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Artigo

DEVELOPMENT OF ORAL NIFEDIPINE-LOADED POLYMERIC NANOCAPSULES: PHYSICOCHEMICAL CHARACTERISATION, PHOTOSTABILITY STUDIES, *IN VITRO* AND *IN VIVO* EVALUATION

Monika Piazzon Tagliari[a], Andrea Granada[a], Marcos Antonio Segatto Silva[a], Hellen Karine Stulzer[a,*], Betina Giehl Zanetti-Ramos[b], Daniel Fernandes[c], Izabella Thais Silva[d], Claudia Maria Oliveira Simões[d], Regina Sordi[e], Jamil Assreuy[e] and Valdir Soldi[f]

[a]Departamento de Ciências Farmacêuticas, Centro de Ciências da Saúde, Universidade Federal de Santa Catarina, Florianópolis – SC, Brasil

[b]Departamento de Pesquisa, Desenvolvimento e Inovação, Nanovetores, Florianópolis – SC, Brasil

[c]Departamento de Ciências Farmacêuticas, Centro de Ciências da Saúde, Universidade Estadual de Ponta Grossa, Ponta Grossa – PR, Brasil

[d]Departamento de Ciências Farmacêuticas, Universidade Federal de Santa Catarina, Florianópolis – SC, Brasil

[e]Departamento de Farmacologia, Universidade Federal de Santa Catarina, Florianópolis – SC, Brasil

[f]Departamento de Química, Centro de Ciências Físicas e Matemáticas, Universidade Federal de Santa Catarina, Florianópolis – SC, Brasil

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In this study, nifedipine (NFP)-loaded polymeric nanocapsules were prepared and characterised with a view to protect the drug from degradation. Nanocapsule suspensions were prepared using two different surfactants (pluronic F68 and polyvinyl alcohol). Physicochemical stability and *in vivo* antihypertensive effect were evaluated. The particle size, zeta potential and entrapment efficiency remained constant during a period of 28 days of exposure under light irradiation. A smaller particle size and a higher zeta potential were obtained for the nanocapsules prepared with Pluronic F68 as surfactant. The solid drug and the nanocapsules were submitted to light exposure for 28 days. After this period of time, the percentage of drug remaining in the PF68NFP and PVANFP nanocapsules was 28.1% and 21.3%, respectively. In contrast, the solid drug was completely degraded after 4 days, suggesting that the nanocapsule suspensions promoted significant protection of the drug against light exposure. In addition, *in vivo* studies were carried out, which demonstrated that the formulations with polyvinyl alcohol exhibited a very rapid onset of action after oral administration in rats and led to faster drug release. The nanoparticles developed can be considered as an alternative for improving NFP stability in liquid formulations.

Keywords: nifedipine; nanocapsule; polymeric material; stability.

INTRODUCTION

Nanoparticles, which are defined as solid colloidal particles, include both nanocapsules and nanospheres. Nanocapsules can be defined as vesicular systems in which a drug is confined to a cavity consisting of an inner liquid core surrounded by a polymeric membrane.[1-5] Over the past few decades, there has been considerable interest in the development of biodegradable nanoparticles to be used as effective drug delivery systems. These colloidal carriers have shown the following advantages: (i) drug protection against *in vivo* degradation; (ii) enhanced bioavailability of drugs with poor water solubility; and (iii) the ability to control drug release.[6,7]

Nifedipine (NFP) (1,4-dihydro-2,6-dimethyl-4-(2--nitrophenyl)-3,5-pyridine dicarboxylic acid dimethyl ester) is a calcium channel blocker that has been widely used for the management of hypertension, angina and myocardial infarction. It has a very low bioavailability, and is thermally unstable.[8] It is also highly sensitive to chemical oxidation depending on the source of irradiation. The degradation of this drug is faster in normal sunlight than under exposure to a light bulb, generating nitrophenylpyridine and nitrosophenylpyridine as products, respectively.[9,10] It has also been reported that ethanolic solutions of NFP submitted to light irradiation were converted to fully aromatic nitro derivatives.[11] Consequently, there have been many reports in the literature aimed at improving the solubility of the drug and its stability in different formulations. These examples include microparticles, lipid nanoparticles, solid dispersions, inclusion complexes, nanocrystals, gelatin microcapsules, dry powder aerosols, osmotic pumps, and systems including the micronized drug.[12-26]

The decreasing of the particle size of a drug particle as a nanoparticulate system results in better dissolution and solubilisation due to the increase in surface area. The "topdown" technique used to prepare nanostructured materials results in an increase in the effective surface area (the surface area available for medium interaction). Moreover, the enhanced surface-to-volume ratio further allows the effective attachment of targeting moieties onto the surface of nanoparticles. Thus, the drug molecules are safely carried to the target site without undergoing any chemical modification.[27]

However, the main challenge is to maintain the mean particle size and zeta potential of nanoparticles for longer periods of time. Thus, a crucial issue in the development of nanoparticles is to assure their physicochemical stability during storage.[28]

There is a lack of information in the literature regarding the chemical stability of NFP in a polymeric nanoparticle system. Consequently, the aim of this study was to develop and characterise stable NFD NFP-loaded polymeric nanocapsules in order to protect the NFP from light-induced degradation. In addition, the *in vivo* antihypertensive action and the *in vitro* cytotoxicity effects of the prepared formulations were evaluated.

*e-mail: hellen.stulzer@gmail.com

EXPERIMENTAL

Materials

Nifedipine (NFP) and poly (ε-caprolactone) (PCL, M_w 65000 Da) were purchased from Sigma-Aldrich (Steinheim, Germany). Soybean hydrogenated lecithin (LIPOID S 75-3N) and purified castor oil were obtained from Lipoid GmbH (Ludwigshafen, Germany) and Via Farma Importadora Ltda. (São Paulo, Brazil), respectively. Poly(ethylene oxide)–b–poly(propylene oxide)–b–poly(ethylene oxide) block copolymer (Pluronic F68) was kindly donated by BASF Chemical Company (Germany) and polyvinyl alcohol P.S. (Mn = 92000 g mol^{-1}, 86.5–89.5% hydrolysis degree) (PVA) was purchased from Vetec® (Rio de Janeiro, Brazil). Ultrapure water was obtained from a Milli-Q®Plus apparatus by Millipore (Bedford, USA). All other chemicals were of HPLC or analytical grade.

Preparation of nanocapsule suspensions

The nanocapsule suspensions were prepared using the interfacial deposition process after a solvent displacement, as described by Fessi *et al.*.[29] Briefly, 60.0 mg of PCL (M_w 65000) was dissolved in 4.0 mL of acetone containing 0.250 mL of castor oil and 20.0 mg of NFP. The resulting solution was mixed with 25.0 mg of lecithin, previously dissolved in 10.0 mL of acetone:ethanol (60:40; v/v) solution. This organic phase was poured into an aqueous phase (26.0 mL) containing 1.0% of Pluronic F68 (w/v) or PVA (w/v) at pH 5.0, under magnetic stirring. Acetone and ethanol were then eliminated by evaporation under reduced pressure and the final volume of the suspension was adjusted to 15.0 mL. Finally, the colloidal suspensions were filtered through a 0.8 µm cellulose ester membrane (Millex AA, Millipore, USA) and were denoted as $PF68_{NFP}$ and PVA_{NFP}. Unloaded-PF68 and unloaded-PVA nanocapsule suspensions (Pluronic F68 and PVA, respectively) were prepared and treated in the same manner as the drug nanocapsules.

In vitro characterisation of nanoparticles

High-performance liquid chromatography (HPLC)

The HPLC system consisted of a Shimadzu LC-10A (Kyoto, Japan) liquid chromatograph equipped with a LC-10AD pump, a SPD-10AV variable-wavelength detector, and a SCL-10Avp controller unit; the sample injection was performed via a Rheodyne 7125 valve with 20 µL loop. The chromatographic separation was achieved using a reversed-phase Phenomenex (Torrance, USA) Luna® C18 column (250 mm × 4.6 mm) I.D., with a particle size of 5 µm and pore size of 100 Å. The mobile phase constituted of methanol:water (70:30; v/v) at a flow rate of 1.2 mL min^{-1}. The injection volume was 20 µL and the NFP was detected by UV absorption at 262 nm. The column was maintained at 40 ± 1 °C. Data acquisition was performed using the Shimadzu Class VP_V 6.14 software programme by measurement of the detected peak areas.

Drug entrapment efficiency

The entrapment efficiency (EE) was estimated according to Equation 1.

$$EE(\%) = C_T - C_S/C_T \quad (1)$$

C_T corresponds to the total concentration of NFP present in a methanolic solution containing the polymeric nanocapsule suspensions, and C_S represents the concentration of the drug in the supernatant obtained by applying the suspension ultrafiltration/centrifugation (4500 rpm; 30 minutes) procedure using Amicon Ultra-0.5 membranes (100000 NMWL; Millipore, USA). Finally, aliquots (20 µl) of these solutions were submitted to quantification through HPLC and the drug content was expressed in micrograms of NFP per milliliter of suspension. All the stages of the sample preparation were carried out in the absence of light.

Particle size and zeta potential

The mean particle size and the zeta potential were determined by dynamic light scattering (using volume distribution) and laser--Doppler anemometry, respectively, in a Zetasizer Nano ZS (Malvern Instruments, Worcestershire, UK), equipped with a 173º scattering angle. The measurement of non-filtered particles was carried out at 25 °C after appropriate dilution of the samples in Milli-Q®water. The size distribution of the particles was represented by the polydispersity index (PI).

For the measurement of the zeta potential, the nanocapsule samples were placed in a specific cell, where a potential of ±150 mV was established. The values obtained were calculated from the mean electrophoretic mobility using Smoluchowski's equation.

Morphological analysis

The morphology of the nanocapsules was determined from transmission electron microscopy (TEM) images. This technique was also employed to confirm the particle sizes obtained using dynamic light scattering. The aqueous suspensions (4 µL) were dropped onto copper grids. The grids were maintained at atmospheric pressure and room temperature in order to evaporate the water from the samples. All the samples were observed using a JEOL JEM-1011 microscope operating at an acceleration voltage of 80 kV.

Stability evaluation of solid NFP and NFP-loaded polymeric nanocapsules

A standard methanolic solution of NFP (50 µg mL^{-1}) was analysed in the presence and absence of daylight irradiation to evaluate the effect of the light and methanol on the drug degradation. Also, the NFP in solid state and NFP-loaded polymeric nanocapsules were analysed with and without exposure to daylight in order to analyse the NFP photostability provided by the nanocapsule. At different times (0, 7, 14 and 28 days) all the samples were diluted with methanol, filtered and quantified using the previously described HPLC method. All stages of the sample preparation were performed protected from light to avoid any drug degradation.

The mean particle size and zeta potential of the nanocapsule suspensions were also evaluated.

Determination of *in vitro* cytotoxicity

Cell line

Vero cells (CCL81, American Type Culture Collection, Manassas, VA) were grown in Eagle's minimum essential medium (MEM; Cultilab, Brazil) supplemented with 10% fetal bovine serum (FBS; Gibco, Brazil), penicillin G (100 U mL^{-1}), streptomycin (100 µg mL^{-1}) and amphotericin B (25 µg mL^{-1}) (Cultilab, Brazil). The cell cultures were maintained at 37 °C in a humidified 5% CO_2 atmosphere.

MTT assay

The evaluation of cytotoxicity was performed by MTT [3-(4,5-dimethylthiazol-2,5-diphenyl tetrazolium bromide] assay, with minor modifications.[30] To assess the cytotoxic effects of the $PF68_{NFP}$ and PVA_{NFP} on the Vero cells, 2.5 x 10^4 cells per well were seeded onto 96-well culture plates, and 200 µL of the dilutions, ranging from 0 to 500 µg/mL (1:2 serial dilutions), were added to the confluent cell

monolayers. The isolated components of the formulations were also tested (unloaded-PF68 and unloaded-PVA), including pure NFP (1:2 serial dilutions ranging from 0 to 50 μg mL^{-1}). After 72 h at 37 °C, the medium was removed, 50 μL of MTT solution prepared in MEM (1 mg mL^{-1}; Sigma) were added to each well, and the plates were incubated for a further 4 h. The MTT solution was removed, 100 μL of DMSO (Nuclear, Brazil) were added to each well to dissolve the formazan crystals, and the plates were gently shaken so that the crystals completely dissolved. The absorbance values were read on a microplate reader at 540 nm.

In vivo studies

Animals

Female Wistar rats (weighing 250-300 g) were housed in a temperature and light-controlled room (23 ± 2 °C; 12 h light/dark cycle), with free access to water and food. All procedures were approved by the Ethics Committee of the Federal University of Santa Catarina (Process 23080.034302/2009-81) and the investigation followed the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

Mean arterial pressure measurement

Under anesthesia, heparinised PE-20 and PE-50 polyethylene catheters were inserted into the left femoral vein of the rats for the phenylephrine injections, and into the right carotid artery to record the mean arterial pressure (MAP; mmHg). The animals were allowed to breathe spontaneously and their body temperature was maintained at 37 ± 1 °C. Phenylephrine was diluted in sterile PBS. The mean arterial pressure (MAP) was recorded with a catheter pressure transducer (Mikro-Tip®, Millar Instruments, Inc., Houston, Texas, USA) coupled to a Powerlab 8/30 acquisition system (AD Instruments Pty Ltd., Castle Hill, Australia). The results were expressed as mean ± SEM of the basal and peak changes in MAP following the administration of phenylephrine (30 nmol/kg i.v.) relative to baseline. The rats were then sacrificed with a pentobarbitone overdose.

Experimental protocol

The rats were randomly divided into four groups, each of which received one of the following: vehicle, pure NFP, $PF68_{NFP}$ or PVA_{NFP} (all at 1 mg kg^{-1}, p.o.). At different time periods after treatment (0.5, 1, 2 and 4 h) the animals were instrumented for mean arterial pressure (MAP) recording as described above. The change in MAP in response to phenylephrine (30 nmol kg^{-1}, i.v.) was also obtained.

Data presentation and statistical analysis

Each experiment was carried out in triplicate and the data were expressed as mean ± relative standard deviation (SD). The statistical significance was analysed using one-way ANOVA, followed by Tukey's post hoc test. A p value less than 0.05 was considered to be significant. When necessary, the values were transformed into logarithms in order to achieve normality and homogeneity of variances. These conditions were proved by using the Shapiro-Wilk and Bartlett tests, respectively.

RESULTS AND DISCUSSION

Drug entrapment efficiency, particle size and zeta potential

The nanocapsules showed high entrapment efficiency and zeta potential values (Table 1). Regarding the mean particle size, monodisperse populations of small particle size were obtained using PVA and Pluronic F68 as stabilisers. These results have been previously reported by Granada.[31]

The stability of colloidal suspensions is favoured by eletrostatic repulsion and steric effects, which prevents aggregation provoked by occasional collisions.[32] The electrostatic effect was confirmed by the negative zeta potential. The observed results were in agreement with the negative zeta potential values determined for nanocapsules prepared using polyester polymers (PCL) and non-ionic stabilising agents, reported by Mora-Huertas.[33] In addition, phosphatidic acid obtained from lecithin improves the negative surface charge.[34]

Table 1. Drug content, entrapment efficiency, particle size and zeta potential of NFP-loaded nanocapsule suspensions

Nanocapsule suspensions	Drug content (μg mL^{-1})	Entrapment efficiency (%)	Particle size (nm) and P.I.	ζ-Potential (mV)
$PF68_{NFP}$	1262.2	96.9	185.6 (0.12)	-32.7
PVA_{NFP}	1335.2	97.5	200.9 (0.15)	-17.7

The ability to penetrate physiological drug barriers is increased when nanoparticles of small size are obtained; the stability of colloidal suspensions is also favoured.[35,36] The particle size is affected by the type of surfactants employed. Guinebretière described the effect of PVA and Pluronic F68 on the viscosity and particle size of formulations.[37] PVA was responsible for an increase in suspension viscosity, which led to higher particle sizes, while the use of Pluronic F68 did not seem to alter these parameters.

Morphology analysis

The TEM images of NFP-loaded nanocapsules obtained from PVA and Pluronic F68 are presented in Figure 1.

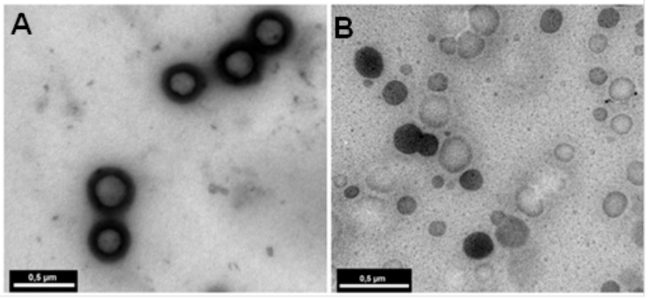

Figure 1. *TEM images of (A) $PF68_{NFP}$ and (B) PVA_{NFP} formulations*

The images of the NFP-loaded nanocapsules indicated a spherical shape of the core/shell. The shell surrounding the oily core was limited by an external polymeric layer (PCL) with surfactants as stabilisers. In general, the thickness of the membrane of nanocapsules is dependent on the polymer concentration.[37] In this study, both formulations were prepared with the same concentration of PCL. According to different authors, shell thickness values are around 10 nm to 20 nm when PCL is selected as the polymer and the nanoprecipitation method is used.[38,39]

The difference in the shell thickness obtained using $PF68_{NFP}$ and PVA_{NFP} was due to the adsorption of the stabilising agent onto the nanocapsule surface (Figure 1). PVA and Pluronic F68 can be considered as polymeric surfactants that stabilise nanocapsule suspensions through the steric effect. These surfactants adsorb onto the nanocapsule surface via their central, hydrophobic moiety. The hydrophilic moiety protrudes into the aqueous environment and

thereby creates a mechanical barrier with a thickness of several nanometers. This layer should be larger than 10 nm to allow for complete steric stabilisation.[38] Pluronic F68 is a triblock copolymer (PEO-PPO-PEO), which consists of hydrophilic (PEO) and hydrophobic (PPO) blocks. The PEO block tends to orient toward the water surface and the PPO is oriented toward the PCL shell. On the other hand, PVA has only one hydroxyl group in its chain, which results in a thinner oil/water interface. In this case, the steric mechanism is more pronounced on the surface of $PF68_{NFP}$.[40] The diameters of the core/shell particles were in agreement with the results obtained by applying the dynamic light scattering technique, which indicates that the nanoparticles are monodisperse. If the particles are agglomerated, the DLS measurement is often much larger than the TEM size and can have a high polydispersity index (large variability in the particle size).

Stability evaluation of solid NFP and NFP-loaded polymeric nanocapsules

The stability of a methanolic solution of NFP was analysed in the presence and absence of daylight irradiation to avoid drug degradation during the preparation of the samples. The results showed that methanol was not able to degrade the NFP in the absence of light.

In order to verify the protective effect of the nanocapsule in terms of the NFP photostability, the formulations prepared were analysed at different times and the results were compared to those of the solid NFP analysed under the same conditions (Figure 2). After 28 days of exposure to light, the NFP concentration was 28.1 ± 1.5% and 21.3 ± 2.1% in $PF68_{NFP}$ and PVA_{NFP}, respectively. On the other hand, under the same exposure conditions the concentration of solid NFP was reduced to 28.5 ± 1.7% after 1 day and was totally degraded in 4 days. These results suggest that the nanocapsules increased the NFP photostability, since the polymeric layers acted as a filter protecting the drug from damaging light. Accordingly to Tukey's test, both formulations were able to provide protection against light when compared to solid NFP.

Moreover, the physical stability of NFP-loaded polymeric nanocapsules was evaluated through the analysis of their average particle size and zeta potential during the 28 days of study (Figure 3).

It was observed that both formulations maintained their initial properties. This stability was probably due to the ionic and steric repulsion forces present on the surface of the nanocapsules, which were provoked by the hydrogenated soybean and non-ionic surfactants, respectively. Nanocapsules can be stabilised either by electrostatic or steric repulsion, depending on the nature of the surfactant. Mixtures of lipophilic and hydrophilic surfactants are generally used

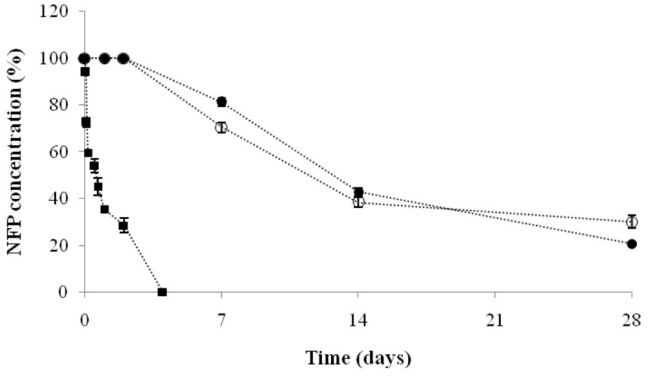

Figure 2. *NFP concentration as a function of time during stability studies: pure solid NFP (--■--), PVA_{NFP} (--●--) and $PF68_{NFP}$ (--○--). Each point represents an average value obtained in triplicate*

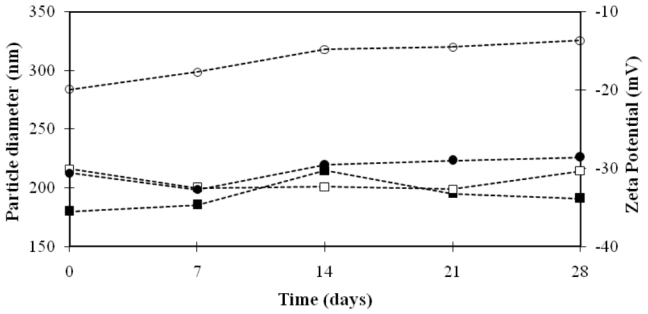

Figure 3. *Average particle size (--□-- PVA_{NFP}, --■-- $PF68_{NFP}$) and zeta potential (--○-- PVA_{NFP}, --●-- $PF68_{NFP}$) of NFP-loaded nanocapsule suspensions as a function of time during stability study. Each point represents an average value obtained in triplicate*

to further reduce the mean particle size and to increase the stability of nanocapsule suspensions.[34]

In vitro **cytotoxicity**

The cytotoxic effects of the samples on Vero cells were investigated employing the MTT assay. This assay has several advantages: it is easy to perform; the evaluations are objective; it can be automated using a personal computer; and the cytotoxicity evaluation can be carried out in parallel with other assays.[41] The $PF68_{NFP}$, PVA_{NFP} and the isolated components of these formulations, such as pure NFP, did not show any cytotoxic effects on the Vero cells (data not shown).

In vivo

In order to investigate the *in vivo* effects, the formulations and NFP alone were administered by gavage to rats. The effects on mean arterial pressure and the response to a vasoconstrictor (phenylephrine) were evaluated for up to 4 hours. As expected, the calcium channel blocker NFP decreased the MAP (Figure 4) and the response to the vasoconstrictor Phe (Figure 5). Interestingly, the effect of the PVA_{NFP} formulation in terms of reducing the MAP (Figure 4) and Phe response (Figure 5) had already started 15 min after oral administration, and these effects were still observed after 4 hours. NFP alone was able to reduce the MAP, and Phe response, only 1 or 2 hours after administration, respectively. The effects of $PF68_{NFP}$ were similar to

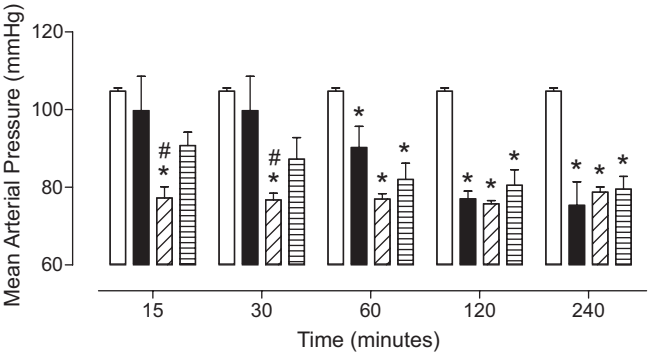

Figure 4. *Effects of NFP and NFP formulations on mean arterial pressure in anesthetized rats. Animals were treated orally with vehicle (open bars), NFP (black bars), PVA_{NFP} (diagonal bars) and $PF68_{NFP}$ (striped bars) and at the times indicated they were prepared for arterial pressure measurement. Each value indicated by a bar represents the mean of 4 animals and vertical lines are the S.E.M. * $p < 0.05$ compared with the control group; # $p<0.05$ compared with the NFP group. Statistical analysis was performed using the ANOVA test followed by Tukey's post-hoc test*

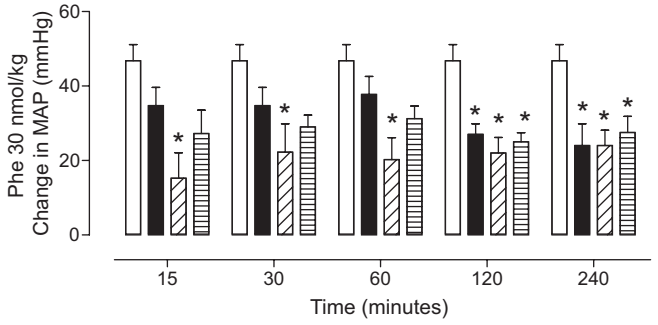

Figure 5. *Effects of NFP and NFP formulations on mean arterial pressure increases after phenylephrine administration to anesthetized rats. Animals were treated orally with vehicle (open bars), NFP (black bars), PVA_{NFP} (diagonal bars) and $PF68_{NFP}$ (striped bars) and at the times indicated they were prepared for arterial pressure measurement. Each value indicated by a bar represents the mean of 4 animals and vertical lines are the S.E.M. * $p < 0.05$ compared with the control group. Statistical analysis was performed using the ANOVA test followed by Tukey's posthoc test*

those of NFP, i.e. they started only 2 hours after oral administration (Figures 4 and 5) and were still observed after 4 hours. Vehicle (PVA and Pluronic F68) administration did not affect the MAP and Phe responses.

Although PCL is more suitable for long-term delivery systems due to its slower hydrolytic degradation, the NFP entrapped in nanocapsules shortens the time before the onset of action.[42] It is probable that the association of the NFP with the oily core of the nanocapsules improves its solubility and consequently it would be more effective in reducing the arterial pressure of rats. Nanosuspensions are also able to increase the number of particles per droplets and a small amount of NFP can be adsorbed onto the nanocapsule surface, leading to a shorter time before the onset of action and increased bioavailability.[27] In a previous study on the *in vitro* release of NFP from nanocapsules, it was found that the nanocapsules prepared using PF68 as a stabiliser released around 90% of the total drug within 96 h, whereas for the PVA-stabilised nanocapsules the release was only 74%.[31] However, the release profiles were not statistically different ($p > 0.05$), suggesting that the faster effect of PVA_{NFP} *in vivo* could be related to its thinner polymeric shell which could favour the diffusion of the drug.

According to Mohanraj and Chen, one important strategy to overcome the gastrointestinal barrier is to deliver the drug in a colloidal carrier system, such as nanoparticles, which is capable of enhancing the mechanisms of interaction between the drug delivery system and the epithelial cells in the GI tract.[43] The influence of a non-ionic surfactant, poloxamer (P-188), on the passive permeability of drugs with poor water solubility was studied by Fischer.[44] The permeation of hydrophobic drugs across the Caco-2 cell monolayers decreased in the presence of poloxamer, varying with the concentration of the surfactant. In the present study we also used a poloxamer (F68) as the stabiliser, which showed only a weak effect in the *in vivo* studies, suggesting that a decrease in permeability occurred.

CONCLUSIONS

The NFP nanocapsules $PF68_{NFP}$ and PVA_{NFP}showed different values of mean particle size, zeta potential and entrapment efficiency. The evaluated parameters remained constant during a period of 28 days under light irradiation. The nanocapsule prepared with Pluronic F68 exhibited smaller particle size and higher zeta potential, which is desirable for the physical stability of the formulation.

Under daylight irradiation, the isolated NFP was considered very unstable. In the solid form, the pure NFP concentration was reduced to 28.5 ± 1.7% after 1 day of exposure.

The stability studies data indicated that $PF68_{NFP}$ promoted a higher drug protection when compared to PVA_{NFP}. The $PF68_{NFP}$, PVA_{NFP} and the isolated components of the formulations, such as pure NFP, showed no cytotoxic effects on healthy cells. Additionally, *in vivo* studies showed that the PVA_{NFP} formulation exhibited a very rapid onset of action after oral administration in rats. The PVA_{NFP} formulation provided a faster drug release and was more efficient when compared to pure NFP and $PF68_{NFP}$. In this sense, the developed nanoparticles can be considered as a successful alternative to protect NFP from light degradation in a suspension system, which represents an evolution in pharmaceutical liquid formulations to improve drug stability. In addition, the *in vivo* studies suggest that the developed nanocapsules are a potential system to improve biopharmaceutical NFP properties.

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