vis spectra of Nile Red and were recorded between 200 and 700 nm. For quantification purposes, Nile Red was detected by absorption at 559 nm, samples of 50 μl being injected into the column. All chromatographic analyses were performed at room temperature (25 °C).

2.3. Calibration standards

Stock solutions of Nile Red were prepared by dissolving the dye in methanol at a concentration of 50 μg/ml. These stock solutions were then diluted with methanol to obtain working solutions containing 14 and 7 mg/l, respectively. To prepare calibration samples, Nile Red-free DMEM buffer solutions were spiked with the appropriate volume of the working solutions to contain Nile Red at concentrations of 3, 15, 50, 200, 800 and 3500 μg/l. The calibration curves were constructed by linear regression using the peak area of Nile Red plotted against the corresponding concentrations. Quality-control samples for Nile Red detection in the cell culture systems were prepared at concentrations of 200, 800 and 3500 μg/l and spiked to C_6 glioma cells in 24 well-plates. Cells were incubated with the dye solution for 1 h and then treated with a microson ultrasonic cell disruptor (Misonix Inc., Fermingdale, NY) at 4 W for 15 min to induce cell disruption and lysis in order to permit maximal recovery of Nile Red from the different lipophilic cell compartments before injecting them into the HPLC system.

2.4. Photobleaching experiments

Nile Red solutions of 100 μg/ml (8:2 methanol–water mixture in order to provide sufficient solubility for the dye) were bleached by laser at 488 nm (Uniphase Laser Systems, San José, CA, USA, Model 2213-75SLYVW) with a laser power of 0.3 W. The different samples were treated for cycles of either 0, 2000 or 3000 s. Thereafter, these solutions were analyzed by the HPLC–UV method or with fluorescence spectrophotoscopy (F-2000 Fluores-

cence Spectrophotoscope, Hitachi Ltd., Tokyo, Japan).

2.5. Fluorescence spectrophotoscopy

With an unbleached Nile Red solution, excitation and emission spectra were recorded. Based on the determined wavelength scans, 1.5 ml of bleached or unbleached Nile Red solutions were analyzed for their fluorescent integrity at excitation wavelengths of 480 nm where the emission signal was recorded at 580 nm.

3. Results and discussion

3.1. Chromatography and UV–Vis detection

A simple, sensitive, and solvent-saving HPLC method was developed and validated. The strategy was to use simple components and to apply an inexpensive mobile phase as well as to decrease the run-time. Since no HPLC method is available in the literature to our knowledge, such an easily accessible quantification system might be of interest to others for the determination of Nile Red.

The retention time for Nile Red was 4.0 min (Fig. 1). The run-time was reducible down to 6 min. The UV–Vis spectrum exhibits two peaks at wavelengths of 265 and 559 nm (Fig. 2a). For detection in cell extracts or tissue samples containing lysed components, the wavelength of 559 nm is the best of the two due its greater capacity for differentiation.

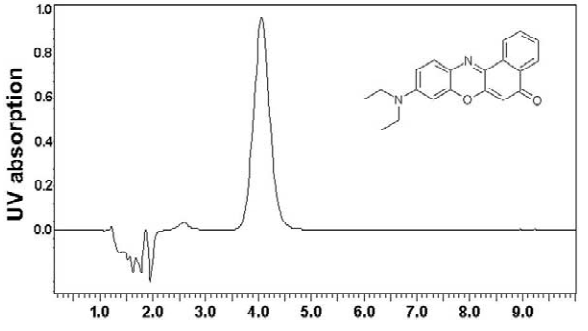

Fig. 1. Typical chromatogram of Nile Red exhibiting a retention time of about 4 min. The molecular structure of Nile Red is given in the insert.

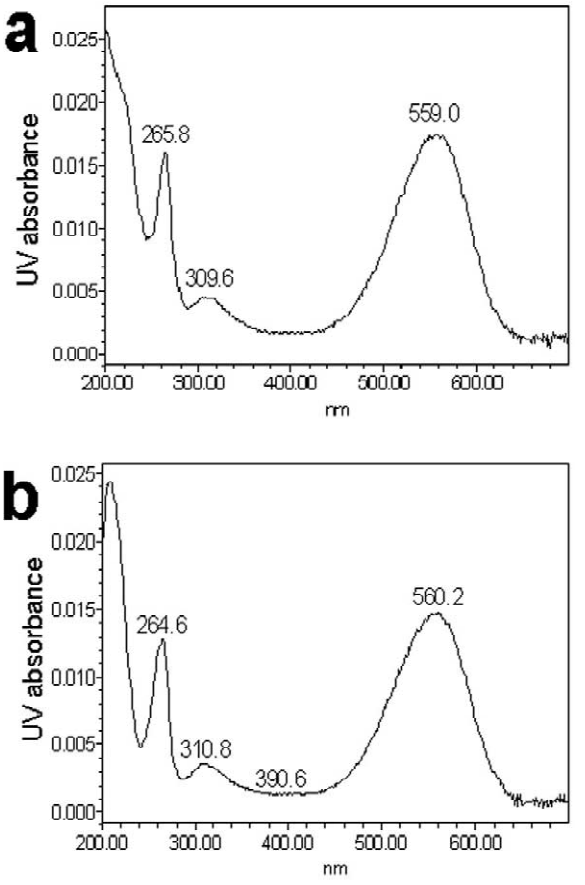

Fig. 2. UV absorption scans from wavelength of 200 to 700 nm at the retention time of 4 min. The two scans compare the spectra of samples before (a) and after 3000 s of bleaching (b).

3.2. Linearity, the limit of detection (LOD) and the limit of quantification (LOQ)

The calibration line for Nile Red was linear in the concentration range investigated. Slopes and intercepts, the standard deviations of the slope and the intercept, and the coefficients of determination were as follows: Signal = $(410.9 \pm 2.5) \times$ [Nile Red] + (11428 ± 3973), $r^2 = 0.9998$.

The limit of detection (LOD), defined as the lowest concentration of Nile Red which can be detected with a signal-to-noise ratio greater than 7:1, was established by serial dilutions with decreasing concentrations of Nile Red. The LOD was 9 μg/l for Nile Red. The limit of quantification (LOQ) was defined as the lowest concentration of Nile Red at

which the coefficient of variation (C.V.) was ≤20% and the accuracy was within ±20% of the true value. The limit of quantification was determined as 50 μg/l.

3.3. Accuracy and precision

In order to evaluate the accuracy and precision of the assay, analyses of the prepared quality control samples of the cultured C_6 cells containing Nile Red at concentrations of 200, 800 and 3500 μg/l were performed. The results are summarized in Table 1. To determine intra-assay accuracy and precision, four replicate analyses were performed at each of the three concentrations. Inter-assay accuracy and precision were determined at each of the three concentrations over a period of 4 days. The three quality control samples were analyzed only once.

The mean and standard deviation for Nile Red were calculated at each concentration. The accuracy of each, referred to as % of target in Table 1, was determined by comparing the mean calculated concentration with the target concentration of the quality control samples. The intra- and inter-assay accuracy for all Nile Red samples was found to be within 94.9 and 100.8%, respectively, of the target values.

The specific detection of Nile Red was facilitated by its determination at 559 nm, whereas interference from components of the lysed cells was observed at 265 nm. Moreover, this allowed the reduction of run-time to a minimum of about 6 min. A final advantage might be the simpler setup of the HPLC equipment for this determination, which does not need a fluorescent detection system, but runs under a standard UV–Vis detector.

Table 1
Inter- and intra-day accuracy and precision for Nile Red at different concentrations extracted from C_6 cell culture samples

	3500 μg/l	800 μg/l	200 μg/l
Intra-assay			
Mean	3320.8	768.9	197.0
SD[a]	281.6	93.8	32.8
% of target	94.9	96.1	98.5
Inter-assay			
Mean	3413.7	758.0	201.6
SD[a]	188.3	35.0	28.8
% of target	97.5	94.8	100.8

[a] Standard deviation.

3.4. Photobleaching experiments

UV–Vis spectra were recorded for the different bleached solutions (Fig. 2b) where no significant variation was observed. Equivalent solutions of Nile Red were analyzed for their photobleaching by fluorescence spectrophotoscopy. These experiments showed the expected degradation of the fluorescent signal after laser impact. As shown in Fig. 3, a significant difference in signal intensity is observed for the two different methods after varying the bleaching periods. The degradation of the fluorescent structure within the Nile Red molecule did not permit an exact quantification throughout the whole bleaching process. However, the UV–Vis signal did not seem to be affected over the same time period.

These results suggest that this HPLC method is applicable for the quantification of Nile Red dyed structures, even if the samples have been previously exposed to intensive photobleaching light sources. Such an analytical method permits, for example, the exact quantification of Nile Red dyed nanoparticles or liposomes in tissue samples even after observation

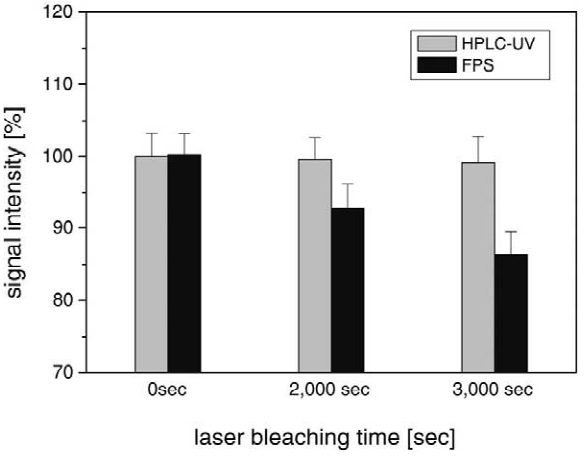

Fig. 3. Comparison of signal intensity loss for a constant concentration of Nile Red solution in HPLC–UV and fluorescence spectrophotoscopy after 0, 2000 or 3000 s of laser bleaching.

of the sample under fluorescence microscopy or confocal laser scanning microscopy.

4. Conclusions

This HPLC method has demonstrated its applicability for the quantification of Nile Red dyed structures after extraction from cultured cells by a simple UV–Vis detection system. Even if photobleaching occurs during a prior microscopic imaging step, an exact determination can be performed with this method which is independent of the fluorescent properties of Nile Red.

Acknowledgements

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