http://dx.doi.org/10.21577/0103-5053.20190205

J. Braz. Chem. Soc., Vol. 31, No. 3, 467-475, 2020
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Article

Application of a New Validated HPLC-PDA Method for Simultaneous Determination of Curcumin and Melatonin in Hyaluronic Acid-Coated Nanoemulsions

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In this study, a high-performance liquid chromatography with photodiode array detector (HPLC-PDA) method was developed and validated to simultaneously determine curcumin (CUR) and melatonin (MEL) in hyaluronic acid-coated nanoemulsions, a novel targetable delivery system to CD44 receptors overexpressed in many types of tumors. Chromatographic analyses were performed in reversed phase mode using a mobile phase consisting of acetonitrile, methanol and 0.1% formic acid (35:15:50, v/v/v) at a flow rate of 1 mL min^{-1}, and detection at 223 and 425 nm. The method was successfully validated according to the parameters of specificity, linearity, limits of detection (LOD) and quantification (LOQ), inter/intra-day precision, accuracy, and robustness. Linearity was demonstrated in the CUR and MEL concentration range of 0.5-20.0 μg mL^{-1} and 1.0-40.0 μg mL^{-1} ($r > 0.999$), respectively. Relative standard deviation (RSD) values for intra-day and inter-day precision were lower than 5%, and mean drug recovery varied from 94.91 to 98.33%. Mean drug content of 85.2 and 501.4 μg mL^{-1} and entrapment efficiency of about 80 and 20% were obtained for CUR and MEL, respectively. These results may be correlated to the differences in the drug solubility of these drugs in the oil and water phases of the nanoemulsion.

Keywords: analytical validation, curcumin, melatonin, nanoemulsions, oral carcinoma

Introduction

Bioactive molecules extracted from diverse natural sources have been considered potential drug candidates for anticancer therapy. About 80% of drugs approved by the United States Food and Drug Administration during the last three decades for cancer therapy either are natural products *per se* or are based on, or mimicked natural products.[1,2] The anticancer and cancer preventive activity of natural products can be explained by multiple cellular and molecular mechanisms, for example, programmed cell death (apoptosis), alteration of cell cycles, anti-angiogenic and anti-inflammatory activity.[1,3]

Curcumin ((1*E*,6*E*)-1,7-bis(4-hydroxy-3-methoxy-phenyl)-1,6-heptadiene-3,5-dione, CUR) (Figure 1a) is a polyphenol derived from the plant *Curcuma longa* L., commonly called turmeric, which has been associated with antioxidant, anti-inflammatory, anticancer, antiviral, and antibacterial activities, as indicated by over 6,000 citations.[4-7] Several clinical trials dealing with cancers have addressed the pharmacokinetics, safety, and efficacy of CUR in humans.[8] Other natural-derived substance with anticancer activity is the indoleamine melatonin (*N*-[2-(5-methoxy-1*H*-indol-3-yl)ethyl]acetamide) (Figure 1b), which is synthesized in the pineal gland and is widely distributed in bacteria, unicellular organisms, algae, plants, invertebrates, and in many organs of vertebrates.[4,9,10] Melatonin (MEL) has a variety of biological properties including anti-inflammatory and antioxidant activity, as well as immune system regulator.[11-15] In cancer cells, MEL has the capacity to induce intracellular reactive oxygen species (ROS), whose accumulation plays an upstream role in mitochondria-mediated apoptosis and autophagy. Moreover, it can act more specifically on cancer cells, but not on normal cells, showing synergistic anticancer activity and reducing undesirable side effects in several chemotherapy regimens.[11,14,15]

Several *in vitro* and *in vivo* studies describe the oncostatic properties for both CUR and MEL against squamous cell carcinoma and other tumors of the oral cavity, through several biological mechanisms, including antiproliferative functions, stimulation of anticancer

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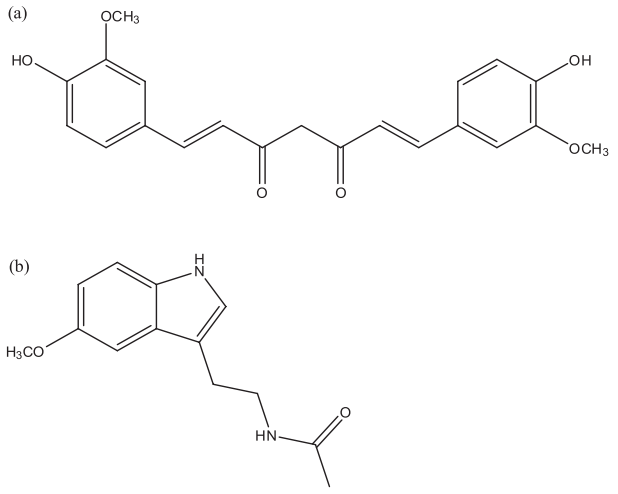

Figure 1. Chemical structure of (a) curcumin and (b) melatonin.

immunity, modulation of the oncogene expression, and anti-antioxidant, inflammatory, antimetastatic, and antiangiogenic effects.[16-21] However, delivering drugs in the oral cavity may be a challenging task, since drugs need to overcome the mechanical effects of the salivary washout that can quickly remove the dosage form from buccal epithelium. In this sense, the use of mucoadhesive nanocarriers has emerged as promising drug delivery systems for buccal administration, since they can interact with the mucus layer, extending contact time and increasing buccal bioavailability.[22] Besides the high drug carrier capability, nanocarriers can accumulate passively into tumor tissue due to its inherited enhanced permeability owed to the presence of larger endothelial fenestrations of the tumor vasculature that allow extravasation of the submicrometric particles.[23]

Considering the above mentioned, we have developed cationic nanoemulsions (NEs) co-encapsulating CUR and MEL intended to the treatment of the oral cavity cancers. Nanoemulsions (also known as miniemulsions) are kinetically stable emulsions with droplets size in the nanometric range. Nanosized droplets lead to improved solubility and bioavailability of drugs and they have the potential to target active molecules to particular tissues or sites. The NEs exhibit unique behavior due to their nanoscopic dimensions, including remarkable stability, droplet-droplet interactions, and rheological properties.[24,25] Besides, aiming to achieve the active targeting of the drugs towards tumor cells, nanoemulsions were coated with hyaluronic acid (HA), a natural polysaccharide found in the extracellular matrix and synovial fluids of the body, that binds to specific cell receptor CD44, which is highly overexpressed in several cancers, including the oral carcinoma.[26-29]

The quantitative determination of the drug incorporated into NEs is usually performed by analytical methods that need to be adequately validated to ensure a reliable quantification of the analyte.[30,31] Literature on analysis of CUR revealed several methods based on different techniques, such as high-performance liquid chromatography (HPLC) with fluorescence detection, which have been used for its quantification in biological samples, and liquid chromatography-mass spectroscopy (LC-MS) for analysis in food products and plasma.[32-34] HPLC methods with UV detection were also used for determining CUR in biological fluids, liquid crystals, tablets and capsules, and liposomes.[8,35-37] Similarly, MEL determination in biological and pharmaceutical samples, such as nanoparticles, have been carried out by HPLC with fluorescence and UV detection.[31,38-40] It is important to consider that, depending on the method used for drug extraction, some components of the nanoemulsions can absorb in UV wavelength range, and thus, impair drug quantification. Moreover, no detailed reports of HPLC methods for simultaneous determination of CUR and MEL in NEs are described. Therefore, the present study aims to develop a simple, sensitive, accurate and reproducible method for simultaneous estimation of CUR and MEL in NEs by HPLC-PDA (photodiode array detector). The PDA enables to analyze one or more wavelengths and it is very efficient in confirming the peak purity of the several reported peaks.[41]

Experimental

Materials

Castor oil, hexadecyltrimethylammonium bromide (CTAB), hyaluronic acid (molecular weight 130-150 kDa), curcumin (≥ 94% curcuminoid content, ≥ 80% curcumin) and melatonin (≥ 98% melatonin) were purchased from Sigma-Aldrich (St. Louis, USA). Hydrogenated soybean lecithin (Lipoid S100, 97.5% phosphatidylcholine) was purchased from Lipoid GmbH (Ludwigshafen, Germany). Analytical grade acetone and ethanol were purchased from Neon Comercial (Suzano, Brazil) and HPLC grade acetonitrile, methanol and formic acid from Honeywell International Inc. (Charlotte, USA). Water utilized in the HPLC analyses was obtained using a Milli-Q purification system from Millipore (Burlington, USA).

Instrumentation and chromatographic conditions

The experiments were conducted using a Shimadzu HPLC system (Japan) equipped with an LC-20AD binary pump, an SIL-20AC HT auto-sampler, a CTO-20A forced air-circulation-type column oven, an SPD-M20 photo diode array UV-Vis detector, and a software LC Solution

1.2 (Shimadzu, Tokyo, Japan). The analyses were carried out in reversed phase mode using a Phenomenex® Luna C18 column (150 × 4.6 mm × 5 µm; USA). The column oven was conditioned at 40 °C. Chromatographic analysis was performed on the isocratic mode with a mobile phase consisting of acetonitrile, methanol and 0.1% (v/v) formic acid aqueous solution (35:15:50; v/v/v), flow rate of 1.0 mL min^{-1}, sample injection volume of 10 µL, and detection at wavelengths of 223 and 425 nm for CUR and MEL, respectively. The run time was 8 min.

Method validation

Validation of the analytical method was performed based on the International Conference on Harmonization (ICH)[42] and ANVISA[43] guidelines, according to the following parameters: linearity, specificity, precision, accuracy, limit of detection (LOD), limit of quantification (LOQ), and robustness.

Specificity was determined by comparing the representative chromatograms obtained for unloaded nanoemulsions and CUR-MEL-loaded nanoemulsions in order to verify the possible interference of the formulation constituents in the HPLC-PDA analysis. The system suitability of the HPLC method was verified by analyzing six replicate samples containing CUR (20.0 µg mL^{-1}) and MEL (40.0 µg mL^{-1}) using the developed method. The parameters of relative retention, repeatability, resolution between the CUR and MEL peaks, theoretical plates of the column, and tailing factor were checked.

Linearity was evaluated by constructing three different calibration curves, with seven points each. For that, a stock solution of CUR and MEL at concentrations of 100 and 200 µg mL^{-1}, respectively, was prepared using acetonitrile as solvent. Subsequent dilutions were carried out to obtain standard solutions containing CUR and MEL at concentrations of 0.5, 1.0, 2.0, 4.0, 10.0, 15.0, 20.0 µg mL^{-1} and 1.0, 2.0, 4.0, 8.0, 20.0, 30.0, 40.0 µg mL^{-1}, respectively, in the same solvent. All the samples were filtered through a 0.22 µm pore size filter (PVDF, Allcrom®, USA) before injection. The linearity of the method was verified through the calculation of the correlation coefficient (r) of the analytical curves using the least squares method. The peak area was divided by the corresponding drug concentration and the resulting values were analyzed by the one-way analysis of variance (ANOVA) to confirm the validity of the linear regression (α = 0.05; 95% confidence). Homoscedasticity was assessed by analysis of the residuals plots. The LOD and LOQ for CUR and MEL were calculated according the following equations:

$$LOD = \frac{3 \times \sigma}{S} \quad (1)$$

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

where σ is the standard deviation of *y*-axis interception values of the calibration curves and S is the angular coefficient of the calibration curve.

Repeatability (intra-day precision) of the method was assessed by testing three solutions of CUR and MEL, in three different levels: low (0.5 and 1.0 µg mL^{-1}), medium (4.0 and 8.0 µg mL^{-1}) and high (20.0 and 40.0 µg mL^{-1}) on the same day. Intermediate precision (inter-day) was evaluated through the analysis of these solutions on three different days, in triplicate. Results were expressed as percentage of relative standard deviation (RSD).

Accuracy of the method was assessed by the recovery test after spiking unloaded nanoemulsions with a CUR and MEL solution in order to obtain theoretical drug concentrations of three different levels: low (0.5 and 1.0 µg mL^{-1}), medium (4.0 and 8.0 µg mL^{-1}) and high (20.0 and 40.0 µg mL^{-1}). The analyses were performed in triplicate.

Robustness of the chromatographic method was evaluated by analysis of a CUR and MEL solution, both at concentration of 40.0 µg mL^{-1}, after small changes in the analytical parameters of column temperature (38 or 42 °C), flow rate (0.9 or 1.1 mL min^{-1}), mobile phase composition (37:13:50 or 33:17:50; v/v/v), mobile phase pH (3.2 or 3.8), and wavelength detection (422/220 or 428/226 nm). The analyses were carried out in triplicate and the results were expressed in percentage of drug recovery and RSD.

Determination of CUR and MEL content and encapsulation efficiency in hyaluronic acid-coated nanoemulsion

Firstly, a cationic nanoemulsion ($NE_{CUR/MEL}$) was prepared by spontaneous emulsification, according to a previously described procedure.[44] The method consists of adding 400 mg of castor oil to an organic phase comprising 60 mg of lecithin, 0.5 mM of CTAB, 2 mg of CUR, and 10 mg of MEL previously dissolved in 20 mL of an ethanol and acetone (1:4, v/v) mixture. This organic phase was added to an aqueous phase (40 mL) under moderate magnetic stirring for 10 min. The formation of the cationic nanoemulsions was instantaneous, which was evident due to the milky appearance of the colloidal dispersion. Nanoemulsions were submitted to evaporation under reduced pressure to eliminate the organic solvents and concentration of the colloidal dispersions. Hyaluronic

acid-coated nanoemulsion (HA-$NE_{CUR/MEL}$) was prepared by adding 2 mg of HA in the aqueous phase, before nanoemulsion formation. Unloaded nanoemulsions were prepared using the same procedure, without adding the drugs. Finally, unloaded and CUR-MEL-loaded nanoemulsions were filtered using filter paper of 8 μm pore size to remove any drug precipitate and the pH of the resulting colloidal dispersions was adjusted to 5-7. Nanoemulsions were characterized according to the droplet size and zeta potential by dynamic light scattering (DLS) and laser-Doppler anemometry, respectively, using a Zetasizer Nanoseries (Malvern Instruments, UK). All formulations were prepared in triplicate.

CUR and MEL content were determined after complete dissolution of nanoemulsions in acetonitrile. The total content of drugs was expressed in μg mL^{-1}. Encapsulation efficiency was estimated as being the difference between the total content of CUR and MEL found in nanoemulsions and the concentration found in the supernatant obtained from the ultracentrifugation of the sample in Optma MX-XP ultracentrifuge (Beckman Coulter, USA) at speed rate of 55,000 × g during 30 min at 25 °C. The samples were appropriately diluted in acetonitrile, filtered through 0.22 μm polyvinylidene difluoride (PVDF) membrane (Merck Millipore, USA), and injected in the chromatographic system. The encapsulation efficiency (EE) was expressed as percentage of each drug associated to the droplets.

Results and Discussion

This study aimed the development of a fast and reliable HPLC method to simultaneously determine CUR and MEL in hyaluronic acid coated-nanoemulsions intended to buccal administration. To find the appropriate HPLC conditions for separation, initial runs were performed using acetonitrile and 0.1% (v/v) formic acid solution as the mobile phase in several proportions and at isocratic mode. Regular shaping and no tailing of MEL peak was observed in most cases (Figure 2). On the other hand, commercially available CUR consists of a mixture of three curcuminoids, with CUR as the main (≥ 80%) constituent and minor amounts of demethoxycurcumin (ca. 17%), and bisdemethoxycurcumin (ca. 3%).[8] Using only acetonitrile and 0.1% formic acid solution, the separation of the peaks corresponding to the three curcuminoids could not be performed and they were co-eluted. To obtain desirable separation of the curcuminoids it was necessary to add methanol in the mobile phase and to determine the most appropriate solvent ratio for separation. The successful separation of CUR, demethoxycurcumin, and bisdemethoxycurcumin peaks was attained using an acetonitrile:methanol:0.1% (v/v) formic acid (35:15:50, v/v/v) mixture as mobile phase, with a reversed phase column equilibrated at temperature of 40 °C, and a flow rate of 1 mL min^{-1}. The curcuminoids peaks could be distinguished from each other based on their retention time, with CUR being the most retained compound.[45] By applying the optimized HPLC method, CUR and MEL exhibited retention times of 7.5 and 2.4 min, respectively (Figure 2). MEL (log P ca. 1.6) displayed the lower retention time, as it was expected, since it is a more polar drug comparing to CUR (log P ca. 3.2).[46,47] The purity of the peaks was confirmed by the comparison of spectra recorded with PDA detector during the registration of chromatographic profile.[48] The λmax values were determined by scanning with PDA detector from 200 to 500 nm; the maximum light absorption for CUR and MEL occurred at 425 and 223 nm, respectively, and these wavelengths were chosen to quantify the drugs in the nanoemulsions.

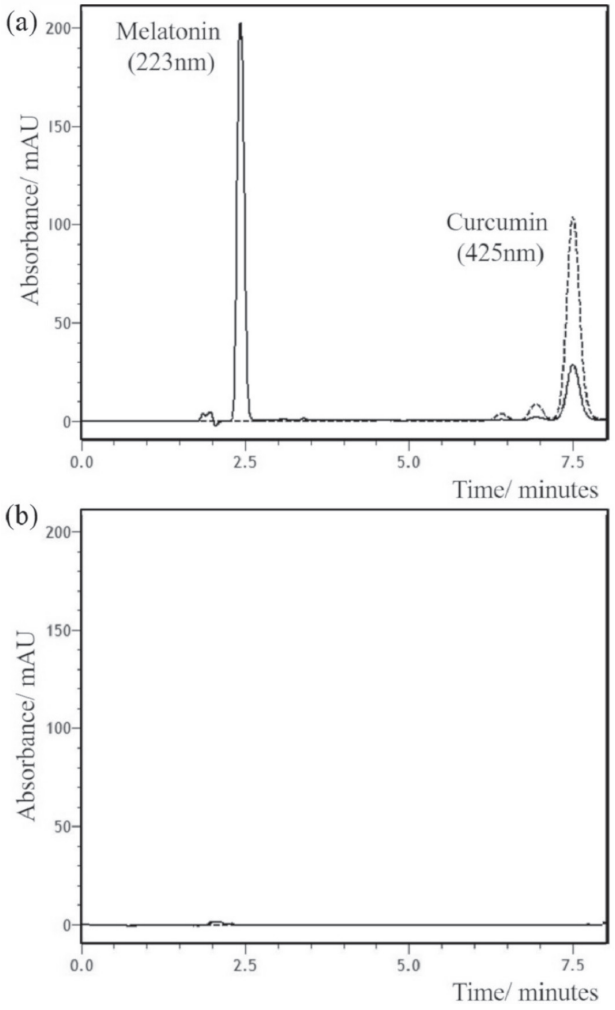

Figure 2. Representative chromatographic profiles obtained for (a) CUR-MEL-loaded HA-acid coated nanoemulsions; (b) unloaded HA-coated nanoemulsions. Solid and dotted lines correspond to the chromatograms obtained with detection at 223 and 425 nm, respectively.

Table 1. Results obtained in the evaluation of the system suitability of the HPLC method (n = 6)

Parameter	Curcumin	Melatonin
Retention time[a] / min	7.55 ± 0.01 (0.14)	2.43 ± 0.02 (0.71)
Peak area[a] / mAU	1868417.1 ± 13839.5 (0.74)	2317736.1 ± 22518.4 (0.97)
Theoretical plate number (N)	8916.5 ± 41.9	6005.6 ± 85.8
Tailing factor	1.22 ± 0.02	1.09 ± 0.01

[a]In parenthesis: relative standard deviation percentage.

The system suitability test was used to verify if the chromatographic system is suitable for performing the analyses. Theoretical plate number more than 2000, tailing factor less than 1.5, and RSD less than 2% for repeatability and retention time were set as acceptance criteria.[42] The results obtained in the evaluation of the suitability of the HPLC method are summarized in the Table 1. The acceptance criteria were met for all parameters evaluated, testifying the system suitability of the method. The resolution between curcumin and the other curcuminoids was considered satisfactory, since values were equal or exceeded 1.5.

Specificity was determined by comparing the representative chromatograms obtained after injection of unloaded nanoemulsions (without drugs) and CUR-MEL-loaded nanoemulsions into the chromatographic system. As can be seen in Figure 2, the other components of the nanoemulsions did not interfere in the peaks corresponding to the drugs, evidencing the specificity of the method.

Linearity was verified by applying the linear regression model to fit the CUR and MEL analytical curves over the concentration range from 0.5 to 20.0 μg mL^{-1} and from 1.0 to 40.0 μg mL^{-1}, respectively. Both calibration curves were demonstrated to be linear over the tested concentration range ($r > 0.999$) (Figure 3). Analyses of variance demonstrated there were no significant differences between the values of peak area to drug concentration ratio obtained for each calibration curve ($F_{calculated} < F_{critical}$, 95% confidence interval) (Table 2). The residue analyses are shown in the Figures 3c (CUR) and 3d (MEL). The residue analysis from each calibration curve exhibited random patterns, thus, the homoscedasticity assumption is satisfied indicating a good fit for the linear model. The LOD and LOQ values for CUR and MEL were 0.09 and 0.23 μg mL^{-1} and 0.30 and 0.85 μg mL^{-1}, respectively, demonstrating that the chromatographic method is suitable enough to detect and quantify these drugs in the HA-coated nanoemulsions.

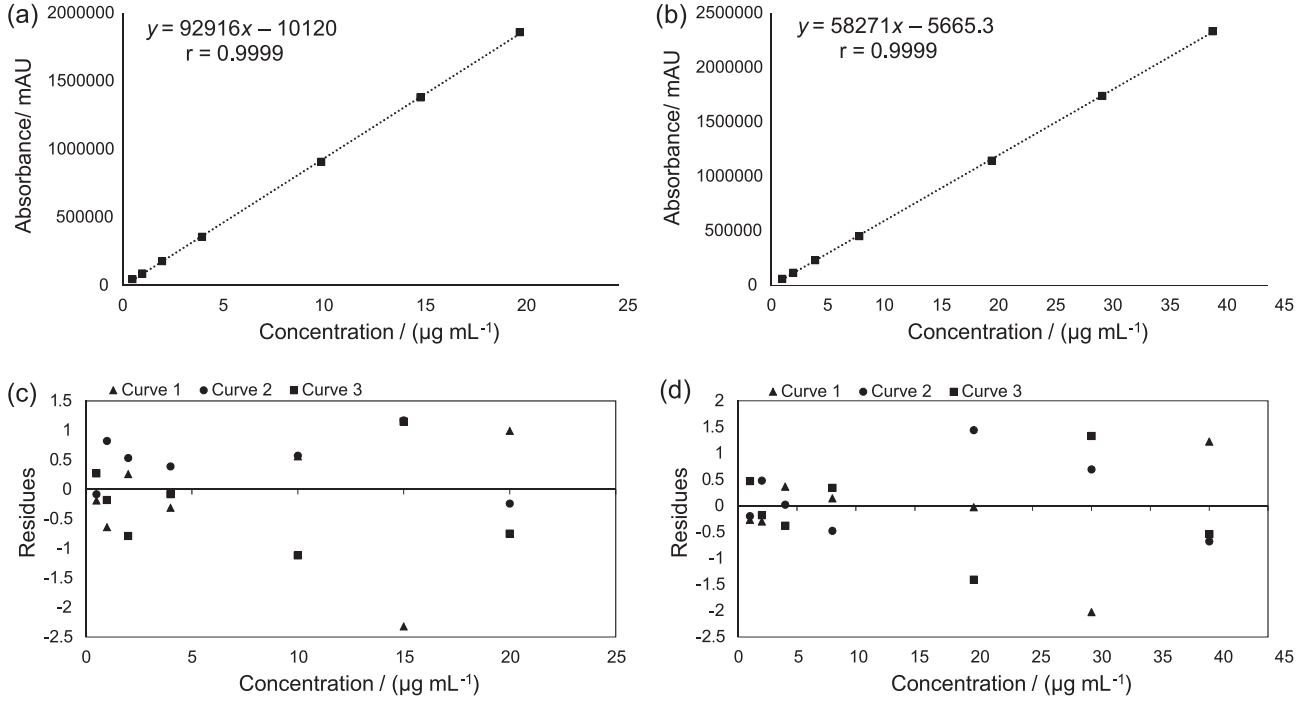

Figure 3. Representative calibration curve obtained for (a) curcumin and (b) melatonin after injection of working standard solutions at the concentration range from 0.5 to 20 μg mL^{-1} (curcumin) and 1.0 to 40.0 μg mL^{-1} (melatonin). Residual plots obtained for (c) curcumin and (d) melatonin.

Table 2. Analysis of variance (ANOVA) for the linear regression analysis

Source of variation	DF[a]	SS[b]	MS[c]	$F_{calculated}$	p-value[d]	$F_{critical}$
Curcumin						
Between groups	2	17118301.7	8559150.8	0.85	0.44	3.55
Within groups	18	181982006.9	10110111.5			
Total	20	199100308.5				
Melatonin						
Between groups	2	2403530.6	1201765.3	0.43	0.65	3.55
Within groups	18	49438102.5	2746561.2			
Total	20	51841633.1				

[a]Degrees of freedom; [b]sum of squares; [c]mean square; [d]$p > 0.05$.

Precision of a method is the closeness of agreement between independent test results obtained under given analytical conditions and includes the measurement of the intra-day (within-day or repeatability) and of the inter-day (between-day) precision. Table 3 shows the results obtained in the evaluation of intra-day and inter-day precision for both CUR and MEL. RSD values lower than 5% were obtained for the two drugs, even in the smallest concentration, assuring a satisfactory precision of the HPLC method.

In order to evaluate the accuracy of the chromatographic method, recovery experiments were performed after spiking unloaded HA-coated nanoemulsions with a CUR and MEL standard solution at three different concentration levels (low, medium and high). Drug recovery values varied from 94.78 to 98.33% with RSD values lower than 3% (Table 4), thereby indicating a low variability and a close agreement between the experimental and theoretical concentration values.

An HPLC method can be considered robust when it remains unaffected after small changes in the analytical parameters. As can be seen in Table 5, small changes in the flow rate, mobile phase composition and pH, column temperature, and wavelength detection did not affect the determination of both CUR and MEL, since the higher RSD value obtained was 4.67%. Then, the developed method proved to be robust for quantification of CUR and MEL in the samples.

Method applicability

Uncoated and hyaluronic acid-coated nanoemulsions were successfully obtained by the spontaneous emulsification method. Both colloidal dispersions displayed size in nanometric range (ca. 200 nm) and monodispersed droplet size distribution (polydispersity index ca. 0.2). Uncoated nanoemulsions exhibited a positively charged surface as expected, due to the presence of the cationic surfactant CTAB at the oil-water interface of droplets. The addition of HA in the nanoemulsion caused not only an increase of the droplet size, but also the reduction of the zeta potential value. HA is negatively charged glycosaminoglycan[49] and therefore, it may be deposited

Table 3. Intra-day and inter-day precision for quantification of curcumin (CUR) and melatonin (MEL)

Drug concentration / (μg mL^{-1})	Intra-day precision[a]		Inter-day precision[b]	
	Measured concentration / (μg mL^{-1})	RSD[c] / %	Measured concentration / (μg mL^{-1})	RSD[c] / %
CUR				
0.5	0.49 ± 0.02	4.98	0.50 ± 0.01	2.29
4.0	4.06 ± 0.05	1.41	4.08 ± 0.07	1.77
20.0	21.33 ± 0.57	2.70	21.5 ± 0.50	2.32
MEL				
1.0	1.05 ± 0.04	4.15	1.07 ± 0.04	3.75
8.0	8.12 ± 0.16	1.98	8.09 ± 0.11	1.36
40.0	40.56 ± 0.92	2.29	40.73 ± 0.64	1.57

[a]Results expressed as mean ± standard deviation of three drug solutions analyzed in the same day; [b]results expressed as mean ± standard deviation obtained after analysis of the drug solution in three different days; [c]relative standard deviation.

Table 4. Curcumin (CUR) and melatonin (MEL) recovery values obtained after spiking unloaded nanoemulsions

Drug added / (μg mL^{-1})	Drug found[a] / (μg mL^{-1})	Recovery / %	RSD[b] / %
CUR			
0.5	0.48 ± 0.01	96. 22 ± 2.03	2.11
4.0	3.90 ± 0.10	97.50 ± 2.50	2.56
20.0	18.95 ± 0.26	94.78 ± 1.33	1.41
MEL			
1.0	0.98 ± 0.01	98.33 ± 1.15	1.17
8.0	7.78 ± 0.07	97.25 ± 0.90	0.92
40.0	37.96 ± 1.01	94.91 ± 2.50	2.63

[a]Results expressed as mean concentration ± standard deviation of three determinations; [b]relative standard deviation.

onto droplet surface provoking a partial neutralization of the charges by the electrostatic interaction of its carboxylated groups with the positively charged quaternary ammonium group of CTAB (Table 6).[50,51]

The chromatographic method once validated was applied to simultaneously estimate CUR and MEL in uncoated and HA-coated nanoemulsions (Table 6). When uncoated nanoemulsion were analyzed, CUR and MEL content were found to be 85.2 ± 1.7 and 501.4 ± 5.5 μg mL^{-1}, respectively, while EE were found to be 83.7 ± 2.1 and 20.9 ± 3.5% for the respective drug. The differences in the drug solubility expressed by their log P can again explain the different EE values; CUR is a more lipophilic drug and, therefore, it is found preferentially associated to the nanoemulsion droplets, while MEL, presenting higher water solubility, is preferentially partitioned towards the aqueous phase. The addition of HA to nanoemulsion formulations caused a reduction in both CUR and MEL content to 63.5 ± 2.9 and 271.9 ± 10.0%, respectively. In fact, we have hypothesized that HA coating changes the oil-water interface properties, reducing the ability of these drugs to adsorb onto droplet surface. However, further studies should be carried out to confirm this hypothesis. Even so, the HPLC-PDA method developed and validated in this study was successfully applied to simultaneously determine CUR and MEL in nanoemulsions, which are promising formulations with antitumor properties.

Conclusions

In this study, an HPLC-PDA method was developed and validated for simultaneous determination of CUR and

Table 5. Effect of changes in the chromatography conditions on the determination of curcumin (CUR) and melatonin (MEL) both at concentration of 40.0 μg mL^{-1} (n = 3)

Parameter		CUR		MEL	
		Recovery / %	RSD[a] / %	Recovery / %	RSD[a] / %
Flow rate / (mL min^{-1})	0.9	96.88	2.41	99.6	2.90
	1.1	95.78	3.40	97.5	3.12
Mobile phase composition (v/v/v)	37:13:50	97.4	3.90	99.6	3.18
	33:17:50	98.0	2.45	100.3	3.41
Mobile phase pH	3.2	95.9	4.67	98.6	2.11
	3.8	96.1	4.44	99.5	1.79
Temperature / °C	37	97.40	3.14	97.4	3.60
	43	96.79	1.45	101.4	2.76
Wavelength / nm	422/220	98.78	2.34	98.78	1.43
	428/226	96.10	3.56	99.98	4.13

[a]Relative standard deviation.

Table 6. Physicochemical properties and curcumin (CUR) and melatonin (MEL) loading of uncoated ($NE_{CUR/MEL}$) and hyaluronic acid-coated nanoemulsions (HA-$NE_{CUR/MEL}$) (n = 3)

Formulation	Size[a] / nm	Zeta / mV	Drug content / (μg mL^{-1})		EE[b] / %	
			CUR	MEL	CUR	MEL
$NE_{CUR/MEL}$	179.7 ± 9.8 (0.116)	41.6 ± 1.0	85.2 ± 1.7	501.4 ± 5.5	83.7 ± 2.1	20.9 ± 3.5
HA-$NE_{CUR/MEL}$	234.8 ± 10.0 (0.220)	17.8 ± 2.2	63.5 ± 2.9	271.9 ± 10.0	95.9 ± 1.2	19.9 ± 2.4

[a]In parenthesis: polydispersity index; [b]encapsulation efficiency.

MEL in nanoemulsions intended to the treatment of cancers of the oral cavity. Peaks corresponding to MEL and CUR could be well separated using an analytical procedure with a chromatographic run time of only 8 min, allowing the analyses of a large number of samples in a short period. This method also proved to be reliable, fast and simple, complying with the requirements of linearity, specificity, precision, accuracy, and robustness. Thus, this method was found to be suitable for determining CUR and MEL loaded into hyaluronic acid-coated nanoemulsions.

Acknowledgments

The authors would like to thank the Coordination for the Improvement of Higher Education Personnel (CAPES) for the financial support.

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Submitted: April 9, 2019
Published online: August 28, 2019