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# Application of a validated HPLC-PDA method for the determination of melatonin content and its release from poly(lactic acid) nanoparticles

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ABSTRACT

Melatonin is a natural hormone and with the advancement of age its production declines and thereby may result in some neurological disorders. Exogenous administration of melatonin has been suggested as a neuroprotective agent. Due to its low oral bioavailability, the loading of melatonin in polymeric nanoparticles could be an important tool to effectively use exogenous melatonin. The quantification of the incorporated drug within polymeric nanoparticles is an important step in nanoparticles characterization. An analytical method using high performance liquid chromatography equipped with photodiode array detector (HPLC-PDA) was developed and validated for melatonin determination in poly (lactic acid) nanoparticles obtained by a single emulsion-solvent evaporation technique. The melatonin in vitro release profile also was determined by the HPLC method. Mobile phase consisted of acetonitrile: water (65:35, v/v) pumped at a flow rate of 0.9 mL/min, in the isocratic mode and PDA detector was set at 220 nm. The method was validated in terms of the selectivity, linearity, precision, accuracy, robustness, limits of detection and quantification. Analytical curve was linear over the concentration range of 10–100 μg/mL, and limits of detection and quantification were 25.9 ng/mL and 78.7 ng/mL, respectively. The mean recovery for melatonin was 100.47% (RSD = 1.25%, $n$ = 9). In the intra- and inter-assay, the coefficient of variation was less than 2%. Robustness was proved performing changes in mobile phase, column temperature and flow rate. The method was suitable for the determination of melatonin encapsulation efficiency in poly(lactic acid) nanoparticles and for the evaluation of melatonin in vitro release profile.

## Introduction

Melatonin (*N*-acetyl-5-methoxy trypamine; MLT) is a natural hormone predominately produced by the pineal gland during the absence of light, exerting various physiological actions, including sleep regulation, neuromodulation, regulation of immune response and blood pressure [1,2]. MLT also exerts potent capture of free radicals, acting directly on reactive oxygen species, such as hydroxyl, alkoxyl, peroxyl, nitric oxide and superoxide and indirectly through the induction of antioxidant enzymes [3,4]. Studies have shown the decline in the production of this hormone with the advancement of age, and it may be related to the emergence of disorders such as insomnia and neurodegenerative diseases [5–7]. The role of MLT as a neuroprotective agent against diseases such as Parkinson's and Alzheimer's and brain lesions from different etiology, such as ischemia and intracerebral hemorrhage, has been suggested [2,6,8]. Furthermore, the administration of exogenous MLT in pharmacological doses can also exert cytotoxicity in tumor cells such as breast adenocarcinoma MCF-7, human osteosarcoma and gastrointestinal tract tumors [9–11]. Therefore, considering the natural decline of MLT and its potential therapeutic effects, the exogenous administration of this molecule might be useful. However, MLT oral bioavailability is lower than 20% due to its poor absorption and/or extensive first-pass metabolism [12–15]. Nanotechnology-based drug delivery systems offer an alternative to circumventing these drawbacks.

Nanotechnology has been applied in pharmaceutical field to improve the efficacy of drugs with biopharmaceutical and pharmacokinetic limitations. Polymeric nanoparticles have been widely exploited due to their great physicochemical and biological properties, offering multiple advantages as drug delivery systems. Besides promoting sustained drug release, protection from degradation and ability to encapsulate a great variety of drugs, nanoparticles improve several pharmacokinetic parameters including drug bioavailability [16–20]. Polymeric nanoparticles can be composed of biodegradable and biocompatible polymers, such as poly (lactic acid) (PLA) and its copolymers with glycolic acid, e.g. poly(lactic-*co*-glycolic acid) (PLGA), which are approved by the Food and Drug Administration (FDA) for biomedical applications [21].

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The quantitative determination of the drug incorporated into polymeric nanoparticles and its posterior in vitro release is usually performed by analytical methods that need to be adequately validated to ensure a reliable quantification of the analyte [22–24]. Various analytical methods are described in literature for MLT determination in biological samples by high-performance liquid chromatography (HPLC) with fluorescence detection [25–27], electrochemical detection [28] and UV detection [29]. Also, liquid chromatography coupled to mass spectrometry (LC-MS)[30] and gas chromatography coupled to mass spectrometry (GC-MS) [31] are reported, but less frequently. Analytical determination of MLT in pharmaceutical formulations is also limited [32–34]. It has been cited for spectrophotometric analysis for evaluation of MLT in nanoparticles [35–38]. However, the higher sensitivity of the HPLC method over UV-spectrophotometry is important for the determination of the drug release profile from nanoparticles, since the amount of drug released over the time is low. Also, depending on the method used for drug extraction from nanoparticles, some matrix components can be present in the sample and absorbed in UV range and thus, interpose in the drug quantification. Moreover, no detailed reports of HPLC methods for determination of MLT in polymeric-based nanoparticles are described. Therefore, the aim of this study was to develop and validate a reversed phase HPLC method for fast and simple determination of the encapsulation efficiency of MLT incorporated in PLA nanoparticles and determination of MLT in vitro release profile from nanoparticles.

## 2. Experimental

### 2.1. Chemicals and reagents

Melatonin (98%), PLA (MW 85–160 kDa) and polyvinyl alcohol (PVA, 31 kDa, 88% hydrolyzed) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Ethyl acetate, ethanol and methylene chloride were purchased from FMaia® (Cotia, São Paulo, Brazil). HPLC-grade acetonitrile was purchased from JTBaker® (Center Valley, PA, USA). Water was purified in a Milli-Q Plus system (Millipore®). All other solvents and chemicals were of analytical or HPLC grade.

### 2.2. Instrumentation

Waters 2695 Alliance HPLC system (Milford,MA, USA) was used equipped with column and sample compartment with temperature control and photodiode array wavelength detector (PDA) (Waters 2998), quaternary pump, autosampler and on-line degasser. Data acquisition, analysis and reporting were performed using Empower chromatography software (Milford, MA, USA). Analyses were performed using a column LiChrospher® 100 RP-18 column (Merck®, Germany), with 5 μm in particle size, 4 mm in internal diameter and 125 mm in length.

### 2.3. Chromatographic conditions

Chromatographic analysis was performed on the isocratic mode with a mobile phase consisting of acetonitrile and water in the proportion of 65:35 (v/v), flow rate of 0.9 mL/min, column temperature set at 30 °C, sample injection volume of 20 μL and PDA detector was set at 220 nm. Method run time was 2 min.

### 2.4. Preparation of standard and sample solutions

Standards and samples used for the analyses were prepared in acetonitrile and water in the proportion of 65:35 (v: v), and filtered through a 0.22 μm pore size filter (PVDF, Allcrom®) before injection. Seven MLT standard solutions (10, 20, 40, 50, 60, 80 and 100 μg/mL) were obtained. MLT samples corresponded to supernatant from the ultracentrifugation of nanoparticles containing MLT, as described further.

### 2.5. System suitability

The system suitability was evaluated by six replicate analyses of MLT standard (50 μg/mL). The system suitability parameters were studied to verify the system performance and factors such as the number of theoretical plates (N), height equivalent to a theoretical plate (HEPT), tailing factor (T) and capacity factor (K) were taken into consideration.

### 2.6. Method validation

Analytical validation followed the recommendations of the International Conference on Harmonization guidelines [39]. The following characteristics were considered for validation: linearity, specificity, precision, accuracy, limit of detection (LOD), limit of quantification (LOQ) and robustness.

Linearity was assessed by calculating a regression line from the plot of peak area versus analyte concentration of the seven standard solutions (10, 20, 40, 50, 60, 80 and 100 μg/mL), using the linear least squares methodology.

Specificity was evaluated by comparing the chromatograms of samples obtained from the supernatant of blank nanoparticles (without MLT) and MLT standard solutions and samples containing MLT.

Precision of the method was determined by repeatability (intra-day precision) and intermediate precision (inter-day precision). Repeatability was assessed analyzing three levels of different standard solutions: low (10 μg/mL), medium (50 μg/mL) and high (100 μg/mL) on the same day. Intermediate precision was evaluated through the analysis of these standard solutions on three different days, in triplicate. Results were expressed as standard deviation (SD) and relative standard deviation (RSD).

Accuracy was assessed by the percentage recovery value. The standard drug solution was added to MLT sample solution (4 μg/mL), in the range of 75%, 100% or 125% of the sample concentration, which originated the final concentration of 7, 8 and 9 μg/mL. Analyses were performed in triplicate.

LOD and LOQ were determined based upon the slope (S) of the calibration curve and least standard deviation obtained from the response (σ), according to Eq. (1) and Eq. (2). The results of LOD and LOQ were posteriorly confirmed.

$$\mathrm{LOD} = \sigma/S \times 3.3 \quad (1)$$

$$\mathrm{LOQ} = \sigma/S \times 10 \quad (2)$$

Robustness was evaluated by small changes in certain analytical parameters such as the proportion of the mobile phase (acetonitrile: water, 63:37, v/v), column temperature (27 °C), increase or decrease in the flow rate of mobile phase (0.95 mL/min or 0.85 mL/min), using standard solutions in low (10 μg/mL), medium (50 μg/mL) and high (100 μg/mL) concentration, in triplicate. Assessment of change in these parameters was based on the percentage of recovery and RSD.

### 2.7. Method applicability

#### 2.7.1. Determination of MLT content in PLA nanoparticles

MLT-loaded nanoparticles were obtained by a single emulsion-solvent evaporation technique. First, MLT was solubilized in ethanol and mixed with the organic phase containing PLA dissolved in methylene chloride and ethyl acetate. The organic phase was slowly poured into the aqueous phase containing 1% PVA (m/v) and emulsified by means of sonication for 5 min, which resulted in an oil-in-water (O/W) emulsion. The organic solvent was rapidly removed by evaporation under vacuum at 37 °C (30 min). After, nanoparticles were recovered by ultracentrifugation (19,975*g*, 30 min, 4 °C). Mean particle size and polydispersity index were determined through the dynamic laser scattering technique (BIC 90 plus equipment – Brookhaven

Instruments Corp). Analyses were performed at a scattering angle of 90° and temperature of 25 °C.

MLT content in nanoparticles was determined by an indirect method [16,23,40,41]. Supernatant obtained from the ultracentrifugation process was diluted in the mobile phase (1:10), filtered through a 0.22 μm pore-size filter and the drug concentration was determined by the validated HPLC method. The MLT encapsulation efficiency (EE) was determined in triplicate, according to Eq. (3).

$$EE(\%) = A_I - A_F / A_I \times 100 \quad (3)$$

where $A_I$ is the amount of MLT initially added to the formulation and $A_F$ is the amount of the non-encapsulated MLT quantified in the supernatant after ultracentrifugation.

#### 2.7.2. Determination of MLT in vitro release profile

The in vitro release profile of MLT from PLA nanoparticles was determined by dissolution followed by ultracentrifugation [42]. An aqueous suspension of nanoparticles containing about 1 mg of MLT was dispersed in 10 mL of phosphate saline buffer (PBS, 0.01 M, pH 7.4) containing 1% tween® 80 (m/v), to maintain sink conditions. The tubes were kept in shaker at 37 °C and 150 rpm. At predetermined time intervals, the suspension was centrifuged at 19,975*g* for 15 min to separate the released MLT from the nanoparticles. The resulting precipitate in each tube was immediately suspended in refresh release medium and incubated until the next sampling. The released MLT present in the supernatant was diluted in the mobile phase (1:10) and analyzed by the HPLC method. The assay was realized in triplicate and over 24 h.

## 3. Results

### 3.1. Chromatography

Initial runs were performed using methanol and water in various proportions as the mobile phase and in the isocratic mode. Irregular shaping and tailing of MLT peak was observed (in all cases the tailing factor, T, was more than 3.0). Then acetonitrile and water in various proportions were tested and the peak irregularity was more evident in the higher water proportions (T value was in the range of 2.0–2.5). Decreasing water ratio, we found the mixture of acetonitrile and water in proportion of 65:35 (v/v), eluted at a flow rate of 0.9 mL/min associated with other parameters such as column temperature (30 °C) sample temperature (25 °C), injection volume (20 μL) and wavelength (220 nm), as the best results obtained with regular and symmetrical MLT peak. In these conditions, MLT was detected at 1.3 min (Fig. 1). The system suitability parameters were obtained to verify the system performance and the number of theoretical plates (N = 1444), tailing factor (T = 1.25), height equivalent to a theoretical plate (HEPT = 0.008) and capacity factor (K = 1.12) are in accordance with specified limits. K values between 2 and 10 are considered ideal, but it is acceptable that this interval may be extended to the range of values from 1 to 20 for isocratic elution. The purity of the peak was confirmed by the peak purity angle (0.120) and threshold angle (0.307).

### 3.2. Method validation

Linearity was evaluated using a calibration curve to check the ability of the analytical method to obtain a proportional response to the analyte concentration in the sample. Based on seven concentrations from 10 to 100 μg/mL, in triplicate, the linearity of the analytical method was evaluated and a calibration curve was constructed. The regression equation of the line was obtained ($Y = 1.388\times10^5X + 1.214\times10^5$), resulting in the correlation coefficient (*r*) of 0.9993, indicating the quality of curve.

To assess the method specificity, blank nanoparticles (without MLT addition) were prepared and the supernatant obtained from their centrifugation was diluted in the mobile phase and analyzed by the HPLC method. The chromatogram obtained was compared to chromatogram of an MLT standard and MLT sample. MLT sample (Fig. 2A) showed the characteristic MLT peak at approximately 1.3 min, which was in agreement with that obtained from MLT standard solution (Fig. 1). The analysis of the supernatant of nanoparticles without MLT revealed no peak at this retention time (Fig. 2B), indicating that the components of nanoparticles formulation did not interfere in the quantitative determination of MLT.

For precision analysis, MLT standard solutions (10, 50 and 100 μg/mL) were prepared in triplicate, and analyzed on the same day (repeatability) or in three different days (intermediate precision). Tables 1 and 2 show both precision did not exceed the required RSD value. The maximum RSD value was 2.04%.

Accuracy was performed by the addition of standard drug solution in MLT sample solution (4 μg/mL), at three different concentration levels, 75%, 100% or 125% of the sample concentration. The percentage of recovery at these three different concentration levels is described in Table 3. The mean percentage of recovery of MLT from the samples was 100.47% (RSD = 1.25%).

In the present study, the lowest concentration at which an analyte can be detected (LOD) or quantified (LOQ) with acceptable precision and accuracy was calculated from the standard deviation of the response and the slope obtained from linear regression of the calibration curve. LOD was 25.9 ng/mL and LOQ value 78.7 ng/mL. To confirm these values, standard solutions in the LOQ and LOD were prepared and submitted to HPLC analysis. The standard solution of 26.0 ng/mL was detected and the standard solution of 79.0 ng/mL was

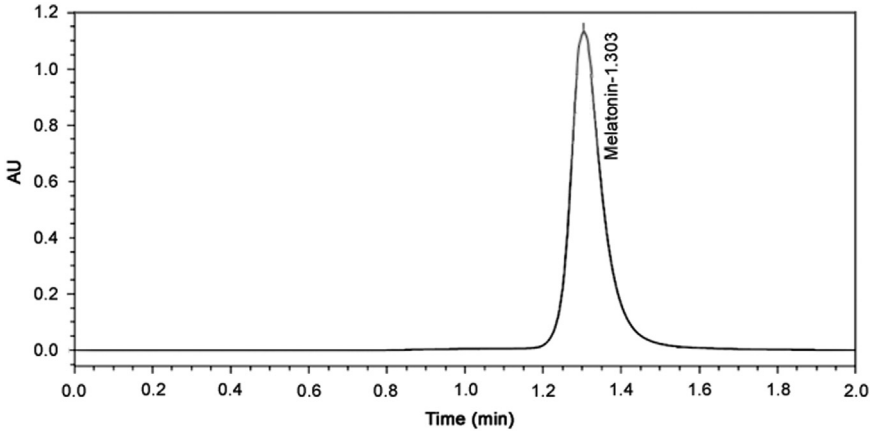


**Fig. 1.** Representative HPLC chromatogram of 50 μg/mL melatonin standard solution. Conditions: mobile phase acetonitrile: water (65:35, v/v); flow rate 0.9 mL/min; PDA detection wavelength 220 nm; column temperature 30 °C; sample temperature 25 °C; injections volume 20 μL.

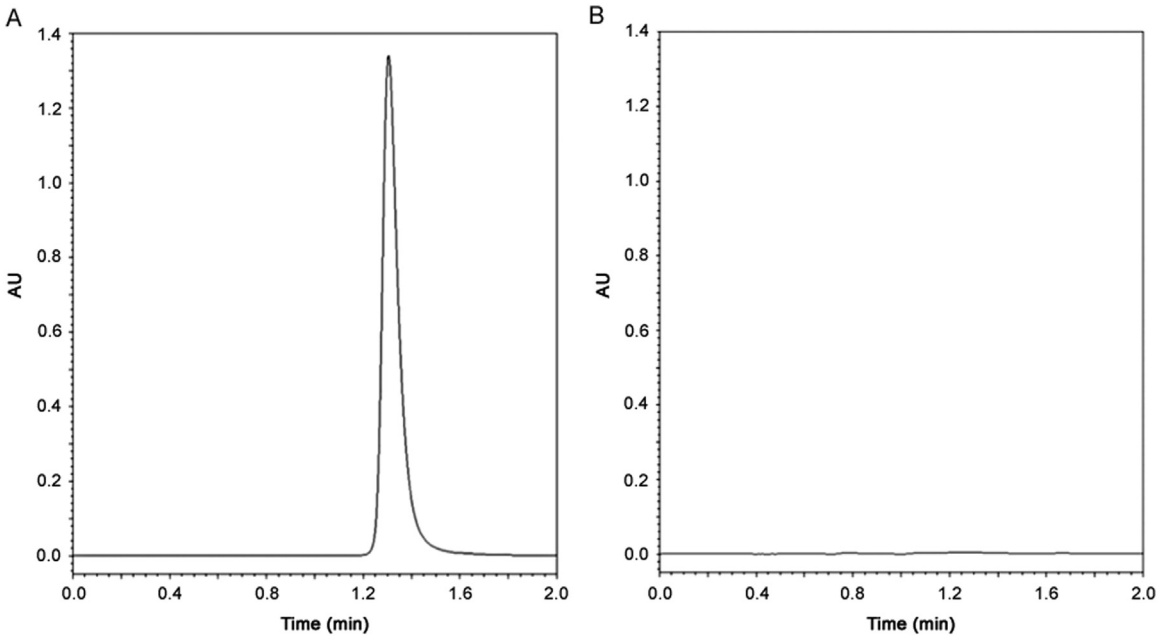


**Fig. 2.** Representative HPLC chromatograms of (A) supernatant from melatonin nanoparticles and (B) supernatant from blank nanoparticles. Conditions: mobile phase acetonitrile: water (65:35, v/v); flow rate 0.9 mL/min; PDA detection wavelength 220 nm; column temperature 30 °C; sample temperature 25 °C; injections volume 20 µL.

**Table 1**
Repeatability for different levels of melatonin ($n$ = 3).

| Melatonin concentration (µg/mL) | Measured concentration ± SD[a] (µg/mL) | RSD[b] (%) |
|---|---|---|
| 10 | 9.24 ± 0.05 | 0.55 |
| 50 | 49.96 ± 0.36 | 0.71 |
| 100 | 98.54 ± 0.03 | 0.03 |

[a] SD: standard deviation.
[b] RSD: relative standard deviation.

**Table 2**
Intermediate precision for different levels of melatonin.

| Melatonin concentration (µg/mL) | Measured concentration[a] ± SD[b] (µg/mL) | RSD[c] (%) |
|---|---|---|
| 10 | 10.18 ± 0.14 | 1.37 |
| 50 | 50.79 ± 1.04 | 2.04 |
| 100 | 101.01 ± 1.52 | 1.50 |

[a] Mean concentration measured on three different days.
[b] SD: standard deviation.
[c] RSD: relative standard deviation.

**Table 3**
Accuracy results for the HPLC method ($n$ = 3).

| Melatonin concentration (µg/mL) | Recovery (%) |
|---|---|
| 7 | 101.96 |
| 8 | 100.56 |
| 9 | 99.89 |

quantified ($n$ = 3).

A method is robust when it remains unchanged even in small variations in the analytical parameters. Robustness was assessed by the percentage of recovery and RSD of the mean concentration of the analyte, at three different concentrations, using different parameters for flow rate of mobile phase, column temperature and ratio of mobile phase. The method proved to be robust considering these changes in chromatographic parameters (Table 4). The maximum RSD obtained was 1.03% when the flow rate of the mobile phase was changed to 0.85 mL/min.

### 3.3. Method applicability

The determination of the encapsulation efficiency of MLT in PLA nanoparticles was performed by an indirect method. The supernatant containing free MLT, separated from the solid nanoparticles by ultracentrifugation, was analyzed by the analytical validated method proposed in this study. The components of nanoparticles formulation (polymer or surfactant) did not interfere in drug quantification, as observed in specificity assay.

Through single emulsion-solvent evaporation method, PLA nanoparticles containing MLT were obtained with mean particle size of 180 ± 18 nm ($n$ = 3), polydispersity index of 0.08 ± 0.01 and monomodal size distribution profile, indicating homogeneity in particle size. The encapsulation efficiency of MLT was (47 ± 7)% ($n$ = 3). The method was adequate to obtain nanoparticles with satisfactory drug loading capacity.

The in vitro release profile of MLT from nanoparticles was evaluated in dissolution medium (37 °C in PBS buffer pH 7.4) over 24 h. The data were plotted in a curve of the cumulative percentage of MLT released over the time. Fig. 3 shows a pronounced prolongation of MLT release over time since in 24 h approximately 23% of MLT was released.

## 4. Discussion

The objective of this study was to develop a reliable and feasible HPLC-PDA method for the determination of the MLT encapsulation efficiency in PLA nanoparticles and then determinate the drug release profile from nanoparticles. The higher sensitivity of the HPLC method over UV-spectrophotometry is important for the determination of drug release profile, since the amount of drug released over the time can be very low. Especially, considering the used model for assessing the drug release in this study, in each sampling all drugs released during each period are removed. The cumulative release was calculated by the sum of each individual sampling; thus, in this assay the HPLC technique was more reliable than spectrophotometry. There are few studies about the development of MLT-loaded nanoparticles in the literature and

**Table 4**
Robustness results at different levels of flow rate, mobile phase proportion and column temperature ($n$ = 3).

| Changes to original method[a] | 10 μg/mL | | 50 μg/mL | | 100 μg/mL | |
|---|---|---|---|---|---|---|
| | Recovery (%) | RSD[b] (%) | Recovery (%) | RSD (%) | Recovery (%) | RSD (%) |
| Mobile phase (acetonitrile: water 63:37) | 104.35 | 0.92 | 98.67 | 0.53 | 97.90 | 0.17 |
| Column temperature (27 °C) | 95.13 | 0.83 | 100.99 | 0.52 | 99.44 | 0.09 |
| Flow rate of mobile phase 0.95 mL/min | 99.04 | 0.82 | 95.32 | 0.23 | 100.78 | 0.35 |
| Flow rate of mobile phase 0.85 mL/min | 96.57 | 1.03 | 102.57 | 0.22 | 100.90 | 0.13 |

[a] Mobile phase acetonitrile:water (65:35, v/v); column temperature 30 °C; flow rate 0.9 mL/min.
[b] RSD=Relative standard deviation.

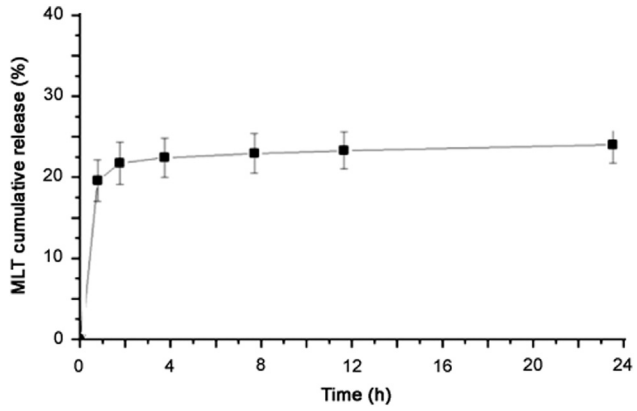


**Fig. 3.** In vitro release profile of melatonin from PLA nanoparticles ($n$ = 3).

thus, there are few reports detailing its quantification in these carriers systems. Musumeci et al. [36] obtained PLGA nanoparticles containing MLT and the encapsulation efficiency was determined by UV–Vis spectrophotometry at 278 nm. Recently, other authors have also obtained PLGA nanoparticles containing MLT and the drug quantitation was carried out by spectrophotometry at 279 nm [38]. In another work, PLA nanoparticles as MLT carriers were obtained by a nanoprecipitation method and the encapsulation efficiency as well as MLT release profile were assessed by UV-spectrophotometry [37]. However, considering the sensitivity, in some situations spectrophotometric methods are not so convenient as HPLC methods. Nanoparticles containing MLT were obtained using Eudragit S100® polymer by nanoprecipitation technique [43] and Hoffmeister et al. [44] obtained MLT-loaded nanocapsules by interfacial deposition. Quantification of MLT in these two studies was conducted by HPLC using acetonitrile and water (55:45, v/v) as mobile phase, but none information about MLT retention time or LOD was given. Albertini et al. [45] quantified MLT from solid lipid microparticles using a mobile phase consisting of ammonium dihydrogen phosphate buffer and methanol (65:35, v/v), and MLT retention time was 7.5 min. In another study, Tursilli et al. [46] determined MLT amount in lipospheres using a mobile phase consisting of sodium acetate buffer (pH 4.0, 0.05 M) and methanol (50:50, v/v), and MLT retention time was 6–8 min. None of these studies reported further details about the chromatographic method such as linearity range or sensitivity.

Therefore, the HPLC-PDA method developed and validated in this work represents an alternative approach for detection of MLT in PLA nanoparticles and determination of its in vitro release profile, filling the requirement for detailed data in the literature for analyzing MLT in nanoparticles via HPLC-PDA detection. The main advantage of the proposed method is the short MLT retention time (1.3 min), allowing the analysis of large number of samples in short period of time.

## 5. Conclusions

An HPLC method for determining the encapsulation efficiency of MLT in PLA nanoparticles and MLT in vitro release profile from nanoparticles was developed and validated in accordance to the ICH guidelines. The method proved to be reliable, fast and simple, complying with the requirements of linearity, specificity, precision, accuracy and robustness in the range of 10–100 μg/mL. Limits of detection and quantification were determined. This method was found suitable for determining MLT amount loaded in PLA nanoparticles and MLT in vitro release profile.

## Conflicts of interest

The authors declare that there are no conflicts of interest.

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