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Validation of an HPLC Method for quantitative Determination of Benzocaine in PHBV-Microparticles and PLA-Nanoparticles

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***SUMMARY*. Benzocaine (BZC) is an ester-type local anesthetic used mainly in topical, dermal and mucosal formulations. The present work consists of the development and validation of analytical methodology for evaluation of benzocaine content in the micro and nanoparticles produced by biodegradable polymers (polyhydroxybutyrate-co-hydroxyvalerate and poly-(L-lactide) by HPLC. The validation was done using the reversed-phase C18 column, using a mobile phase consisting of acetonitrile/water 50:50 (v/v), flow rate of 1.5 mL/min and UV-vis detector at 285 nm. The results here obtained showed that the analytical methodology is accurate, reproducible, robust and linear over the molar range concentration of 10-100 μM of benzocaine. The limit of quantification and detection was 13.06 μM and 3.92 μM, respectively. The encapsulation efficiency of benzocaine in PHBV microspheres and PLA nanocapsule were 40% and 70%, respectively. The method developed was applied in the analysis of benzocaine in micro and nanoparticle systems and showed to be efficient, yielding good results. Here, this method was used to evaluate the encapsulation efficiency of benzocaine and will be used in next studies with different micro/nanoparticle formulations.**

INTRODUCTION

Local anesthetics (LA) are drugs able to induce pain relief by their ability to block the influx of sodium ions and the propagation of the nervous impulse [1]. Benzocaine is an ester-type LA used in dermal, mucosal and topical pharmaceutical preparations, however this anesthetic presents low water solubility. Benzocaine presents a rapid but short effect in relation with the pain duration and its high plasmatic concentrations can presents systemic side effects, such as methahemoglobin formation [2]. When this LA was used in topical formulation, its absorption from the skin is poor, however, when applied to damaged skin its systemic absorption are more effective [3]. Some works showed that the use of micro and nanoparticulated systems can enhance the local anesthetic absorption [4-10].

Literature described many works related to quantification of benzocaine in different biological matrix [11,12] and also for the determination in different pharmaceutical formulations, like as tablets [13-15], colloidal systems [16-18], semisolids preparations [19-21] using high performance liquid chromatography (HPLC). The HPLC has been highly used in the quantification of drugs due of their sensitivity, reproducibility and specificity [22].

Taking into account the possibility to enhance the local action of benzocaine, micro and nanoparticles containing this anesthetic have been developed as described in the literature [23-25], aiming to apply the drug to the topical use,

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however a few methods have been found for the quantification of anesthetics in micro and nanoparticles polymerics by HPLC [26,27].

The most useful biodegradable polymers, poly(l-lactide acid) (PLA) was widely used in the pharmaceutical fields as example for drug delivery systems using polymeric particles due to its biocompatible and biodegradable characteristics [28-30]. Another biodegradable polymer used to prepare particles is poly(hydroxybutyrate-co-hydroxyvalerate) (PHBV) and it is similar to popular synthetic polymers, such as PCL and PLGA [31-34].

When a method has been developed it is important to validate it to confirm that it is suitable for its intended purpose. Therefore the validation tells how good the method is, specifically whether it is good enough for the intended application [35]. This work describes the validation parameters stated either by the ICH guidelines [36] to achieve an analytical method with acceptable characteristics of suitability, reliability and feasibility.

The aim of the present study is to validate an HPLC analytical technique for quantification of benzocaine loaded in the microspheres of PHBV and nanocapsules of PLA. This analytical methodology developed was applied to the analysis of benzocaine in micro and nanoparticle systems in order to evaluate the encapsulation efficiency of different formulations in the future.

MATERIALS AND METHODS

Materials

Benzocaine, polyvinyl alcohol (PVA), poly(DL-lactide acid) (PLA, MW= 173.000 g/mol), Poly(3-hydroxybutyrate-co-hydroxyvalerate) (PHBV, MW= 238.000 g/mol) and sorbitan monostearate (Span 60®) were supplied by Sigma Chem Co. Polysorbate 80 (Tween 80®) was obtained from (LabSynth, Brasil) and caprylic/capric triglyceride (Miglyol® 810) from (Hüls, Alemanha). HPLC-grade acetonitrile (ACN) was obtained from J.T. Baker and deionized water at 18 mΩ from a Waters ultra pure water system. The solutions were filtered through 0.22 µm Millipore® nylon membrane filter (Belford, USA). All other chemicals and solvents were of analytical grade.

Chromatographic system

The chromatographic apparatus consisted of a HPLC Varian ProStar, a PS 325 UV-Vis detector, a PS 210 solvent delivery module and manual injector. For data collection and calculation, Galaxy Workstation Software was used. The chromatographic conditions were optimized using a column C18 (Varian, 250 x 4.60 mm x 1/4"). The mobile phase consisted of acetonitrile:water, 50:50 v/v. The mobile phase was filtered through a 0.22 µm Millipore® nylon membrane filter. The flow rate was 1.0 mL/min. The monitoring wavelength was 285 nm and the injection volume was 20 µL. Peaks areas were measured and HPLC analysis was conducted at room temperature.

Stock and working solutions

Standard solution of benzocaine was prepared at a concentration of 200 µM was accurately weighed (3.3 mg) of BZC and transferred to a volumetric flask of 100.0 mL. The volume was made up with Milli-Q® water. Working standard solutions for the validation of the analytical methods were prepared by dilution of the stock solutions with water to the concentration range between 10 and 100 µM.

Validation study

The objective of validation of an analytical procedure is to demonstrate that it is suitable for its intended purpose (ICH guideline definition) [36]. Validation was performed following the International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use guideline [36]. The method was validated by its linearity, limit of detection and quantification, accuracy and precision. The validation of samples analyzed was investigated after three consecutive days.

Specificity

The specificity of the method was tested by running solutions containing the formulation components in the same quantities and conditions of the samples (placebo sample) to show that there are no peaks in the retention time corresponding to benzocaine (ICH guideline definition) [36]. The specificity of the method was evaluated onto three placebo samples and standard solutions of benzocaine.

Linearity

The linearity of an analytical procedure is defined by its ability (within a given range) to obtain test results which are directly proportional to the concentration (amount) of the analyte in the sample (ICH guideline definition) [36]. Linearity was studied for benzocaine in a concentration range of 10–100 µM, with six different concentration levels in each curve. This study was conducted in three different days, and each

solution was injected in triplicate into the HPLC system. The mean peaks area versus concentration data was treated by least-squares linear regression analysis. The relative standard deviations (RSD) value for the slop and Y-intercept of the calibration curve was calculated.

Limit of detection (LOD)

The LOD of a method is the lowest analyte concentration able to produce a detectable response above the noise level of the system, typically three times the noise level (ICH guideline definition) [36]. To determine this parameter was determined by calibration curve method [37]. Solutions were prepared in the range of 10-100 µM and injected in triplicate by three different days. Average peak area of nine analyses was plotted against concentration. Apart from this, a linear regression was also calculated to obtain the calibration curve *(Y = a+bX)*. The value obtained for the slope *(b)* and residual variance was used to calculate (Eq. **1**, where *S* is residual variance due to regression and *b* is slope).

This parameter needs to be determined only for impurity methods, in which chromatographic peaks near the detection limit will be observed [27].

$$LOD = \frac{S \times 3.3}{b} \qquad [\mathbf{1}]$$

Limit of quantification (LOQ)

The LOQ is the lower level of analyte that can be accurately and precisely measured and is used particularly for the determination of impurities and/or degradation products (ICH guideline definition) [36]. Similarly to LOD assay, a battery of different concentrations diluted was prepared. Prepared concentrations ranged from 20 to 90 µM. The response factor was calculated (relationship between the area and concentration) for each point studied (Eq. **2**, where *S* is residual variance due to regression and *b* is slope). Afterwards, the concentrations in relation to the RSD obtained for the response factors from each of the concentrations were plotted. The first point which does not fulfill this RSD. corresponds to the LOD, and the first point which fits into this specified value corresponds to the LOQ.

$$LOQ = \frac{S \times 10}{b} \qquad [\mathbf{2}]$$

Accuracy (recovery method)

The accuracy of an analytical procedure expresses the closeness of agreement between the value which is accepted either as a conventional true value or an accepted reference value and the value found (ICH guideline definition) [36]. To check the accuracy of the method, BZC samples were prepared with known concentrations of reference standard at 3 different levels (low, intermediate and high). Aliquots of 5, 12.5 and 22.5 mL of the standard sample (200 µM) were transferred into 50 mL volumetric flasks. The volume was brought to 50 mL with ultrapure water, obtaining final concentrations of 20, 50 and 90 µM. The samples were analyzed and the concentrations were recalculated from the corresponding calibration straight line (experimental concentration) and were compared with the theoretical concentrations. These studies were performed in triplicate on two different days.

Precision

The precision of an analytical procedure expresses the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions (ICH guideline definition) [36]. Precision may be considered at three levels: repeatability, intermediate precision and reproducibility. To evaluate the within- and between-day precision, three replicates of standard solutions at three different concentrations (20 µM, 50 µM and 90 µM) for each validation range were assayed on the same day and on two different days, being the precision calculated based on the RSD of the results obtained on the analytical curves. This study was carried out on basis of the samples described above.

Preparation of PHBV microparticle encapsulating benzocaine

Microparticles (MP) were prepared by an oil-in-water (o/w) emulsion/solvent evaporation method [23]. Briefly, 100 mg of PHBV and 10 mg of benzocaine were dissolved in 10mL of chloroform. An aqueous phase (200 mL) containing 0.5% (w/v) PVA was also prepared. The aqueous phase was added into the organic phase at 50 °C under magnetic stirring (1000 rpm, 15 min) [24]. The solvent was evaporated from the emulsion at 40 °C at reduced pressure. The suspension was filtered to collect the PHBV microparticles with or without benzocaine. The filtered microspheres were washed with deionized water and after that the microspheres were dried with Na_2SO_4.

Characterization of microspheres by Scanning electron microscopy

Scanning electron microscope (SEM, Model Jeol JSM-6700F, Japan) was used to observe the morphology of PHBV-MP and BZC-PHBV-MP. The size distribution of the MP was determined on images obtained using scanning electron microscope.

Preparation of PLA nanoparticle encapsulating benzocaine

Empty and BZC loaded poly (DL-lactide) nanocapsules (PLA-NC) were prepared by interfacial deposition [25]. Briefly, an organic solution composed of benzocaine (40 mg), 200 mg of oily phase (capric/caprylic triglyceride mixture), 40 mg of sorbitan monooleate (Span 60), 50 mg of the polymer (poly (D,L-Lactide)) and acetone was added to an aqueous solution containing polysorbate 80 (Tween 80) under moderate magnetic stirring (10 min). Then, acetone was eliminated and the aqueous phase concentrated by evaporation under reduced pressure (40 °C) to a final volume of 10 mL (0.40% of drug). All preparations were carried out protected from the light.

Particle size and ζ-potential measurements of PLA nanocapsules

The average size and ζ-potential of the nanocapsules were determined by dynamic light scattering using a Zetasizer instrument model Nano-ZS (Malvern Instruments, England) at a fixed angle of 90° and at 25 °C. The analyses were carried out after dilution of formulations (1:10) in water (MilliQ®) and made in triplicate [38-41].

Drug loading and encapsulation efficiency

The drug loading was determined by dissolving accurately weighed amounts of micro/nanoparticles (approximately 10 mg) in 50mL of chloroform for microparticles (PHBV) and 50 mL of acetonitrile for nanocapsules (PLA) as previously published [42]. Blank samples (PHBV microparticles and PLA nanocapsules without benzocaine) were dissolved in chloroform and analyzed by HPLC.

RESULTS AND DISCUSSION

Validation study

From the benzocaine chromatogram, we could observe that the drug eluted at a retention time of 5 min (Fig. 1). The blank samples (PHBV microspheres and PLA nanocapsules) chromatogram don't present any absorption in the retention time of benzocaine.

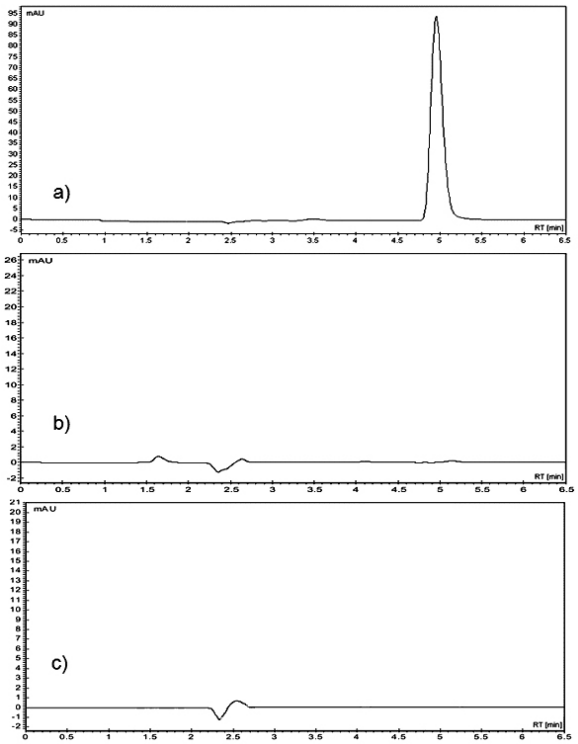

Figure 1. HPLC chromatogram for **a**) benzocaine, **b**) PHBV microspheres and **c**) PLA nanocapsules. Column C18 (Varian, 250 x 4.60mm x 1/4"), mobile phase: acetonitrile:water (50:50 v/v), flow: 1.0 ml min^{-1} and detection: 285 nm.

We investigated the specificity of the method by observing the absence of interferences of the excipients for micro and nanoparticle preparation and the absence of impurity interferences provided by the supplier of the raw material, since none of the peaks appeared at the same retention time as the benzocaine peak (Fig. 1).

Linearity of the method was investigated using six standard solutions of benzocaine that were freshly prepared in the concentration range of 10-100 µM. The calibration curve obtained by plotting the BZC peak area *versus* the concentration of standard solution was linear in the above mentioned concentration range (Fig. 2). The mean (± RSD) values of slope and Y-intercept were 0.14085 (± 0.00534) and 0.09082 (± 0.01840), respectively. The correlation coefficient was > 0.997 for the benzocaine drug (n = 9).

The LOD was determined by the analysis of samples with known concentrations of BZC and by establishing the minimum level at which this analyte can be reliably detected [36]. Visual observations of the sample chromatograms showed

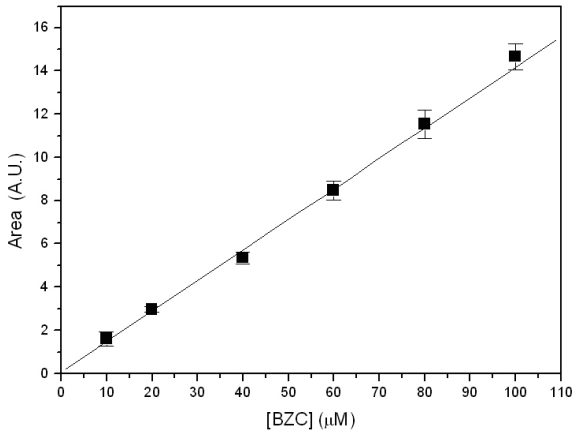

Figure 2. System linearity corresponding to the concentration range of 10-100 μM of the BZC standard solution.

that the lowest drug concentration which produces a signal different from the noise signal emitted by the HPLC equipment was 3.92 μM. On the other hand, the LOQ was based on visual evaluation, as recommended the ICH guideline [36]. This parameter is generally determined by the analysis of samples with known concentrations of analyte and by establishing the minimum level at which the analyte can by quantified with acceptable accuracy and precision. The results obtained for LOQ was 13.06 μM.

The accuracy was demonstrated by the recovery of known amounts of BZC. Recoveries from 95.0 to 105.0% of the added amounts are recommended in dissolution tests [21,36]. The mean recovery percentages for two different days ranged from 95.2 to 104.4% for BZC (Table 1), corroborating the accuracy of the method. The intra-day precision was evaluated at three different concentration levels. The intermediate precision was evaluated in the same solutions at different days. Values presented in Table 2 show the good precision of the method with R.S.D. lower than 2%.

Microparticle drug loading, encapsulation efficiency and particle size

The drug loading and encapsulation efficien-

BZC concentration added (μM)	Sample (Day)	BZC concentration found (μM)	BZC concentration found (μM) ± RSD (n = 6)	Recovery rate mean (range) (%) (n = 6)
20	1a	20.6	19.7 ± 0.6	98.5 (95.5 –101.5)
	1b	19.2		
	1c	19.2		
	2a	19.4		
	2b	19.5		
	2c	20.3		
50	1a	47.3	48.0 ± 0.4	96.0 (95.2 – 96.8)
	1b	48.3		
	1c	48.5		
	2a	47.6		
	2b	48.3		
	2c	47.7		
90	1a	91.6	92.7 ± 1.3	103.0 (101.5– 104.4)
	1b	94.4		
	1c	91.7		
	2a	92.5		
	2b	91.8		
	2c	94.3		

Table 1. Accuracy studies for benzocaine analytical method validation.

BZC concentration added (μM)	Day 1	Day 2	Inter-day (n=6)
	RSD (%)	RSD (%)	RSD (%)
20	1.89	1.94	1.91
50	1.90	1.89	1.89
90	1.04	1.00	1.02

Table 2. Precision studies for benzocaine analytical method validation.

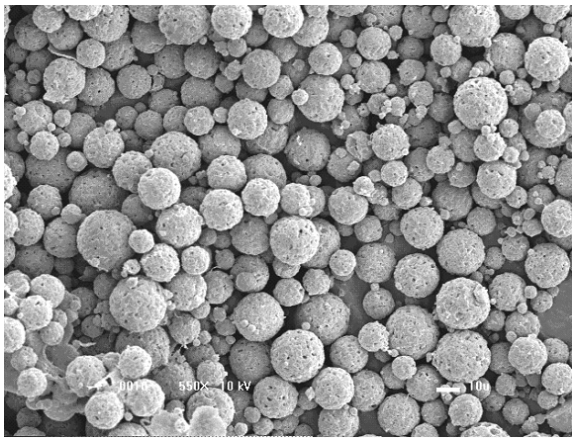

Figure 3. SEM image (10 kV, bar = 10?m) of BZC loaded PHBV microparticles.

cy was determined as described in the Method Section and using the analytical methodology of this study. Benzocaine-PHBV microparticles presented drug loading of 40 mg/g (drug/polymer ratio) and the encapsulation efficiency was about 40%.

In order to investigate the Benzocaine-PHBV microparticles characteristics, SEM images of microparticles were collected and the images were presented in Figure 3. This figure showed that all particles presented fine spherical shape with smooth surface with no aggregations or adhesions. Importantly, the presence of a very porous surface was observed in all spheres with BZC, what is a desirable characteristic for drug release systems. The size distribution of BZC-PHBV microparticles are in the size range of 2-20 µm. The same results were observed for others drugs loaded in PHBV microparticles [26,43].

Nanoparticle drug loading, encapsulation efficiency and particle size

As determined for benzocaine PHBV microparticles, the drug loading and encapsulation efficiency for benzocaine-PLA nanoparticles was 0.28 mg/g (drug/polymer ratio) and 70%, respectively. The characterization of PLA nanocapsules containing BZC was determined based on diameter, polidispersivity index and zeta potential. The mean diameter value was 140 ± 12 nm with a 0.176 of polydispersivity index, in agreement with the characteristics of the colloidal dispersions obtained by the nanoprecipitation technique using PLA as a polymer [44]. The zeta potential presented values around –33.7 mV, what is enough to assure the physical stability of the systems [45].

CONCLUSION

A new, simple and quick analytical method has been developed to be routinely applied in the determinate of benzocaine in micro and nanoparticles. The proposed high-performance liquid chromatographic method has been evaluated over the specificity, linearity, precision, accuracy and proved to be convenient and effective for the quality control of benzocaine in micro and nanoparticulated systems. The methodology application showed that local anesthetic benzocaine was successfully incorporated into micro and nanoparticles.

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