Abstract: Curcumin (CUR) shows potential use for treating cancer. However, CUR has low solubility and reduced bioavailability, which limit its clinical effect. Therefore, the development of nanocarriers can overcome these problems and can ensure the desired pharmacological effect. In addition, it is mandatory to prove the quality, the efficacy, and the safety for a novel nanomedicine to be approved. In that sense, this paper aimed (a) to prepare CUR-loaded polyethylene glycol-poly(ε-caprolactone) nanocapsules; (b) to validate an analytical method by high performance liquid chromatography (HPLC) for quantifying CUR in these nanoformulations; (c) to evaluate the physicochemical stability of these formulations; and to investigate their cytotoxicity on NIH-3T3 mouse fibroblast cells. The HPLC method was specific to CUR in the loaded nanocapsules, linear (r = 0.9994) in a range of 10.0 to 90.0 µg.mL\(^{-1}\) with limits of detection and quantification of 0.160 and 0.480 µg.mL\(^{-1}\), respectively. Precision was demonstrated by a relative standard deviation lower than 5%. Suitable accuracy (102.37 ± 0.92%) was obtained. Values of pH, particle size, polydispersity index, and zeta potential presented no statistical difference (p > 0.05) for CUR-loaded nanoparticles. No cytotoxicity...
was observed against NIH-3T3 mouse embryo fibroblast cell line using both the tetrazolium salt and sulfurphodamine B assays. In conclusion, a simple and inexpensive HPLC method was validated for the CUR quantification in the suspensions of nanocapsules. The obtained polymeric nanocapsules containing CUR showed suitable results for all the performed assays and can be further investigated as a feasible novel approach for cancer treatment.

**Keywords:** curcumin; HPLC method; mouse embryonic fibroblast cell; MTT assay; nanotechnology; physicochemical stability testing; polymeric nanocapsules; polyphenol; SRB assay; zeta potential.

**INTRODUCTION**

Curcumin (CUR) is a hydrophobic polyphenol obtained from the Curcuma longa L. rhizomes [1,2]. It is generally used as seasoning and food coloring due to its strong yellow color [3]. CUR has been recommended for several diseases, such as cancer, neurological disorders, infections, inflammations, atherosclerosis, joint and liver diseases [1,2]. Clinical evidences have been showed that CUR presents antitumor effect against a wide diversity of cancer types, including gastrointestinal, melanoma, genitourinary, breast, lung, neck, and sarcoma [4]. In general, CUR acts by suppressing the initiation, the promotion, and the metastasis of tumor cells when used alone or in combination with other chemotherapeutic agents [5]. This drug affects the cell cycle and leads to apoptosis and reduced invasion, proliferation, angiogenesis, and metastasis [6,2]. Besides these attractive properties, CUR demonstrates a remarkable tolerability at high doses and a very low toxicity [5].

In spite of its promising use against cancer, CUR has some problems regarding its physicochemical and pharmacokinetic properties as low water solubility, reduced photostability, rapid presystemic metabolism and low bioavailability that decrease its pharmacodynamic potential for clinical use [7,3]. In that sense, the development of nanocarriers appears as a suitable strategy for CUR in order to provide protection against degradation and to ensure improved drug solubility and bioavailability [8]. However, the use of nanocarriers as polymeric nanocapsules, even as any commercial pharmaceutical medicine, needs to present quality, efficacy, and safety [9]. In this context, an analytical method previously validated is required for quality assurance purposes [10]. When an advanced search was performed in ScienceDirect and Google Scholar on April 10, 2020, using title mode and English language, only two papers were devoted to investigate the keywords “validation” AND “HPLC method” AND “curcumin” AND “nanoparticles”. The first paper developed and validated an HPLC method using fluorescence detection for the quantitative determination of curcumin in poly(lactic-co-glycolic acid) (PLGA) and PLGA-polyethylene glycol (PEG) nanoparticles [6]. Although it showed interesting results, the fluorescence use is less popular and less cost-effective in HPLC. The second paper reported a validated HPLC method for the simultaneous estimation of curcumin and pipeline in Eudragit E 100 nanoparticles [11]. This study used as experimental conditions a mobile phase composed by 0.1% ortho phosphoric acid aqueous solution and acetonitrile (45:55, v/v) in an isocratic mode elution with a flow rate of 1.2 mL.min⁻¹ at the column oven temperature of 35°C and at a wavelength of 262 nm. Although a photodiode array detector is more usual, this work used acetonitrile in the mobile phase composition, which is more expensive than methanol [10]. In that sense, the current literature lacks in providing a validated HPLC method for quantifying CUR in polymeric nanocapsules by a simple, widely available and inexpensive HPLC method.

Several reports have been demonstrated that colloidal suspensions of polymeric nanocapsules take a long time to start the phase separation process since sedimentation/flotation is reduced by the Brownian movement of the nanosized particles [12]. However, particle aggregation process can occur over time [13]. For this reason, the physicochemical stability studies are essential since several factors can influence the system properties, such as adsorption of active molecules on the surface of nanoparticles and/or presence of adsorbed surfactants [14]. Moreover, to the best of our knowledge, this is the first report concerning to the stability of CUR-loaded PEG-PCL nanocapsules.

Regarding to the safety aspects, the NIH-3T3 mouse embryonic cell line is the standard cell line of fibroblasts, which has been widely used for evaluating the cytotoxic potential of nanocarriers and other polymeric materials [15]. Fibroblasts are the most fundamental cells of connective tissue, which produce the amorphous substance and fibers. Hence, these cells are important in the development and differentiation of the connective tissue and its derivatives such as bone, cartilage, and blood. Furthermore, these mesenchyme-derived cells have versatile functions, including responding to inflammation by recruiting immune cells, generating cytokines/chemokines, and modifying tissue architecture during wound healing.
[16]. In that sense, the use of NIH-3T3 cell cytotoxicity assay can provide crucial and basic information about the safety of polymeric nanocapsules containing CUR.

In this context, this paper aimed to prepare PEG-poly(ε-caprolactone) (PCL) nanocapsules containing curcumin and to answer three fundamental research questions in pharmaceutical nanotechnology: (a) is there a simple and inexpensive HPLC method for quantifying CUR in PEG-PCL nanocapsules?; (b) how long are CUR-loaded PEG-PCL nanocapsules physicochemically stable? (c) Do CUR-loaded PEG-PCL nanocapsules show cytotoxicity on NIH-3T3 cell line?

MATERIAL AND METHODS

Chemicals and reagents

Curcumin (CUR, ≥ 94% curcuminoid content, Sigma-Aldrich, St. Louis, MO, USA), poly(ε-caprolactone) (PCL, $M_w$ 10,000-14,000 g.mol$^{-1}$, Sigma-Aldrich, St. Louis, MO, USA), poly(ethylene glycol) 6000 (PEG, $M_w$ 5,400-6,600 g.mol$^{-1}$, Cromato Produtos Químicos, Diadema, Brazil), sorbitan monooleate (Span® 80, Oxiteno, Mauá, Brazil), polysorbate 80 (Tween® 80, Delaware, Porto Alegre, Brazil), medium chain triglycerides (MCT, 99% pure, Focus Química, São Paulo, Brazil), methylthiazolyldiphenyl-tetrazolium bromide (MTT, Sigma-Aldrich, St. Louis, MO, USA), sulphorhodamine B (SRB, Sigma-Aldrich, St. Louis, MO, USA), penicillin-streptomycin (Sigma-Aldrich, St. Louis, MO, USA), and acetone (≥ 99.9% pure, Vetec Química, Rio de Janeiro, Brazil) were used as received. HPLC-grade methanol was purchased from Tedia (Rio de Janeiro, Brazil). RPMI 1640 medium and fetal bovine serum were obtained from Vitrocell (Campinas, Brazil). Water was purified in a Milli-Q Plus water purification system (Millipore, Bedford, MA, USA). All other solvents and reagents were analytical grade. The NIH-3T3 cell line, number CRL-1658, was obtained from American Type Culture Collection (ATCC) and was provided by Dr. Patrícia Mathias Döll-Boscardin.

Equipment

A Merck-Hitachi Lachrom HPLC system (Tokyo, Japan) was used for method development. The HPLC system was equipped with an Interface D-7000, UV detector module L-74000, equipped with pumps L-7100 and an integral degasser, controller software (Chromquest, Thermo Fisher Scientific, Incorporated, Pittsburgh, PA, USA), and manual injector (Rheodyne, Rohnert Park, CA, USA) equipped with a 20 µL injector loop and a 100 µL syringe (Microliter 710, Hamilton, Bonaduz, Switzerland).

Preparation of polymeric nanocapsules

Suspension of polymeric nanocapsules were obtained from the PEG and PCL polymers, and CUR (Nano_CUR). This formulation was prepared by the interfacial deposition of the preformed polymer method as described by Fessi and coauthors [17]. Briefly, PEG (20 mg) and PCL (80 mg) were dissolved in 27 mL of acetone in the presence of Span 80® (77 mg), CUR (30 mg), and MCT (300 mg). The organic phase was added to the aqueous phase containing 77 mg of Tween® 80 and 53 mL of distilled water by dripping and under vigorous magnetic stirring at 40°C. The organic solvent was then evaporated under reduced pressure in a rotary evaporator to the final volume of 10 mL. For comparative purposes, a suspension of nanoparticles was prepared with no CUR (Nano_0). All formulations were prepared in triplicate and protected from light.

HPLC method validation

Preparation of standard solutions

A stock standard solution (500.0 µg.mL$^{-1}$) was daily prepared by dissolving 50 mg of CUR into a 100 mL volumetric flask using methanol. This solution was further diluted in methanol:water acidified with 0.5% acetic acid (50:50 v/v) to prepare seven different working standard solutions ranging from 10.0 to 90.0 µg.mL$^{-1}$. These solutions were filtered through a polytetrafluoroethylene filter (PTFE, Cromafil® Xtra, 0.45 µm x 22 mm, Macherey-Nagel GNBH & Co. KG, Düren, Germany) before injection into the HPLC system. All procedures were carried out in dark conditions.

Preparation of sample solutions

The amount of drug into Nano_CUR was indirectly determined. The suspension of nanocapsules was submitted to a combined ultrafiltration/centrifugation using centrifugal devices (Amicon® 10.000 $M_w$, Millipore,
Bedford, MA, USA) at 2200 x g during 30 minutes in triplicate. Free CUR was determined in ultrafiltrate after its suitable dilution and filtration through a poly(vinylidene fluoride) filter (PVDF, Cromafil® Xtra, 0.45 µm x 22 mm, Macherey-Nagel GNBH & Co. KG, Düren, Germany) by injection into the HPLC system.

**Chromatographic conditions**

Experiments were performed in the previously described HPLC system using a GL Sciences Inertsil® ODS3 reverse phase analytical column (Torrance, CA, USA) (150 mm x 4.6 mm, 5 µm) and a GL Sciences Inertsil® ODS3 protection cartridge system (Torrance, CA, USA) (10 mm x 4 mm, 5 µm) at room temperature (20 ± 2°C) using UV detection at 261 nm. Gradient elution was performed using a mobile phase composed by methanol:water acidified with 0.5% acetic acid at a flow rate of 1.0 mL.min⁻¹. The mobile phase consisted of a gradient initiated at 44% MeOH for 3 min, increased to 90% MeOH in 5 minutes, maintained constant for 11 minutes and then returned to 44% MeOH in 12 minutes. The method run time was 12 minutes and all experiments were carried out in triplicate.

**Method performance parameters**

For validation of this analytical method, the guidelines established by the International Council on the Harmonization (ICH) of technical requirements for the registration of pharmaceuticals for human use guideline [18] was used. The following parameters were assessed: specificity, linearity, detection and quantification limits, precision, accuracy, and robustness.

The specificity was determined by analyzing the chromatograms of non-loaded nanocapsules (Nano_0) and those obtained for CUR-loaded PEG-PCL nanocapsules (Nano_CUR) aiming at confirming that excipients had no interference in drug quantitation.

The linearity was obtained by calculating a regression line from the plot of peak area versus concentration of the working standard solutions prepared at five concentration levels (10.0, 20.0, 30.0, 60.0, and 90.0 µg.mL⁻¹) using least-squares linear regression analysis. The linearity test was performed for three consecutive days at the same concentration range. The slope and other statistics from the calibration curves were calculated by linear regression and analysis of variance (ANOVA).

The limit of detection (LOD) and limit of quantification (LOQ) were calculated based on the standard deviation (SD) and the slope (S) of the calibration curve based on Equations 1 and 2, respectively.

\[
LOD = \frac{3.3 \times SD}{S}
\]

\[
LOQ = \frac{10 \times SD}{S}
\]

The precision was investigated at two different levels: repeatability and intermediate precision. For the repeatability, three concentrations (15.0, 45.0, and 70.0 µg.mL⁻¹) were determined in triplicate. The intermediate precision was assayed by the standard deviation (SD) and relative standard deviation (RSD) of six injections at 30.0 µg.mL⁻¹ that were analyzed intra-day, inter-day, and by a different analyst.

The accuracy was evaluated by recovery analysis, adding a known amount of CUR (3 mg) to 10 mL of the CUR standard solution at 500.0 µg.mL⁻¹. An aliquot of the CUR standard solution at 500.0 or 800.0 µg.mL⁻¹ was properly diluted with mobile phase to obtain samples at 400.0 µg.mL⁻¹ in triplicate. The accuracy of the method was calculated by the ratio between the experimental concentrations obtained and then multiplied by 100.

The robustness was determined when changes in flow rates (0.995 and 1.005 mL.min⁻¹) and mobile phase concentration [methanol:water acidified with 0.5% acetic acid 43:57/89:11 (v/v) and 45:55/91:09 (v/v)] were performed. The results were studied using RSD compared to the values obtained for standard condition [flow rate of 1.000 mL.min⁻¹ and mobile phase concentration of methanol:water acidified with 0.5% acetic acid 44:56/90:10 (v/v)].

**Method applicability: analysis of drug-loading efficiency**

Considering the CUR content in drug-loaded PEG-PCL nanocapsules, the encapsulation efficiency (EE) was calculated using Equation 3.

\[
EE(\%) = \frac{\text{theoretical drug loading} - \text{free drug content}}{\text{theoretical drug loading}} \times 100
\]
Physicochemical stability

The nanosuspensions were stored at room temperature (25 ± 2°C) and protected from light into amber glass bottles. The formulations were monitored over 60 days of storage by checking the pH using a previously calibrated digital potentiometer (Hanna, HI 2221 model, São Paulo, Brazil). The particle size, the polydispersity index (PDI) and the zeta potential was determined by the Zetasizer Nanoseries equipment (Malvern Instruments, NANO ZS 90 model, Malvern, United Kingdom) after a 1:500 dilution using ultrapure water. All samples were assayed in triplicate. All data resulting from stability testing were analyzed using the GraphPad Prism software, 6.01 version for Windows.

In vitro cell culture-based assays

Cell culture

The NIH-3T3 cell line was incubated into culture flasks containing RPMI 1640 medium supplemented with 2 mmol.L⁻¹ L-glutamine, 24 mmol.L⁻¹ NaHCO₃, 10% fetal bovine serum, penicillin (100 U.mL⁻¹), and streptomycin (100 μg.mL⁻¹) under 5% CO₂ at 37°C. After 2–4 days, cells were scraped from each culture flask and centrifuged for 5 minutes at 125 x g. Cells were re-suspended in fresh medium and plated into appropriate multi-well plates. The experiments were performed when cells reached about 70% confluence.

NIH-3T3 cell cytotoxicity assay

Cell viability was determined via reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to formazan [19] and via protein staining test using Sulforhodamine B (SRB) [20]. The NIH-3T3 cells (8x10³ cells.well⁻¹) were seeded in 96-well plates. After overnight adhesion, cells were incubated for 72 h at 37°C in culture medium containing free CUR or Nano-CUR at concentrations equivalent to 2.5, 5.0, 25.0, 50.0 and 100.0 μmol.L⁻¹ CUR. CUR was previously solubilized in culture medium containing 0.05% DMSO while Nano-CUR was dispersed only in supplemented RPMI 1640 medium. After incubation, supernatant cells were removed and 200 μL of the MTT solution at a final concentration of 0.5 mg.mL⁻¹ was added to cells for 2 hours at 37°C and protected from light. The supernatant was removed. Then, the formazan crystals were dissolved in DMSO and absorbance was spectrophotometrically measured at 550 nm using a Synergy H1 hybridmulti-modemicroplate reader (Bio-Tek Instruments, Winooski, VT, USA). Non-treated cells in culture medium were used as control. NIH-3T3 cells in culture medium containing 0.05% DMSO were tested as vehicle. To calculate cell viability, Equation 4 was used.

\[
\text{Cell viability(\%) =} \frac{\text{absorbance of test}}{\text{absorbance of control}} \times 100
\] (4)

For SRB analysis [20,21], NIH-3T3 cells were treated at the same concentrations described above. After 72 hours, the supernatant was discarded. The cells were washed with 200 mL of sodium phosphate buffer solution at pH = 7.4. Then, 200 μL of 10% trichloroacetic acid were added and the plates were placed in the refrigerator for 30 minutes to fix the cells. Subsequently, the cells were washed three times with 200 μL of distilled water and maintained for 24 h at room temperature (20 ± 2°C) until dry. Then, 200 μL of 0.2% SRB solution was added and maintained for 30 minutes. The plates were washed five times with 200 μL of 1% acetic acid and dried again for 30 minutes. Finally, 150 μL of 10 mmol.L⁻¹ TrisBase were added and the spectrophotometric absorbance reading was performed at a wavelength of 432 nm. Equation 4 was also used to calculate cell viability.

Statistical analysis

All data were expressed as mean ± standard deviation (SD) or standard error. The linearity data were evaluated by simple linear regression. Relative standard deviation (RSD) was shown as required. Validation, physicochemical, and cytotoxicity data were compared by Student's t-test or analysis of variance (ANOVA) with Tukey's post-hoc test at a significance level of 5% (α = 0.05). GraphPad Prism software version 5.03 (San Diego, CA, USA) was used for statistical analysis.
RESULTS AND DISCUSSION

Preparation of polymeric nanocapsules

The suspension of non-loaded nanocapsules (Nano_0) showed a liquid aspect with a slightly bluish-white opalescent coloring as typically reported in literature [17]. However, the suspension of CUR-loaded PEG-PCL nanocapsules (Nano_CUR) presented a yellow liquid aspect due to the original intense yellow color of CUR [4].

HPLC method validation

The proposed method was validated by determining its performance characteristics regarding specificity, linearity, limit of detection, limit of quantification, precision, accuracy, and robustness. Specificity was demonstrated by comparing the chromatograms of non-loaded and CUR-loaded nanocapsules prepared as per the test method (Figure 1). The results showed that there was no interference at the retention time of CUR from the other formulation components. In that sense, it is possible to confirm the specificity of the studied HPLC method.

![HPLC chromatograms obtained for non-loaded and CUR-loaded nanocapsules: Nano_CUR (A) and Nano_0 (B). Chromatographic conditions: (a) gradient elution mode; (b) mobile phase: methanol:water acidified with 0.5% acetic acid; (c) gradient program: started at 44% MeOH for 3 min, increased to 90% MeOH in 5 minutes, maintained constant for 11 minutes and then returned to 44% MeOH in 12 minutes; (d) flow rate: 1.0 mL.min⁻¹; (e) UV detection wavelength: 261 nm; (f) column temperature: 20± 2°C; and (g) run time: 12 minutes](image_url)

The linearity of the HPLC method at five concentration levels was determined and the results are presented in Figure 2. A linear relationship between peak area and concentration of CUR at a concentration range from 10.0 to 90.0 µg.mL⁻¹ was observed. The linear equation obtained by the least-square method was \( y = 32449 x - 34557 \) \((n = 3)\), where \( y \) is the peak area and \( x \) is the standard solution concentration at µg.mL⁻¹. A suitable correlation coefficient \((r = 0.9994)\) was recorded, which demonstrates that the proposed method is linear. In general, \( r \) values near to 1 are indicative of linearity [22].

However, the literature [23] reports that a regression coefficient value close to 1.0 is not certainly the result of a linear relationship and, consequently, a more careful test should be applied. Therefore, the analysis of variance (ANOVA) for the linearity data is presented in Table 1. The value of \( F \) for the lack of fit was lower than the value of \( F \) tabulated for the 95% confidence interval \((\alpha = 0.05)\). Thus, linear regression was confirmed due to it did not show lacking of fit.

The lowest concentration where CUR can be detected (LOD) and quantified (LOQ) with acceptable precision and accuracy was 0.16 and 0.48 µg.mL⁻¹, respectively. These values are lower than the minimum concentration of calibration curve (10.0 µg.mL⁻¹), which represents a remarkable sensitivity.
Figure 2. Mean calibration curve obtained for CUR using working standard solutions at the concentration range of 10.0 to 90.0 µg.mL\(^{-1}\) (n = 3) (λ = 261 nm)

Table 1. ANOVA results for linearity test

| Source     | SS          | df | MS             | F         | F\(_{\text{tab}}\) |
|------------|-------------|----|----------------|-----------|-----------------|
| Model      | 1.351975E+13| 1  | 1.351975E+13   | 2969.202  | 4.667           |
| Residual   | 5.919326E+10| 13 | 4.553327E+09   | Linear    | –               |
| Lack of fit| 1.498533E+10| 3  | 4.995111E+09   | 1.129913  | 3.708           |
| Pure error | 4.420792E+10| 10 | 4.420792E+10   | No lack of fit | –               |

Legend: SS: sums of squares; df: degrees of freedom; MS: mean squares; F: F value of the test; F\(_{\text{tab}}\): fixed F value)

The results for precision (repeatability and intermediate precision) are summarized in Table 2. The RSD for the repeatability was 0.90% and for the intermediate precision was 4.15%. These values are less than 5%, which prove that the developed method is precise [24] within the concentration range and the standard conditions used.

Table 2. Repeatability and intermediate precision data for curcumin analysis using loaded nanocapsules

| Theoretical amount (µg.mL\(^{-1}\)) | Experimental amount (µg.mL\(^{-1}\), mean ± SD) | RSD (%) |
|-------------------------------------|-------------------------------------------------|---------|
| Repeatability (n = 9)               |                                                 |         |
| 15.0                                | 14.92 ± 0.06                                    | 0.37    |
| 45.0                                | 44.74 ± 0.25                                    | 0.57    |
| 70.0                                | 69.17 ± 1.22                                    | 1.76    |
| Intermediate precision              |                                                 |         |
| Intraday (n = 3)                    | 30.0                                            | 0.94    |
| Interday (n = 3)                    | 30.0                                            | 3.87    |
| Different analyst (n = 3)           | 30.0                                            | 3.42    |
| Mean ± SD (n = 9)                   | 30.0                                            | 4.15    |

Legend: SD = standard deviation; RSD = relative standard deviation
The accuracy was investigated by the recovery test in which an exact amount of CUR was added to an analytical solution of known concentration. The mean recovery was 102.37 ± 0.92% and showed a RSD of 0.90%. This result proves that the method is accurate since it is in agreement with the limits recognized by ICH [18].

The robustness of an analytical method indicates the reliability of the method against small variations of the analytical parameters [25]. The RSD values obtained after these changes did not exceed 5%, which represented that the proposed HPLC method is robust [26]. There were no significant differences (p > 0.05) in the area under curve and the retention time of CUR when the flow rate varied to 0.995 and 1.005 mL.min⁻¹ (RSD = 4.45 and 0.67%, respectively) and the mobile phase changed to 43:57/89:11 and 45:55/91:09 (RSD = 1.68 and 0.97%, respectively). Thus, the method was recognized as robust for determination of CUR in polymeric nanoparticles.

**Method applicability: analysis of drug-loading efficiency**

The proposed HPLC method was used to determine the drug-loading efficiency of CUR in PEG-PCL nanocapsules. The Nano_CUR formulation showed a drug-loading efficiency of 98.70 ± 0.66%. Considering this result, a drug concentration very close to the theoretical value was achieved, which represents that there was low drug loss during the preparation procedure. In addition, the formulation presented a high drug-loading efficiency that is related to the low aqueous solubility of CUR (< 8 µg.mL⁻¹ at 20 °C), which resulted in increased drug concentration into the polymeric nanocapsules.

**Physicochemical stability**

The results obtained for pH, particle size, polydispersity index (PDI), and zeta potential immediately after preparation are depicted in Table 3.

| Formulation | pH       | Particle size (nm) | Polydispersity index | Zeta potential (mV) |
|-------------|----------|--------------------|----------------------|---------------------|
| Nano_0      | 6.16 ± 0.29 | 313.30 ± 48.20   | 0.351 ± 0.15        | −39.83 ± 2.46       |
| Nano_CUR    | 5.86 ± 0.20 | 315.96 ± 10.30   | 0.351 ± 0.002       | −41.20 ± 7.20       |

Legend: mean (n = 3) ± standard deviation

In general, these physicochemical data were in accordance to those recommended for polymeric nanocapsules considering the preparation method and the nature of polymers used [27]. The interfacial deposition of the preformed polymer method have usually provided nanocapsules of mean diameters between 200 and 300 nm and PDI between 0.2 and 0.3, mainly when poly(lactic-co-glycolic acid) and PCL are chosen as polymers [28]. However, polymeric nanocapsules of larger diameter can be related to the presence of PEG in their composition. PEG chains create a more viscous organic phase, which affects its dispersion into the aqueous phase during stirring and leads to higher particle sizes with broader polydispersity [29]. Moreover, the negative values observed for zeta potential of formulations NANO_0 and Nano_CUR were associated with the anionic nature of PCL due to the presence of carboxylic acid functional groups in this polyester [14]. The statistical analysis verified that the pH values, the mean diameters, the PDI values and the zeta potentials were similar between the nanoformulations with or without CUR (p > 0.05).

Considering the stability testing, Figure 3 compares the results obtained for pH, particle size, PDI, and zeta potential immediately after preparation and after 60 days of storage. The formulation Nano_0 presented a significant decrease only in pH (p < 0.01) after the time interval assayed. However, no statistically significant change was found for the formulation Nano_CUR after 60 days of storage. Taking all these into account, CUR-loaded PEG-PCL nanoparticles had a suitable stability within 60 days of preparation and can be further used for cancer treatment during this time interval.
The pH reduction for control nanocapsules after 60 days can be attributed to three possible phenomena. Tween® 80 can show ester hydrolysis and can then generate free fatty acids (oleic acid) which can decrease pH value of colloidal nanosuspension [30]. Moreover, the ester functional group of PCL can be hydrolyzed and fragmented into shorter polymeric chains with lower molecular weight. The acidification can be then associated with the carboxylic groups from PCL fragments or monomers [31]. Furthermore, MCT can be released from oily core over time and can itself degrade by ester hydrolysis to form free fat acids which leads to a lower pH value for nanosuspensions [32]. Considering the loaded formulation, CUR has antioxidant potential [2] and may have contributed to a decrease of these hydrolysis reactions.

**In vitro cell culture-based assays**

In order to assess the Nano_CUR cytotoxicity on the NIH-3T3 mouse embryo fibroblast cell line, cell viability experiments were performed by the MTT and SRB assays within 72 hours after incubation. These two strategies to predict cytotoxicity were used since MTT evaluates the mitochondrial and nonmitochondrial enzymatic activity and SRB investigates the dye’s bind ability to protein components in cells [33].

The results for the MTT and SRB assays are presented in Figure 4. The results of cell viability by MTT-reducing activity for Nano_CUR did not show any cytotoxicity on the NIH-3T3 cells at the concentrations tested. However, the same formulation demonstrated a significant reduction (p < 0.001) in cell viability at the highest concentration tested when evaluated by the SRB assay. This cytotoxic effect at the high concentration of 100 µmol.L⁻¹ provided by the SRB assay is above the criteria of cytotoxicity activity as established by the US National Cancer Institute (NCI) [34]. In that sense, it is possible to claim that no relevant cytotoxicity was observed for CUR-loaded PEG-PCL nanocapsules against NIH-3T3 mouse embryo fibroblast cells. Moreover, the decrease in cell viability demonstrated by the SRB assay occurred because this technique showed to have more sensitivity than MTT for detecting changes in cell metabolism [35].

These results are in agreement with the data reported in the literature. Cheng and coauthors [36] revised 25 studies and showed that CUR was no toxic when orally administered to animals and humans using a dose up to 8 grams per day for 3 months. Addala [37] studied the effect of CUR on HCT-116 human colon cancer cell line and CRL-1790 normal human colon epithelial cell line using the SRB assay and calculated the selectivity index (SI). CUR had a SI of 16.75, which suggests that CUR is more selective for tumor cells and presents a lower effect on normal cells as NIH-3T3 fibroblasts.
Figure 4. Effect of the formulation Nano_CUR on NIH-3T3 cell viability after the 72-hour period by the MTT (A) and SRB (B) assays. The results are expressed as mean and standard error obtained from 4 independent tests with n = 4 per test. The symbol *** represents a significant difference in relation to the control obtained by the one-way ANOVA followed by the Tukey’s post-hoc test (** p < 0.001).

CONCLUSION

Curcumin-loaded polyethylene glycol-poly(ε-caprolactone) nanocapsules were successfully obtained by the interfacial deposition of the preformed polymer method. The analytical method by high performance liquid chromatography was suitable validated for quantifying curcumin in these formulations. All polymeric nanocapsules demonstrated appropriate pH, particle size, polydispersity index, and zeta potential. The physicochemical stability was demonstrated over 60 days after their preparation. The polymeric nanocapsules containing curcumin presented no cytotoxicity on NIH-3T3 mouse embryo fibroblast cell line.

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Conflicts of Interest: The authors declare no conflict of interest.

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