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Formulation, evaluation and microbiological activity of ampicillin and amoxicillin microspheres

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Abstract
The aim of this study is to improve properties of two antibiotics, ampicillin (AM) and amoxicillin (AMO), for controlled delivery. Microspheres loaded with (AM) or (AMO) were prepared by oil-in-water (O/W) emulsion solvent evaporation method. Ethylcellulose (EC) and poly (ε-caprolactone) (PCL) were used to prepare the microspheres with tween80 (T80) and gelatin (GE) as emulsifiers. These systems were characterized by SEM and FTIR spectroscopy and the size distribution was also determined. The results suggest that the entrapment in the microspheres was more than 70%. Data obtained from in-vitro drug release from microspheres were fitted to various kinetic models. Drug release kinetics corresponds to Higuchi model. The antimicrobial activity of the released (AM) and (AMO) was confirmed by Escherichia coli and Klebsiella bioassay.

Keywords: Ampicillin; Amoxicillin; Microparticles; Emulsion-Solvent Evaporation; Higuchi's kinetic model.

1. Introduction
Drug delivery has become increasingly important mainly due to the awareness of the difficulties observed with a variety of active pharmaceutical ingredients. Several approaches have been proposed to improve drug delivery systems, such as microencapsulation, which represents one of the most interesting fields in the area of pharmaceutical technology. Microparticles, prepared from microencapsulation, are able to protect active pharmaceutical ingredients against degradation, to reduce toxicity and to control their release from the site of administration. In some particular cases, it is also possible to improve the passage through biological barriers [1]. A number of methods have been reported for the microencapsulation of flavors, such as interfacial polymerization [2], spray drying [3-4], complex coacervation [5], interfacial solvent exchange [6], and oil-in-water (o/w) emulsion solvent extraction [7-10].
Solvent evaporation method is commonly used among various microsphere preparation techniques, This method can be influenced by many parameters [1, 11-14], ie solvent evaporation rate, polymer solubility, drugs and excipients in both emulsion phases, dispersion stirring rate, viscosity, solubility, polymer and drug quantities, and the physico-chemical properties and concentration of the stabilizers. A few examples of drugs have been encapsulated using solvent evaporated preparation including piroxicam [15], dexamethasone [16], zidovudine [17], mefenamic acid [18], azithromycin [19]. In this method, microspheres can be formed by evaporation of the organic solvent from the dispersed oil droplets containing both polymer and drug. This process has significant impacts on the characteristics of drug loaded microspheres such as the surface morphology, encapsulation efficiency, particle size and in vitro release profiles.
In the present study, ampicillin and amoxicillin microspheres were prepared by solvent evaporation technique. Ethylcellulose and poly (ε-caprolactone) were used as matrix. Ethylcellulose, a non-biodegradable and biocompatible polymer, can be used to sustain drug release from oral delivery systems either by formation of a matrix or an insoluble but permeable film [20-22]. Poly (ε-caprolactone) is a biodegradable and non-toxic polymer. It has been used in different applications such as drug delivery devices, surgical implants or in disposable materials [23]. Ampicillin (6- [2– amino– 2– phenyl acetamide] penicillanic acid) and amoxicillin

(α -amino-p-hydroxybenzyl-penicillin) are two of the most widely prescribed β-lactam antibiotics [24-32]. Ampicillin is sparingly soluble in cold water (1g in 50mL) [24], and amoxicillin is slightly soluble in both water and alcohol [33-34]. They have been used extensively to treat enterococcal infections and commonly-occurring gram positive and gram negative bacteria infections including Haemophilus influenza, Neisseria gonorrhoeae, Escherichia coli, Salmonella, and Shigella infections [24-28, 32]. They present a broad-spectrum, high activity, and they are stable orally absorbed antibiotics. These drugs inhibit the protein synthesis on ribosomes in bacteria by causing misreading of the genetic code [27-29].
Literature survey reveals several encapsulation methods for ampicillin and amoxicillin or/and structurally related antibiotics, such as sulbactam sodium [35], gemifloxacin mesylat [36], ertapenem and meropenem [26]. Tween80 and gelatin were used as processing medium to solidify the microspheres. These microparticles were formulated in order to study the release rate of active agent and to improve its protection. The medicament release kinetic studies were performed in acid aqueous medium at pH 1.2, and analysed according to Higuchi [37] and Korsmeyer-Peppas [38-39] equations for calculation of the release rate constants. The stability of the (AM) and (AMO) drugs (Figure 1) after release was also confirmed by antimicrobial activity studies with Escherichia coli and Klebsiella.

(AM) (AMO)

Figure 1: Chemical structure of ampicillin (AM) and amoxicillin (AMO).

2. Materials and Methods

2.1. Chemicals

Ethylcellulose was obtained from Fluka Analytical (product of United States). Ampicillin (99% purity), was purchased from Ningbo Samreal Chemical (China). Amoxicillin was purchased from Sigma (St. Louis, MO, USA). Dichloromethane was purchased from BIOCHEM Chemopharma (UK). Tween80 (Polyethylene Glycol sorbitan monooleate) was obtained from Sigma-Aldrich (USA). Gelatin, from bovine skin was purchased from Sigma-Aldrich (USA) and Poly (caprolactone) was synthesised in our laboratory (Mv= 16830).

2.2. Microspheres preparation:

All microspheres were obtained by the (O/W) emulsion solvent evaporation method using dichloromethane (DCM) as the organic solvent. Initially, 0.6 g of active agent (AM or AMO), 1.2 g of polymer (EC or PCL) were dissolved in 50 mL of (DCM), and heated under slight reflux (30–35°C) and stirred to allow homogenization (600 rpm). At the same time, 0.5mg of tween80 (T80) or gelatin (GE) was dissolved in 50mg of deionized water under stirring. The organic phase was emulsified with the aqueous continuous phase in cylindrical glass reactor (volume of 1000 mL, external diameter = 80 mm) under mechanical stirring with four-bladed turbine impeller (blade length = 50 mm, blade width = 08 mm, type: IKA RW20 digital, UK) for 4 h to complete solvent evaporation. The solidified microspheres were filtered, washed three times with distilled water and were vacuum-dried in a desiccator in the presence of $CaCl_2$.
The microspheres were prepared by varying different parameters as summarised in Table 1. The effects of process variables on the particle size and the surface of the microspheres, drug loading efficiency, and drug release were studied. The results are presented in Table 2.

Table 1: (AM) and (AMO) microspheres prepared by the emulsion solvent evaporation methods.

Code	*Active agent*	*Polymer*	*Emulsifier*
TA1	*AM*	*EC*	*T80*
TA2	*AMO*	*EC*	*T80*
TA3	*AMO*	*PCL*	*T80*
TA4	*AMO*	*EC*	*GE*

2.3. Microspheres characterization:

Drug amount in microspheres was determined by dissolving 100 mg of the microspheres in a sealed bottle containing buffer solution pH=7.7 (100mL) under stirring for 24 h at T=40°C. The resulting solution was analyzed by UV-VIS spectroscopy (Shimadzu UV-2401 PC, Shimadzu, Japan) at 203 nm for ampicillin and 230 nm for amoxicillin. The drug

concentration was determined from the standard curve. The drug loading (%) and the encapsulation efficiency (Yield) of the microspheres were calculated using the equations 1 and 2, respectively.

$$\text{Drug loading \%} = \frac{\text{Masse of active agent in microparticle}}{\text{Masse of microparticles}} * 100 \quad \textbf{(1)}$$

$$\text{Yield \%} = \frac{\text{Masse of active agent en microparticles}}{\text{Initial masse of active agent}} * 100 \quad \textbf{(2)}$$

The mean particle size of the active agent microspheres was determined by optical microscopy (OPTIKA 4083. B1). At least 500 microspheres were analyze**d** for each preparation and the mean diameter was calculated. Each sample was measured in triplicate. The particle-size distribution was calculated from various equations [40] (see Table2).

Table 2: Microparticles characteristics and encapsulation results.

Code	*Drug loading %*	*Yield %*	$d_{10}\,\mu m$	$d_{30}\,\mu m$	$d_{32}\,\mu m$	$d_{43}\,\mu m$	*Dispersion*[a]
TA1	*23.30 ± 0.16*	*70.60 ± 0.46*	*294.6*	*339.6*	*380.6*	*401.0*	*1.4*
TA2	*23.50 ± 0.03*	*71.21± 0.08*	*221.8*	*274.1*	*326.4*	*358.0*	*1.6*
TA3	*21.60± 0.25*	*65.48± 0.25*	*212.2*	*258.2*	*303.2*	*330.8*	*1.6*
TA4	*24.39± 0.08*	*73.92± 0.17*	*38.6*	*42.9*	*46.8*	*48.8*	*1.3*

The arithmetic mean: $d_{10}=\sum ni\,di/\sum ni$, The volume mean: $d_{30}=(\sum ni\,di^3/\sum ni)^{1/3}$,The volume-surface mean:$d_{32}=\sum ni\,di^3/\sum ni\,di^2$,The volume-moment mean:$d_{43}=\sum ni\,di^4/\sum ni\,di^3$,Dispersion[a] was calculated as Dispersion[a] = d_{43}/d_{10}. i represent an index of the population, and di is the particle diameter of population i.

The microspheres were characterised by infrared spectroscopy. The infrared spectra of pure active agent, polymer and corresponding microparticles were compared. The FTIR spectra were recorded using an FTIR-8300 Shimadzu spectrophotometer (Shimadzu, Japan). Samples were prepared in KBr disks and transmittance was measured from 400 to 4000 cm^{-1}.

Surface morphology of microparticles was characterized by SEM (Figures 2, 3 and 4**)** using Quanta 200 (FEI, France) at Bordeaux Center Imaging, University Bordeaux-1. The samples were mounted on a double-scotched carbon film fixed on a metal support. A sample (TA1) after 10 hours of release in reconstituted acid medium (pH=1.2, T=37°C) was also magnified to observe the variation of surface morphology of the microspheres (figure 9).

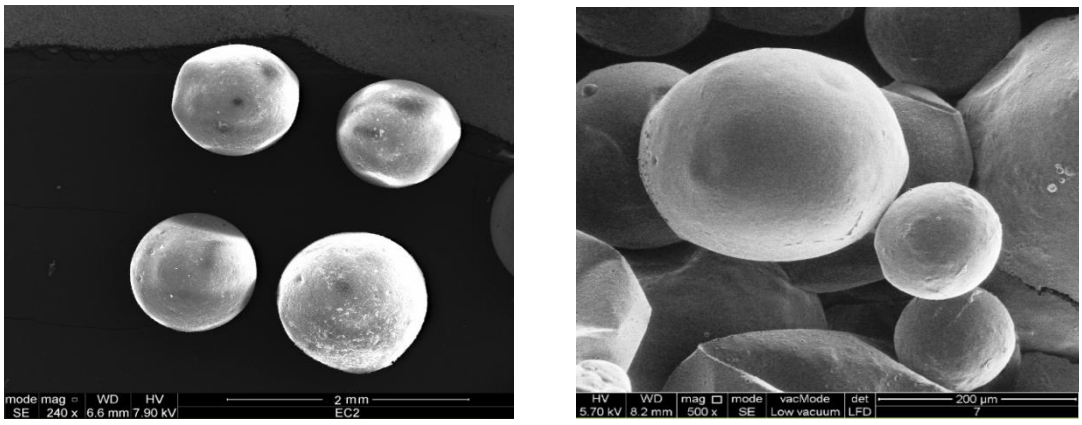


Figure2: SEM of spherical microspheres; TA1 and TA2.

2.4. In vitro drug release studies

The in-vitro release study from the microspheres to the solution was carried out using a cylindrical double-wall glass reactor equipped with fritted glass extremity immersed in the solution. This allows the ascent of the solution without passage of microparticles. The release kinetics of active agent from microparticles were followed by using an UV-Vis spectrometer 2401PC SCHIMADZU with a cell compartment thermostat at 37°C. A sample of microparticles (100 mg) was soaked in 100 mL of buffer solution (pH=1.2). The dispersion medium was stirred magnetically at a rotation speed of 500 rpm; the dosage of released active agent is made on taking out of 1mL of acid solution containing the support (reading of the optical density). The UV apparatus was beforehand calibrated at the maximum wavelength of active agents.

2.5. Microbiological assay for ampicillin and amoxicillin

Microbiological tests on the stability of the extracted ampicillin and amoxicillin released from the microspheres during diffusion were performed by the test tube serial dilution [41].Test bacteria were Escherichia coli (E.coli) and Klebsiella

(K). The antibiotics were extracted from microspheres in buffer solution (pH 7.7). The extracted active agents (AM) and (AMO) were diluted in phosphate buffer saline solution and serial six-fold series for each active agent were diluted in liquid Mueller–Hinton broth. The tubes containing 1ml of each dilution were inoculated with 1×105 bacterial cells and they were then incubated at 37 ◦C for 24 h. Bacterial growth was observed by spectrophotometer at 620 nm. The minimum inhibitory concentration (MIC) expresses the antibiotic activity. It is recorded as the highest dilution showing no bacterial growth. The results are shown in the figure 10.

3. Results and discussion

The microspheres containing the active agent were prepared by the solvent evaporation method of the (O/W) emulsion system in order to control the concentration of drugs in living microspheres. The SEM analysis of various batches was carried out. The study indicated that the surface of the microspheres prepared with EC were spherical shape, smooth and rigid. Few drug crystals were also observed in the field (figure 2). With gelatin emulsifier, microspheres were spherical with a wrinkled surface (figure 4), but with PCL matrix, they were non spherical with great pores (figure 3). The mean diameter of the microspheres was kept in average 40 to 400 µm (table2). It can be noted that smaller microspheres particle sizes were obtained with gelatin emulsifier. It is well known that the surfactant reduces the surface tension of continuous phase, avoids the coalescence and agglomeration of drops and stabilizes the emulsion [12].

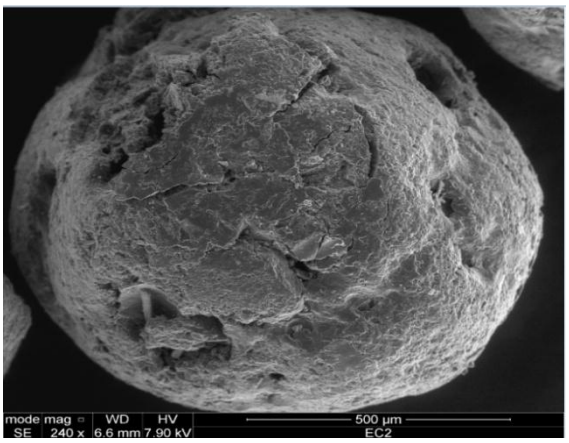


Figure 3: SEM of spherical microspheres; TA3.

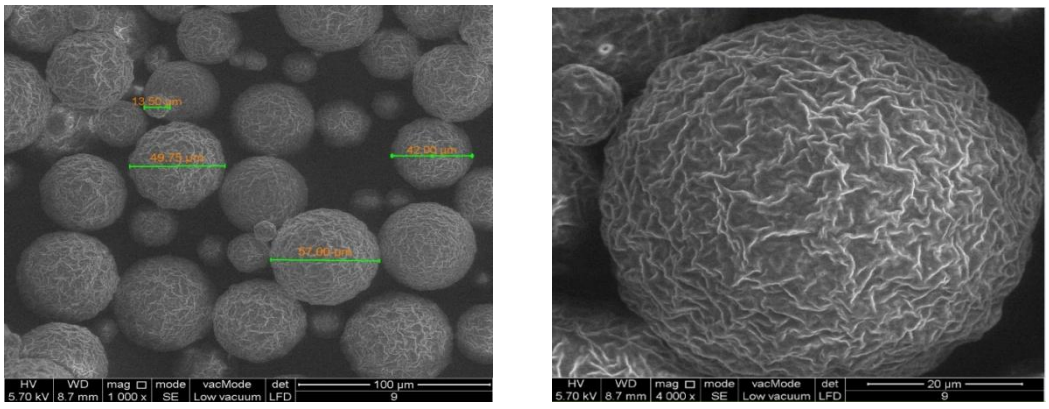


Figure 4: SEM of spherical microspheres; TA4.

The value of the dispersion[a] is not more than 1.6, indicating adjacent sizes of microspheres. Maximum drug load for microspheres is 73.92% (TA4) and minimum drug load is 65.48 % (TA3). Thus it is remarkable to note that microencapsulation with evaporation method gives a good percentage entrapment efficiency and practical yield consistent with current research [15-18].

The IR spectrum of microparticles (TA1) was compared with the etylcellulose and pure ampicillin spectra. We identified the presence of important significant IR bands of ampicillin in the microparticles spectrum at the

expected wave number: 1375 cm^{-1} for the N—C aromatic bond, 1610 cm^{-1} for aromatic C=C vibration, 1775 cm^{-1} and 3208 cm^{-1} for C=O and O—H vibration of carboxylic acid, 2090 cm^{-1} bending of S-C and 3450-3500 cm^{-1} for amine groups. The microparticle spectrum appears as the sum of (AM) and (EC) spectra, so the FTIR analysis confirms the presence of ampicillin in the microparticles (figure 5). We also confirmed the presence of amoxicillin in (TA2) and (TA3) when we compared the spectrum of (AMO), (EC) and (PCL). For (TA3), we identified the IR bands of (AMO) in microparticles at the same wavelength: 1370 cm^{-1} for the N—C aromatic bond, 1730 cm^{-1} and 2950 $cm^{-1}$for C=O and O—H vibration of carboxylic acid, 2090 cm^{-1} bending of S-C, at 3500cm^{-1} of amine function and 3400 cm^{-1} vibration of alcohol functions. We found also the characteristic bands of PCL, at 1725 cm^{-1} the ester function, at 2940 the O-H of carboxylic acid and the fine band of external chain observed at 1470 cm^{-1} (figure 6).

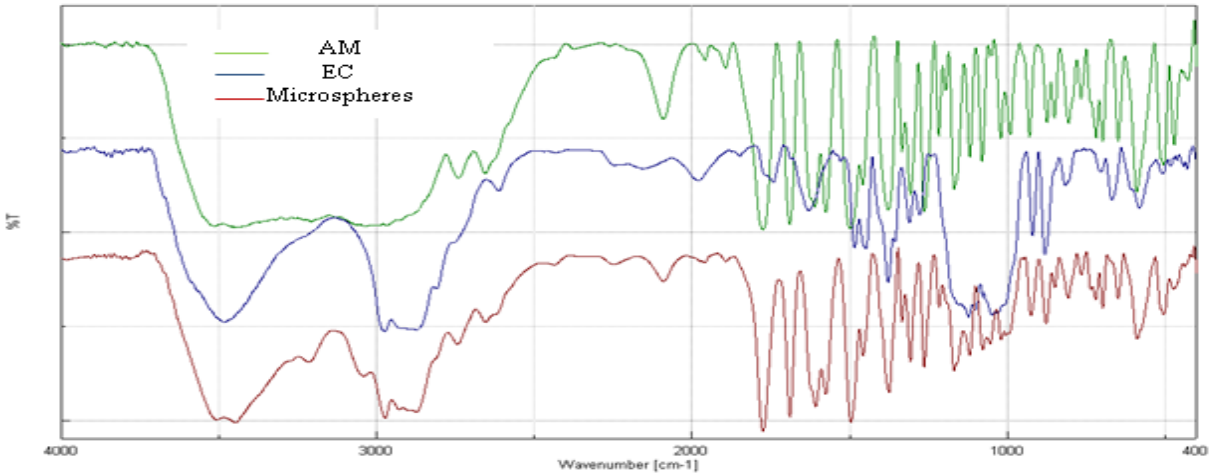
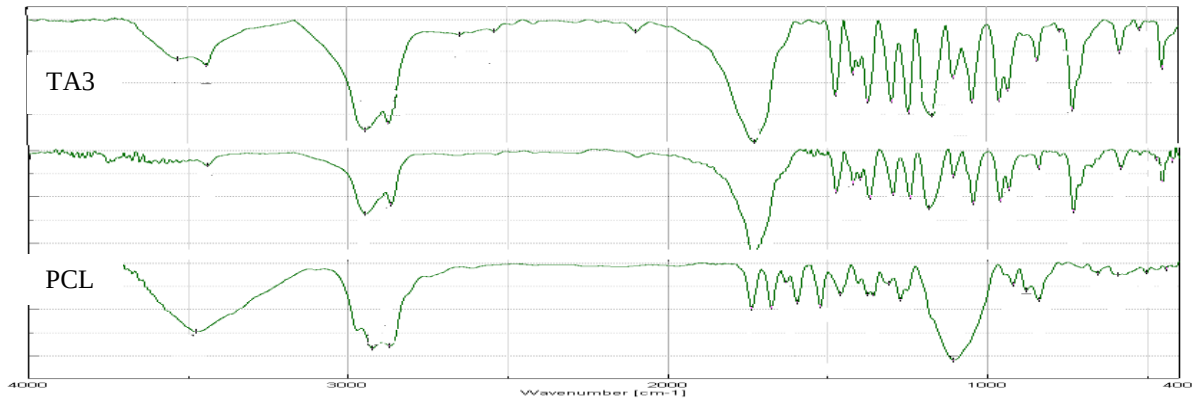


Figure 5: Infrared spectra of ampicillin (AM), ethylcellulose (EC) and microspheres(TA1).



AMO

Figure 6: Infrared spectra of amoxicillin (AMO), polycaprolactone (PCL) and microspheres (TA3)

3.1. Study of drug release from microparticles:

The process of matter transfer implying microparticles in contact with synthetic gastric liquid is complex. The ionization (pK), solubility (log S) and lipophilicity (log P) of the drug are the important physicochemical parameters. Their knowledge is of fundamental importance in drug discovery in order to facilitate the screening of drug-like candidates [42-43]. As reported [42, 44], ampicillin has two pK value: 2.50 and 7.05 due to acid and amine functions respectively. At pH=1.2, the protonic form [(AMH^+): $C_{15}H_{15}N_2O_2S$ (COOH) (NH_3^+)] is favored [42]. Amoxicillin presents three different pK values: 2.4 (carboxyl), 7.4 (amine) and 9.6 (phenol) [44]**,** and the protonic form is also favoured in acidic pH.

In vitro, release of the prepared microspheres was performed in phosphate buffer solution pH 1.2. Figure 7 shows the release profiles obtained. The percentage yield of all the formulation was found to be more than 70%. This percentage is released after 10hours fromTA2 and TA4 when it is released after 2 hours from TA1 and TA2, this is explain by the high porosity of the surface in TA1 and TA3. The difference in the drug release was

not statistically significant when varying active agent (TA1, TA2) at short time (figure 8), it may be returned to the equality in yields, drug loading. With PCL (TA3), the microspheres liberate more drugs than the amounts observed with EC (TA2), this is due to the porosity and surface morphology of (TA3) and the distribution of internal active agent explain the high percentages observed. It was also shown that drug release in (TA4) is great than (TA2). This is related with the surface of microsphere, (TA4) having the smaller microspheres sizes which represent a greater surface of diffusion.

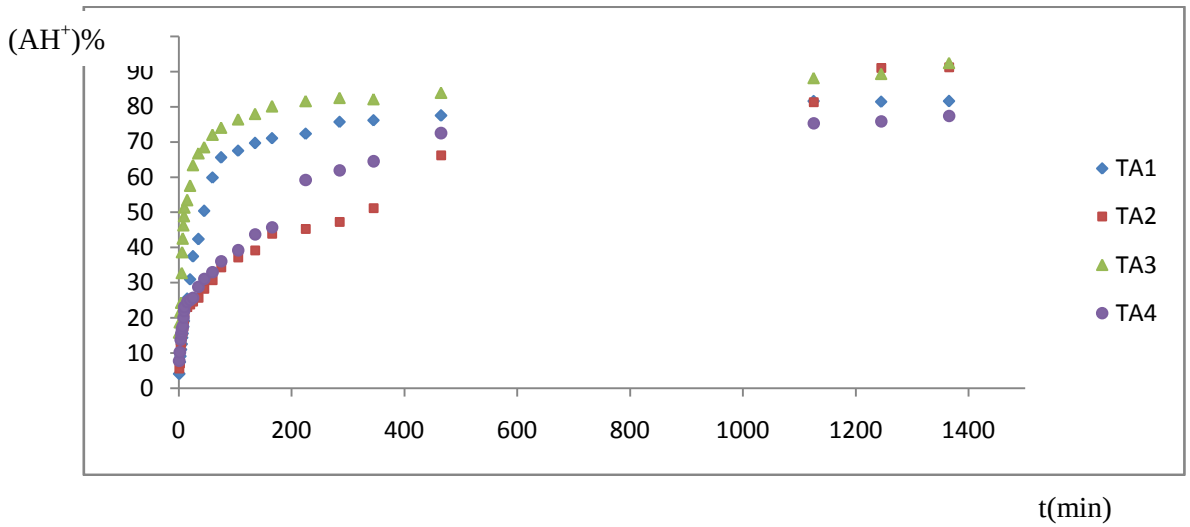


Figure 7: Release profiles of (AM) and (AMO) from microspheres (TA1, TA2, TA3 and TA4) in pH=1.2 at 37°C.

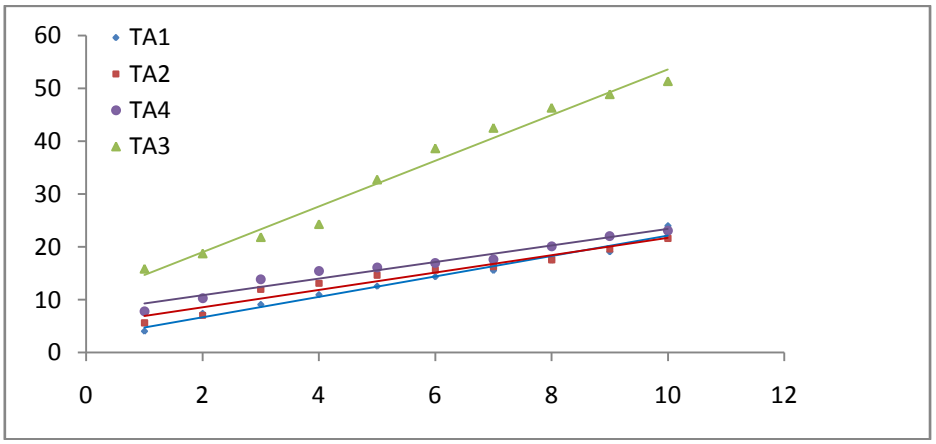


Figure 8: Cumulated percentage of (MAH^+) and ($AMOH^+$) released from microspheres as a function of square root of time

A (TA1) study with SEM analysis after 10 hours of release in acid medium (pH=1.2, T=37°C) was also carried out. This analysis indicates that the surface of microspheres sustains a collapse of the surface, once dispersed in the acid medium as shown in (Figure 9). After a period of 10h, the surface of the microparticles appears particularly collapsed by loss of ampicillin into solution. The collection of microparticles shows clearly the subsidence for 6 microspheres over thirteen. This difference can be explained by the heterogeneity of the "ethylcellulose-ampicillin" composition of internal matrix system.

3.2. Data analysis:

Different active agent delivery systems cannot be described by a classical kinetic equation (n=0, 1 or 2). This process is related with phenomenon of mass transfer controlled by diffusion according to the curves shown in Figure 7. Thus a vertical tangent is observed at the beginning of the process. A linear effect is observed (at short time). Drug release was evaluated by other kinetic models including Higuchi square root and Korsmeyer-Peppas equation. Higuchi [37] describes drug release from a matrix system by a simple relationship:

$$Pa\% = k_H \sqrt{t} \quad \textbf{(3)}$$

where, Pa% is the amount of drug released vs time and k_h is the Higuchi dissolution constant.
Korsmeyer and Peppas [38, 39] develop a different equation to describe the drug release:

$$M_t/M_\infty = k_{K\text{-}P}\, t^n \quad \textbf{(4)}$$

where M_t/M_∞ is the fraction of drug released, t is the release time, k is the kinetic constant and n is the diffusional exponent. It is known that 'n' value could be used to characterize different release mechanisms. The interpretation of n values was done in the following manner:
• n<0.5 (0.45) - quasi-Fickian Diffusion
• n=0.5 (0.45) - Diffusion mechanism
• 0.5<n<1 - Anomalous (non-Fickian) Diffusion - both diffusion and relaxation (erosion)
• n=1 (0.89) - Case II transport (zero order release)
• n>1 (0.89) - Super Case II transport (relaxation)

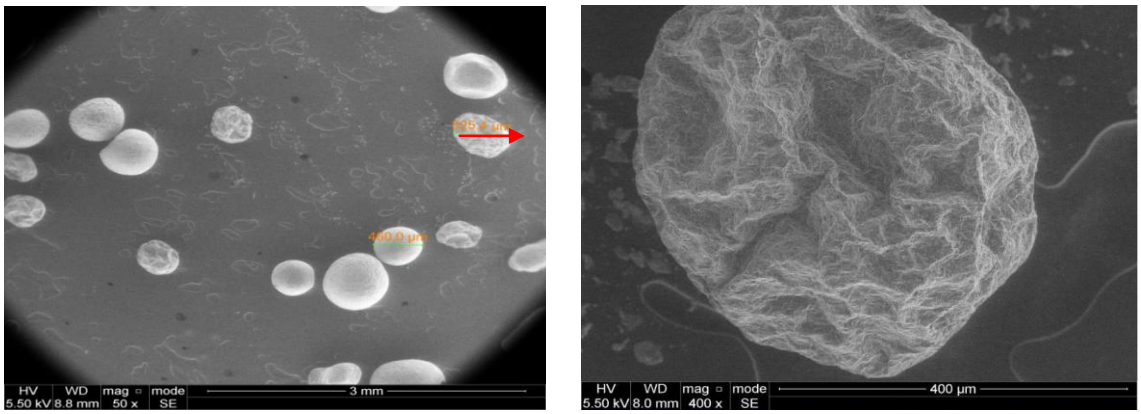


Figure9: SEM of (TA1) immediately and after 10 hours in the medium pH=1.2.

The results for the mathematical modeling of the in-vitro drug release data for the floating microspheres have been compiled and the R^2 values shown in the table 3. The R^2 values are above 0.95 in Higuchi model. The mass transfer with respect to square root of time (figure 8) shows a linear graph with a regression value close to n<0.5 stating that the release from the matrix was through diffusion. From the results of the Korsmeyer–Peppas equation, the values of n is in average of 0.2-0.5, confirming that the transfer of matter was governed by diffusion.

Table 3: Coefficients of correlation and release rate constants of (AM) and (AMO) from microparticles.

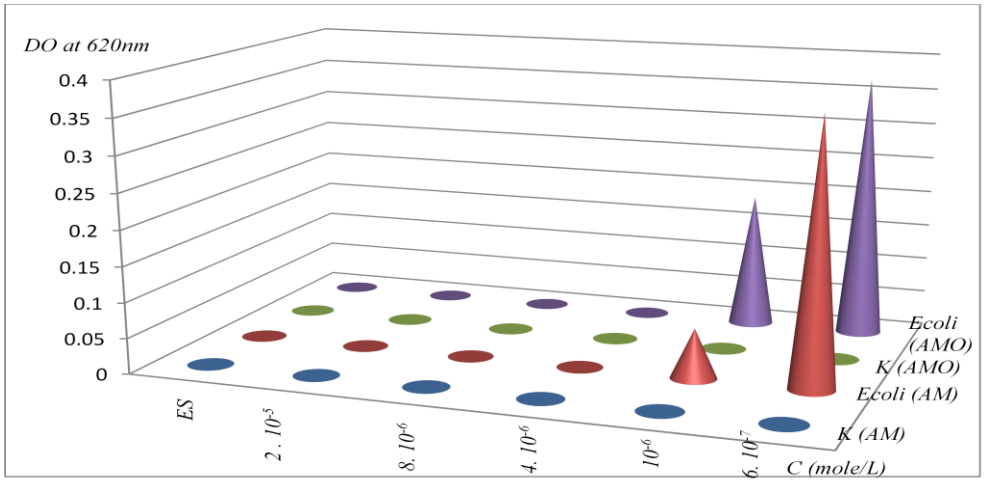
Code	*Higuchi's equation*		*Korsmeyer–Peppas's equation*		
	$k_H\ (min^{-1/2})$	R^2	*n*	$k_{K\text{-}P}\ (min^{-n})$	R^2
TA1	*1.929*	*0,990*	*0.483*	*0.458*	*0,806*
TA2	*1.640*	*0,959*	*0.491*	*0.364*	*0,976*
TA3	*1.565*	*0,973*	*0.216*	*0.713*	*0,909*
TA4	*4.324*	*0,975*	*0.463*	*0.399*	*0,964*

In addition, it was shown that dispersion was not the main cause for the percentage of drug release. The data prove that disperse populations of microspheres (Table 2) can be used to study drug release kinetics. In the case of the microspheres containing the drug, the Higuchi and Korsmeyer-Peppas models are successfully tested for the transport of the drug. The ions (AH^+) are important in the process of matter diffusion due to their pK in pH=1.2.

3.3. Microbiological assay for ampicillin and amoxicillin

For a medical application of these microspheres, it is necessary to confirm the activity of the compounds released from microparticles. This was realized by assessing the drug effectiveness to inhibit the microbial

growth. The bactericidal action of extracted active agent was tested by measuring the minimum inhibitory concentration (MIC) of (AM) and (AMO) against Escherichia coli (E.coli) and Klebsiella (K). As presented in figure 10, the concentrations: ES, 2.10^{-5}, 8.10^{-6} and 4.10^{-6} showing no bacterial growth against E.coli. The highest dilution represents the minimum inhibitory concentration (MIC); it has values of 1.46 µg/l (AMO) and 1.39 µg/l (AM). Against (K), all diluted concentrations inhibit the bacterial growth.



ES: extracted solution

Figure 10: Histograms of the minimum inhibitory concentration of (AM) and (AMO)

Conclusions

Microspheres of (AM) and (AMO) were successfully prepared by solvent evaporation method, using ehylcellulose and PCL as matrix and the tween80 or Gelatin as emulsifiers. From the results it can be concluded that biocompatible, biodegradable and cost-effective polymers can be used to formulate an efficient floating microparticulate system with good percentage entrapment efficiency and practical yield. These microparticles were prepared to delay the release and to achieve required liberation profile. The dispersion of medical agent was chosen to study this drug release in gastric medium by determining the kinetics of the release of the drug. The matter transfers were controlled by transient diffusion, and can be described by a simple mathematical model. These results can help in the quantitative prediction of the rate of medical agent release from the microspheres. Drug release kinetics of this formulation corresponds mainly to Higuchi model. Further bacterial tests were realized to confirm stability of the drugs in these microparticles.

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