International Journal of Drug Delivery 5 (2013) 224-232
http://www.arjournals.org/index.php/ijdd/index

Original Research Article

ISSN: 0975-0215

Development and validation of an HPLC method for the quantification of a cytotoxic dihydropyranoxanthone in biodegradable nanoparticles

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Abstract

Context and purpose 3,4-dihydro-12-hydroxy-2,2-dimethyl-2*H*,6*H*-pyrano[3,2-b]xanthen-6-one (compound 1) is a cytotoxic dihydropyranoxanthone exhibiting antiproliferative effects, inducing S-phase cell cycle arrest, and increasing the percentage of apoptotic cells in leukemia cell lines. Nevertheless, the poor aqueous solubility of compound 1 is a major drawback not only for its potential use in therapy but even for the *in vitro* assessment of its biological activity. Polymeric nanoparticles formulations were developed as potential carriers to overcome problems related with low water solubility of compound 1. The objective of this work was to develop and validate a specific, sensitive and simple HPLC method for the quantitative analysis of the prenylated xanthone (compound 1), which was entrapped in PLGA nanoparticles for the first time.

Main findings Chromatographic separation was performed with a reversed-phase C18 column, using methanol: water (85:15, v/v) containing 1 % (v/v) acetic acid as a mobile phase at a flow rate of 1 ml/min and quantification was made by UV detection at 254 nm. The isocratic system required 10 minutes of chromatographic run. The method was shown to be linear (r> 0.999) over the concentration range of 0.50-3.00 µg/ml and precise at the intra-day and inter-day levels as reflected by the relative standard deviation values (lower than 1.5% and 1.6%, respectively). The mean recovery ranged from 97.53 to 104.28 % (RSD: 0.027%) and from 98.49 to 101.81% (RSD: 0.019 %) for nanospheres and nanocapsules, respectively.

Brief summary and potential implications A simple, linear, sensitive, accurate, and precise HPLC method suitable for the quantification of compound 1 incorporated in polymeric nanoparticles was developed and validated.

Keywords: dihydropyranoxanthone, HPLC, polymeric nanoparticles, xanthones.

Introduction

Prenylated xanthones have shown interesting pharmacological properties, which has aroused great interest in the search for new bioactive derivatives [1]. Among them, 3,4-dihydro-12-hydroxy-2,2-dimethyl-2H,6H-pyrano[3,2-b]xanthen-6-one (compound 1) (Figure. 1), a cytotoxic dihydropyranoxanthone, exhibited interesting effects such as antiproliferative, inducing S-phase cell cycle arrest, and increasing the percentage of apoptotic cells in leukemia cell lines [2]. This cytotoxic dihydropyranoxanthone also enhanced the growth inhibitory action of partial anti-estrogen 4-hydroxytamoxifen against the estrogen sensitive breast adenocarcinoma cancer cell line ER+ MCF-7 [3].

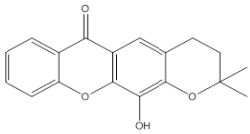

Figure1.3,4-Dihydro-12-hydroxy-2,2-dimethyl-2*H*,6*H*-pyrano[3,2-b]xanthen-6-one (compound 1).

Using adequate software (ACD/Labs Software, Toronto, Canada), the calculated maximum water solubility of compound 1 was determined (16 µg/ml). The poor aqueous solubility of 1 is a major drawback not only for its potential use in therapy but even for the *in vitro* assessment of its biological activity. From the study of the permeability of compound 1 across Caco-2 cells, it was possible to conclude that this prenylated xanthone presented favorable apparent permeability coefficient (unpublished work).

Many reports have demonstrated the increase in solubility of poorly soluble small molecules by modifying the size and functional groups present on the molecule. The entrapment of drugs in nanoparticles can provide a carrier-driven cellular entry mechanism in certain situations that would be irrespective of the solubility or permeability of the entrapped drug. The unique properties of these carrier systems could be used for the drugs that belong to class II (low solubility-high permeability), class III (high solubility-low permeability), or class IV (low solubility-low permeability) of the Biopharmaceutics Classification System [4].

Over the past couple of decades, the field of drug delivery has been revolutionized with the advent of nanoparticles, acting as inert carriers for drugs and genes to target cells or tissues. The development of biodegradable nanoparticles with their small size, ability to encapsulate drug of poor solubility and permeability, controlled drug release manner, and long circulation half-life, are a dominant platform for drug delivery [5]. Particularly, in polymeric nanoparticles comprised of poly(D,L-lactide-co-glycolide) (PLGA), the particle matrix degrades slowly *in vivo* and the by-products like lactic and glycolic acid are easily metabolized and excreted [6]. As shown previously by our group, the incorporation of xanthones in polymeric nanoparticles led to physical stable formulations with adequate physico-chemical properties and, in some cases, with improvement of biological activity [7, 8].

In order to test the usefulness of PLGA nanoparticles as potential carriers to overcome problems related with low water solubility of compound 1, we are carrying out in vitro studies with different formulations containing this prenylated xanthone. An analytical method for the quantification of drug in nanoparticles is necessary during the pharmaceutical development of new nanoparticle formulations. Hence, the objective of this work was to develop and validate a fast, simple, and optimized HPLC method to quantify the content of compound 1, belonging to class II of the Biopharmaceutics Classification System, incorporated in PLGA nanoparticles.

Materials and Methods

Reagents and chemicals

PLGA 50:50 (MW 50 000–75 000), *Pluronic*® F-68, and D-(+)-Glucose were purchased from Sigma-Aldrich Química (Sintra, Portugal). Compound 1 was synthesized as described in literature [2], and possessed a 98.6% purity determined by HPLC-DAD analysis. HPLC grade reagents methanol, acetonitrile and acetic acid were obtained from Carlo Erba Reagents (Val de Reuil, France) and ultra purified water was produced by Simplicity® UV Ultrapure Water System, Millipore Corporation, (Billerica, USA). Miglyol® 812 was purchased from Acofarma (Barcelona, Spain). Prior to use, mobile phase solvents were degassed in an ultrasonic bath for 15 min. Other chemicals were of analytical grade.

Apparatus and chromatographic conditions

The HPLC analysis was performed in a Finnigan Surveyor – Autosampler Plus and LC Pump Plus, Thermo Electron Corporation (Ohio, United States of America), equipped with a diode array detector TSP UV6000LP, Thermo Electron Corporation (Ohio, United States of America) and using a C-18 column (5 µm, 250 mm 4.6 mm I.D.), from Macherey-Nagel (Düren, Germany). The injected volume was 20 µL and the eluent was monitored at 254nm. Xcalibur® 2.0 SUR 1 software, Thermo Electron Corporation (Ohio, United States of America) managed chromatographic data.

Preparation of standard solutions

Stock standard solutions of compound 1 (100 µg/ml) were prepared in acetonitrile. Standard solutions were obtained by dilution of a freshly prepared stock standard solution with acetonitrile to give six different concentrations over the range of interest (0.50 to 3.00 µg/ml).

Nanoparticle preparation and characterization

Nanospheres were prepared according to Fessi *et al*, 1988 [9] with some modifications. In brief, 50 mg of polymer was dissolved in 10 ml of acetone. To this organic solution, 0.75 ml of a solution of compound 1 (1 mg/ml) and 0.50 ml of methanol were added and poured into an aqueous solution of *Pluronic*® F-68 (0.25 %, w/v) (10 ml), under magnetic stirring. Nanosphere dispersion was concentrated to 5 ml under reduced pressure. Separation of non-encapsulated compound was performed first by filtration through a 0.45 µm membrane and then by centrifugation at 2000×*g* for 30 min (Sigma, Osterode am Harz, Germany) after solubilization of a certain amount of glucose for achieving a 5% (w/v) concentration. The supernatant was discarded and the pellet containing the nanospheres was redispersed in water to complete the initial volume of nanosphere dispersion submitted to centrifugation. Empty nanospheres were prepared according to the same procedure but omitting compound 1 in the organic phase.

Nanocapsules were prepared according to a modified procedure of Bernardi *et al,* 2009 [10]. In brief, an organic phase of 50 mg of PLGA, 0.55 ml of a solution of compound 1 in Miglyol® 812 (3.50 mg/ml) was poured into an aqueous solution of *Pluronic*® F-68 (0.385 %, w/v) and submitted to stirring for 5 min. Then, acetone was removed and the colloidal dispersion of nanocapsules was concentrated to 5 ml by evaporation under reduced pressure (Rotavapor Basic R-210, Buchi, Flawil, Switzerland). The amount of non-encapsulated compound was separated by ultrafiltration/centrifugation technique using centrifugal filter devices (Centricon YM-50, Millipore, Lisboa, Portugal) at 2276×*g* for 30 min (Beckman UL-80 ultracentrifuge, Albertville, USA), and the volume completed with Milli Q water. Empty nanocapsules were prepared according to the same procedure but omitting the addition of compound 1 in the organic phase. Particle size and zeta potential of nanoparticles were assessed by Dynamic Light Scattering (DLS) using Zetasizer BI-MAS90Plus (Brokhaven Instruments, USA).

Preparation of sample solutions for determination of compound 1 in nanoparticles

Sample solutions were prepared by dissolving an aliquot of the dihydropyranoxanthone 1 nanosphere or nanocapsule dispersions in acetonitrile (corresponding to a dilution of 1:50 and 1:500, respectively) and subjected to HPLC analysis. Considering 100% of entrapment for compound 1 in nanoparticles, the obtained sample solutions had a maximum theoretical concentration (MTC) of 3 µg/ml and 0.77 µg/ml for nanospheres and nanocapsules, respectively. All analyses were performed in triplicate and the mean results (± standard deviation - SD) are reported. Incorporation efficiency (IE) was calculated as follows:

IE (%) = A/B x 100

where A is the drug concentration (µg/ml) in final nanoparticle dispersions and B is the theoretical drug concentration (µg/ml).

HPLC method validation

Specificity

The specificity of the analytical method was determined in samples of compound 1 submitted to thermal, acidic, and alkaline stress conditions and in samples containing the compound and nanoparticle excipients (i.e. spiked with empty nanoparticles). For the evaluation of thermal degradation, a known amount (1 mg) of compound 1 was placed in an oven at 80ºC for 24 h. Afterwards, the sample was dissolved in acetonitrile and subjected to HPLC analysis. For the evaluation of compound 1 degradation in acidic and alkaline conditions, a known amount (1 mg) of compound 1 was mixed with 25 ml of 1N HCl or with 25 ml of 1N NaOH. Following 36 h of stirring at room temperature, the samples were filtered, diluted with acetonitrile and subjected to HPLC analysis. Control samples (without compound 1) were also prepared and assayed.

Sensitivity

The detection limit (DL) was expressed as:

DL =3.3 /S

where is the standard deviation of the response and S is the slope of the calibration curve. The DL was studied using samples from the calibration curve, containing the analyte in the range of DL.

The quantification limit (QL) was expressed as:

QL =10 /S

where is the standard deviation of the response and S is the slope of the calibration curve. The DL was studied using samples from the calibration curve, containing the analyte in the range of QL, according to according to ICH guidelines [11].

Linearity

Linearity was determined by calculation of a regression line from the peak area *vs.* concentration plot for six standard solutions (0.50, 0.75, 1.00, 1.50, 2.00, and 3.00 µg/ml) [11]. This range corresponds to 17-100 % and 65-390% of the MTC of compound 1 in nanosphere and nanocapsule formulations, respectively. Calibration curves were constructed using linear least squares methodology. The overall procedure was repeated three times on different days.

Accuracy

Accuracy is often calculated as percent recovery by the assay of known, added amounts of analyte to the sample. Accuracy should be assessed using a minimum of nine determinations over a minimum of three concentration levels and by determining the relative standard deviation (RSD)[11, 12].

Recovery experiments were performed by analyzing empty nanoparticle dispersions (nanospheres and nanocapsules) spiked with three standard solutions (1.00, 2.00 and 3.00 µg/ml) (n=9).

Precision

Repeatability (intra-assay precision) of the chromatographic method was determined by the analysis on the same day of five standard solutions of compound 1 in the concentration range tested (three replicates each). Intermediate precision of the chromatographic method was determined by the analysis of the same standard solutions on three different days, and reported as SD and RSD (relative standard deviation).

The repeatability of the total analytical method was investigated by performing six replicate samples of the same batch of nanospheres and of nanocapsules containing compound 1.

Calculations and statistics

Anova for Microsoft® Excel was applied for statistic determinations.

Results and Discussion

HPLC method development

Based on our previous experience with other xanthones [8, 13-15], several proportions of mobile phase compositions were investigated in order to obtain satisfactory retention factors. Best conditions were achieved with methanol: water (85:15, v/v) with 1 % (v/v) of acetic acid was used as mobile phase for the HPLC analysis of compound 1. A flow rate of 1 ml/min gave an optimal signal-to-noise ratio and a reasonable separation time. Retention time of compound 1 was 5.45 (±0.11) min and the total time required for analysis was 10 min. The maximum absorption of the compound in the experimental conditions was found to be 254 nm, which was the selected wavelength for the analysis. Acetonitrile was used for preparation of sample solutions of nanospheres and nanocapsules dispersions containing compound 1. A 50 and 500 fold dilution of aliquots of nanosphere and nanocapsule dispersions were used.

Specificity

Specificity of the method was evaluated by comparing the chromatograms of compound 1 and those of potential interfering formulation components (namely the excipients used on its preparation) and also with the chromatograms resulted from the stress conditions (temperature, 1N HCl, and 1N NaOH). Figure 2 shows a representative chromatogram of a standard solution of compound 1 (1.50 µg/ml) with the retention time of 5.45 min. Another peak with the retention time of approximately 2.5 min, due

to the solvent front, was observed. As shown in Figure 2B-D, when submitting compound 1 to thermal (B), acidic (C), and alkaline (D) stress conditions, no degradation products were formed. Also, the presence of the excipients did not interfere with the analytical method, as can be observed in Figure 2 E-H by the absence of any peak in the region where compound 1 elutes.

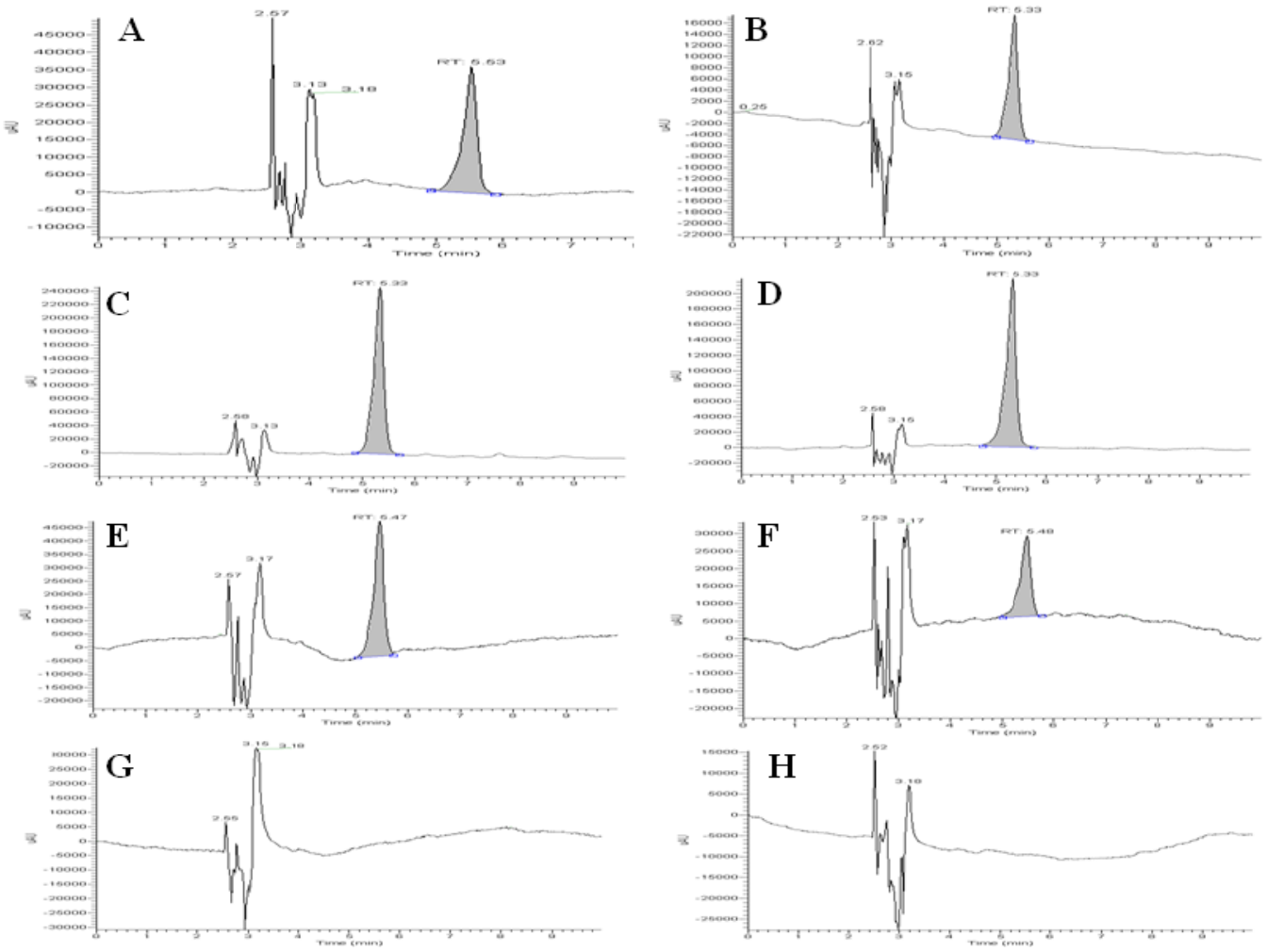

Figure 2. Representative chromatograms obtained following injection of: (A) standard solution of 1 (1.50 µg/ml), (B) compound 1 subjected to thermal degradation (80 ºC, 24 h), (C) acidic degradation (1N HCl, 24 h) and (D) alkaline degradation (1N NaOH, 24 h) (E) standard solutions of 1 (1.00 µg/ml) spiked with empty nanospheres and (F) standard solutions of 1 (1.00 µg/ml) spiked with empty nanocapsules (G) and spiked with empty nanospheres (H).

The peak purity of the samples subjected to thermal, acidic and alkaline stress conditions was, in all cases, higher than 96.9%, indicating that there were no degradation products co-eluting with the dihydropyranoxanthone 1 (Figure 2B-D). This demonstrates the stability-indicating capability of the analytical method. In Figure 3A the UV-spectra of compound 1, after subjecting the samples to thermal, acidic, and alkaline stress conditions, showed a similar profile to the spectra of the standard solution. Furthermore, the absence of any peak in the region of the chromatogram where the dihydropyranoxanthone 1 elutes (Figure 2) and similar profiles of the UV-spectra of loaded nanoparticles and of compound 1 (Figure 3B), indicates the specificity of the developed method, regarding nanoparticle excipients.

In general, the obtained data provide evidence that the method can be regarded as specific since no interfering peaks were observed.

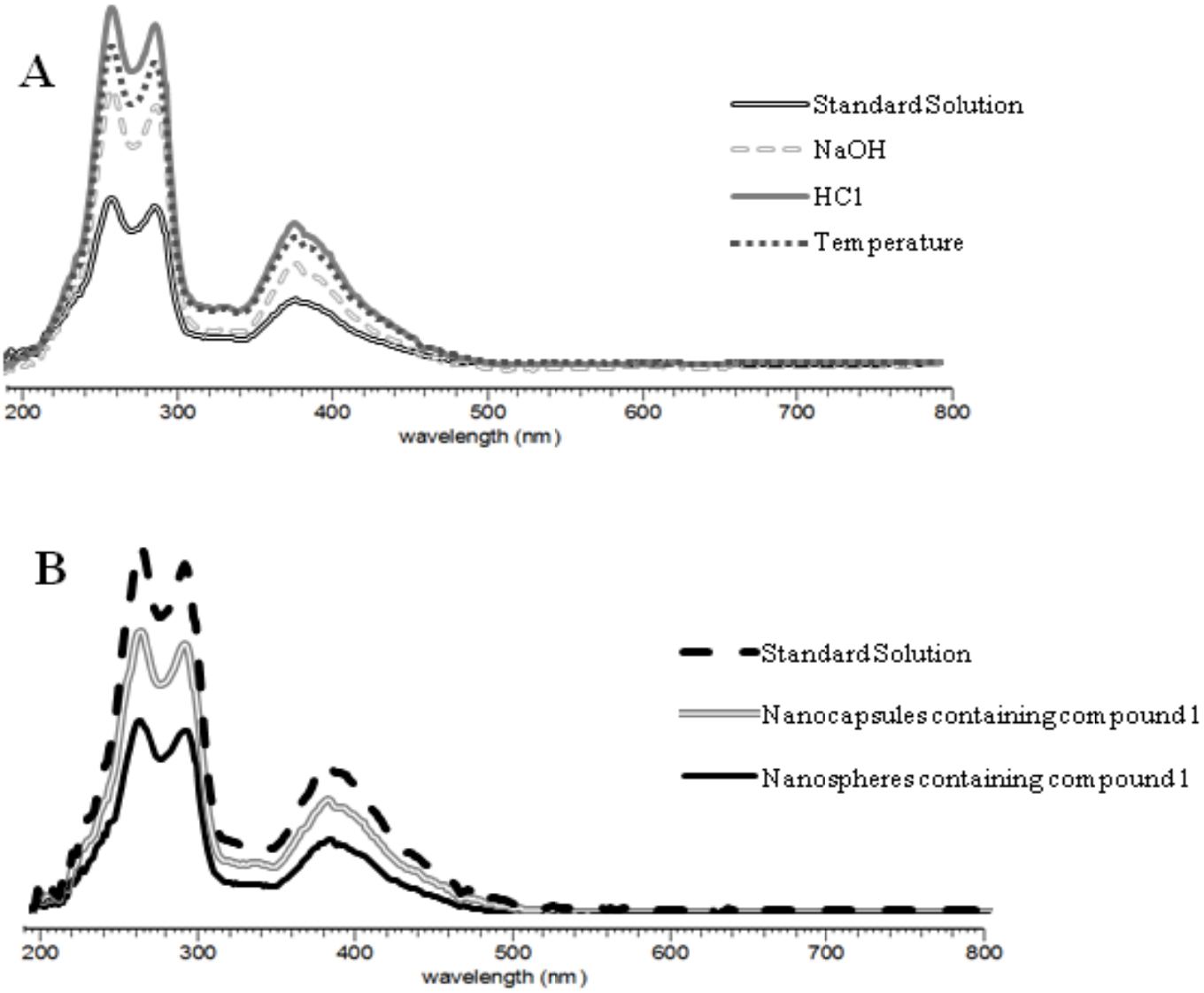

Figure 3. UV spectra of compound 1 standard solution and samples (A) submitted to alkaline (1N NaOH for 36 h), acidic (1N HCl for 36 h) and thermal (80 ºC for 24h) degradation (B) entrapped in nanocapsules and nanospheres.

Sensitivity and linearity

The QL of compound 1 was observed to be 0.177 µg/ml. The DL of compound 1 was 0.058 µg/ml.

Table 1 summarizes the data extracted from the calibration curves prepared with standard solutions of compound 1.

The method has an adequate linearity over the range of 0.50–3.00 µg/ml. The mean regression equation from three replicate calibration curves was y= 563189 (±8410.33)x-74645(±7394.22). The square of mean correlation coefficient (r^2) was 0.999.

The coefficient of correlation can be subjected to misinterpretation and may give a distortion of linearity, since different datasets can yield identical regression statistics. Analysis of the residuals provides further support that the calibration curve would be deemed linear if the residual response shows a normal distribution with a zero mean [16]. The graphic of residuals plotted *vs.* concentration values in shown in Figure 4. The residuals should be randomly distributed around a true mean of zero.

Table 1. Summary of standard curve results

Theoretical concentration(µg/ml)	Average peak area(mV)	SD	RSD(%)
0.50	208116	3461	1.66
0.75	358850	4456	1.24
1.00	485752	6068	1.25
1.50	773268	12265	1.59
2.00	1024718	14153	1.38
3.00	1629325	19406	1.19
Y intercept	-74645.5[a]		
Slope	563188.7[a]		
Correlation coefficient (r)	0.99960		
Coefficient of determination (r^2)	0.99921		

[a] Confidence limits (P=0.05)

As shown in Figure 4 the residual plot shows a random behavior in a constant range and it is not biased in one direction or the other and the mean value is close to zero ($1.33x10^{-6}$). For all stated above, the method proved to be linear for the range of concentrations tested.

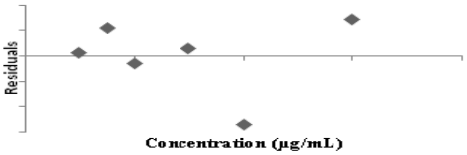

Figure 4. Residual plot *vs.* concentration

Accuracy and precision

Detailed results for the three tested concentration levels are presented in Table 2. The overall recovery was found to be 100.32±0.03 % and 100.12±0.02 %, for nanospheres and for nanocapsules, respectively, showing strong agreement between experimental and theoretical values.

Table 2. Accuracy results for different levels of compound 1

Nanospheres			Nanocapsules		
Theoretical concentration (µg/ml)	Experimental concentration (µg/ml) (n=3)	Recovery (%)	Theoretical concentration (µg/ml)	Experimental concentration (µg/ml) (n=3)	Recovery (%)
1.00	1.0428	104.28±0.05	1.00	1.0007	100.07±3.02
2.00	1.9506	97.53±1.28	2.00	2.0361	101.81±1.75
3.00	2.9749	99.16±1.07	3.00	2.9547	98.49±0.59
Mean recovery (%)		100.32	Mean recovery (%)		100.12
RSD (%)		0.027	RSD (%)		0.019

Repeatability (intra-assay precision) and intermediate precision of the chromatographic method can be consulted in Table 3.

Table 3. Results of precision determinations

Repeatability (intra-assay precision)		
Concentration (µg/ml)	SD	RSD (%)
0.50	0.007	1.50
0.75	0.004	0.58
1.00	0.011	1.15
2.00	0.027	1.40
3.00	0.039	1.28
Intermediate precision (different days)		
Concentration (µg/ml)	SD	RSD (%)
0.50	0.008	1.55
0.75	0.001	0.05
1.00	0.016	1.54
2.00	0.022	1.11
3.00	0.014	0.46

Application of the developed method for the quantification of compound 1 in nanoparticles

The analytical method is effective, fast, and meets all criteria for method validation and can be applied for the quantification of compound 1 incorporated in different batches of nanoparticle formulations. Figure 5A-B shows representative chromatograms of nanospheres and nanocapsules, respectively, containing compound 1 (total scan and discrete channel at 254nm). None of these chromatograms show any interfering peaks. Furthermore, peak purity was close to 97% indicating that the nanoparticle preparation method did not produced degradation of the dihydropyranoxanthone 1. The physicochemical characteristics of the PLGA nanoparticle formulations, namely incorporation efficiency (IE), mean particle size, polidispersity index (PI), and zeta potential are presented in Table 4.

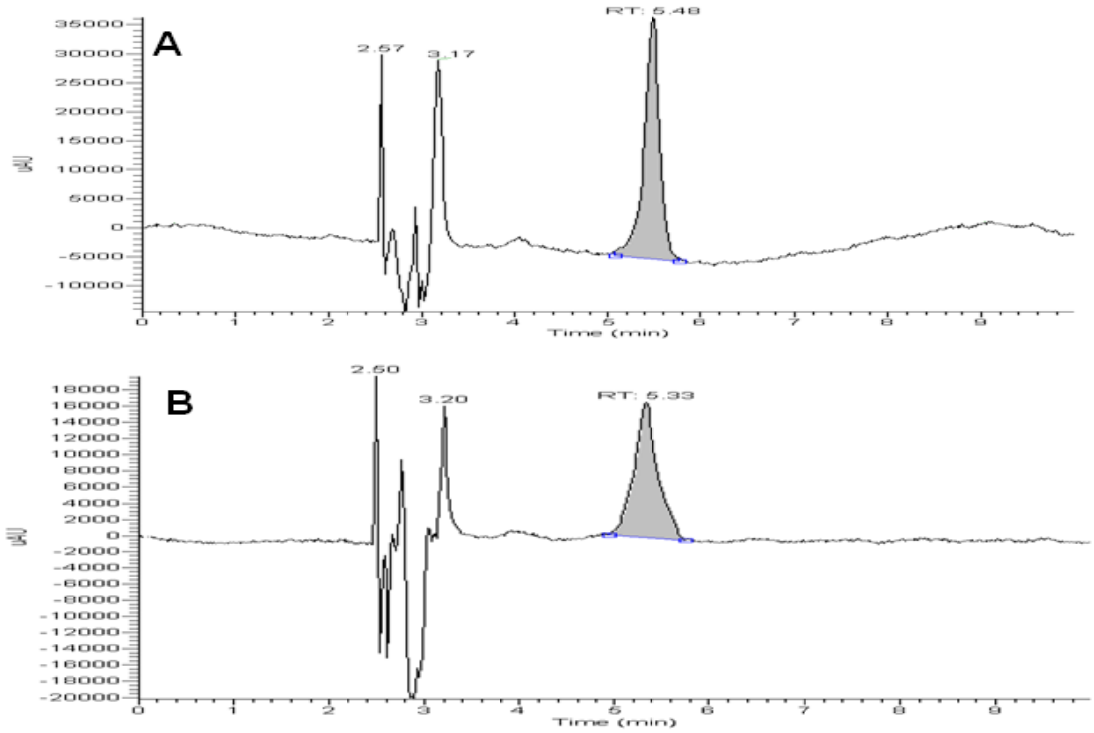

Figure 5. Representative chromatograms of nanospheres (A) and nanocapsules (B) containing compound 1.

Nanoparticles presented mean particle size in the nanometric range (149-219 nm), with polydispersity index between 0.08 and 0.15 and a negative zeta potential (between 33.17 and 24.57 mV). Higher incorporation efficiency values were observed for nanocapsules (56.05%) than for nanospheres (25.67%), prepared by solvent displacement technique. These result is in accordance with the data obtained in our previous work with other xanthone derivatives [14].

Table 4. Incorporation efficiency (IE), mean diameter, polydispersity index (PI) and zeta potential of nanosphere and nanocapsule formulations containing compound 1

Nanospheres					
Theoretical concentration (µg/ml)	Final concentration (µg/ml)	IE (%)	Diameter (nm)	PI	Zeta potential (mV)
150	38.51±4.73	25.67±3.15	149.22±13.22	0.08±0.02	-33.17±1.38
Nanocapsules					
Theoretical concentration (µg/ml)	Final concentration (µg/ml)	IE (%)	Diameter (nm)	PI	Zeta potential (mV)
385	196.18±24.16	56.05±6.90	219.3±3.3	0.15±0.04	-24.57±4.3

Conclusions

A simple, linear, sensitive, accurate, and precise HPLC method was developed and validated for the quantitative determination of a cytotoxic dihydropyranoxanthone (1) in polymeric nanoparticles. Simple sample preparation procedure and retention time lower than 6 min allow the quantification of several samples in a short period of time. The proposed method is free from interferences of excipients used in nanoparticle formulations. No degradation of the compound in analysis was found upon nanoparticle preparation by the adopted solvent displacement method. The developed and validated method was successfully applied to the quantification of compound 1 in polymeric nanoparticles and can be adopted for the routine quality control of finished products.

Authors´ Contributions

AMP carried out the development and validation of a HPLC method and the development and characterization of nanoparticle formulations.

MT conceived the study, and participated in its design and coordination and helped to draft the manuscript.

RP and CMB participated in the coordination of the study and helped to draft the manuscript.

ES and MP participated in the design and coordination of the study and helped to draft the manuscript.

Acknowledgements

To FCT – Fundação para a Ciência e a Tecnologia under the project CEQUIMED - PEst-OE/SAU/UI4040/2011 and PTDC/SAU-FAR/110848/2009, FEDER through the COMPETE program under the projects FCOMP-01-0124-FEDER-011057 and FCOMP-01-0124-FEDER-015752; and to U.Porto/Santander Totta for financial support. A.M. Paiva thanks for a grant (PTDC/SAU-FCT/100930/2008) in the scope of the project. To Sara Cravo for technical support.

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