ORIGINAL ARTICLE

PREPARATION AND CHARACTERIZATION OF CHITOSAN MICROSPHERES FOR VANCOMYCIN DELIVERY

OLIMPIA DUMITRIU BUZIA[1#], CRISTIAN DIMA[2#], ŞTEFAN DIMA[3*#]

[1] *"Dunărea de Jos" University of Galati, Faculty of Medicine and Pharmacy, Al. I. Cuza Str. 3, Galaţi, Romania*
[2] *"Dunărea de Jos" University of Galati, Faculty of Food Science and Engineering, Domneasca Str. 111, Galaţi, Romania*
[3] *"Dunărea de Jos" University of Galati, Faculty of Sciences and Environment, Domneasca Str. 111, Galaţi, Romania*

**corresponding author: dimastefan@yahoo.com*
[#] *All authors had equal contribution to designing and writing the presented paper.*

Manuscript received: February 2015

Abstract

This study aimed the preparation and characterization of vancomycin chitosan microspheres by internal gelation method using sodium tripolyphosphate (TPP) and KOH as cross-linked agents. The chitosan-KOH microspheres were smooth, spherical with a regular shape, while the chitosan-TPP microspheres had a brain-like structure. The entrapment efficiency was in the range of 66.8 ± 3.7% to 78.18 ± 2.8% for chitosan-TPP microspheres while for chitosan-KOH microspheres, the entrapment efficiency was in the range of 48.8 ± 3.1% to 52.6 ± 2.3%. It was also studied the kinetics profile of vancomycin releasing from chitosan microspheres in simulated gastro-intestinal fluids. The release rate demonstrated that the chitosan-TPP microspheres can be used for the oral delivery of vancomycin. The antibacterial activity of vancomycin microspheres, against *Streptococcus pyogenes* and *Streptococcus faecalis* was investigated.

Rezumat

Această lucrare a avut ca scop prepararea şi caracterizarea microsferelor de chitosan încărcate cu vancomicină. S-a aplicat metoda gelifierii interne, folosind ca agenţi de reticulare tripolifosfatul de sodiu (TPP) şi KOH. Microsferele din chitosan obţinute prin reticulare cu TPP au formă regulată sferică şi suprafaţa netedă, în timp ce microsferele din chitosan obţinute prin precipitare cu KOH au formă neregulată cu suprafaţa striată. Eficienţa încapsulării vancomicinei în microsferele obţinute din chitosan prin reticulare cu TPP a fost cuprinsă între 66,8 ± 3,7% şi 78,18 ± 2,8% în timp ce pentru cele obţinute prin precipitare cu KOH a fost cuprinsă între 48,8 ± 3,1% şi 52,6 ± 2,3%. S-a studiat, de asemenea, activitatea antibacteriană a microsferelor obţinute experimental faţă de *Streptococcus pyogenes* şi *Streptococcus faecalis*.

Keywords: vancomycin (VANCO), chitosan microspheres, internal gelation

Introduction

Vancomycin (VANCO) is a tricyclic glycopeptide antibiotic. VANCO is an amphoteric molecule containing three phenolic groups (pKa1 = 10.6; pKa2 = 10.3; pKa3 = 9.4), a carboxyl group (pKa4 = 2.5) and two amino groups (pKa5 = 8.6; pKa6 = 6.8) [14].

VANCO is active against a large number of multi-resistant Gram-positive bacteria such as staphylococci, enterococci, diphtheroid bacilli and clostridia [10-13]. Because it is poorly absorbed after oral administration, VANCO is given intra-venously for therapy of systemic infections. VANCO presents nephrotoxicity, ototoxicity, and poor venous tolerance [14].

Also, the bactericidal activity of VANCO administered parenterally is slow and decreases in time due to the survival of bacteria between 24-48 hours. VANCO is used to treat infections of the intestines due to *Clostridium difficile*, which can cause watery or bloody diarrhoea [9].

In the last years there were prepared different drug delivery systems, such as: emulsions, multiple emulsions, microemulsions, nanoemulsions, liposomes, nanoparticles etc. [1, 6, 11, 12, 17]. A drug delivery system is used to transport, protect and deliver therapeutic agents to specific organ, tissue or cells (targeted drug delivery systems), and provide a drug release profile (controlled-release drug delivery systems) [15].

In this work, the preparation and characterization of the chitosan microspheres loaded with VANCO, using the emulsification/internal gelation method were studied.

Chitosan is a linear heteropolysaccharide composed of a glucosamine unit (2-amino-2-deoxy-D-gluco-pyranose) and an acetylglucosamine unit (2-acetamido-2-deoxy-D-glucopyranose). Chitosan is obtained from chitin by deacetylation. It has a degree of deacetylation (DDA) of more than 50% and it is soluble in aqueous acidic media [18].

Chitosan is a very attractive biomaterial, used as a drug delivery carrier, due to its special chemical

and biological properties: it is biocompatible, biodegradable, nontoxic, non-immunogenic, bio-active, antibacterial [1-3].

Due to positive charges at physiological pH, chitosan has bioadhesive properties and improves the retention of drug delivery systems at the site of administration.

The interaction between polycationic chitosan and proteins (collagen, α-keratose), anionic poly-saccharides (carboxymethyl cellulose, alginate, pectin, k-carrageenan, hyaluronic acid, heparin, chondroitin sulphate etc.) generates new biomaterials used for the preparation of drug delivery systems for oral, parenteral, transdermal and trans-mucosal administration, encapsulation of bioactive compounds and immobilization of enzymes and cells [18]. For the preparation of hydrogels, films, fibres and microspheres, chitosan may be crosslinked with chemical reagents (glutaraldehyde, epichlorohydrin, diisocyanate, tripolyphosphate (TPP), oxalic acid etc.) [2, 15]. Some studies were focused on the preparation of chitosan nanoparticles by increasing the ionic strength of chitosan solution at high pH values. Thus, the addition of salts ($CH_3COO^-Na^+$, Na^+Cl^-, K^+Cl^-) and hydroxides (KOH, NaOH) in chitosan solution precipitates and allows the formation of chitosan nanoparticles [2, 3].

This study aimed the preparation and characterization of VANCO chitosan microspheres by internal gelation method using TPP and KOH as cross-linked agents. It was also studied the kinetics profile of VANCO releasing from chitosan microspheres in simulated gastro-intestinal fluids.

The antibacterial activity of encapsulated VANCO, against *Streptococcus pyogenes* and *Streptococcus faecalis* was investigated.

Materials and Methods

Materials

Chitosan, glutaraldehyde solution and Span 80 were purchased from Sigma. Chitosan had the deacetylation degree (DDA) of 75% determined by the potentiometric method and the average molecular weight M_w = 387 Kg/mol, determined through the viscometric method [9].

Vancomycin hydrochloride Mylan S.A.S (France), sodium tripolyphosphate, KOH and n-octanol were purchased from Merck, Germany.

Simulated gastro-intestinal fluids were prepared as described in US Pharmacopoeia [1].

Simulated gastric fluid (SGF pH 1.2) containing 7 mL HCl, 2 g NaCl and 3.2 g pepsin, diluted to 1 L and simulated intestinal fluid (SIF pH 7.5), containing 6.8 g of K_2HPO4 and 190 mL of 0.2 N NaOH and 10 g pancreatin.

The antimicrobial test for *Streptococcus pyogenes* and *Streptococcus faecalis*, were performed at the Pneumology Hospital Galati, Romania.

Ultrapure water was used in the preparation process of all solutions (Milly Q, conductivity < 0.1 $\mu S \cdot cm^{-1}$). All the other chemicals and reagents used were of analytical grade.

Methods

Preparation of chitosan microspheres

The chitosan microspheres loaded with VANCO were prepared by the emulsification/internal gelation method [4].

The chitosan solution (1%; 2%; 3% w/v) was prepared through electromagnetic stirring of a chitosan suspension in an aqueous solution of acetic acid 1% (v/v) at room temperature for 6 hours. In order to eliminate the gas bubbles, the solutions of chitosan were ultrasonicated for 2 minutes and were kept overnight [7].

The powder of VANCO was dissolved into chitosan solution to a final concentration of 8% (w/w of chitosan), under magnetic stirring.

The chitosan-VANCO solution (25 mL) was added dropwise into 75 mL sunflower oil containing 1% w/w Span 80, under stirring at 400 rpm for 30 min (Ika Laboratory Equipment, Staufen, Germany). The cross-linked agent (10 mL) (TPP aqueous solution 5%, 10%, 15% w/v of chitosan or KOH n-octanol solution 2%, 4%, 6%, w/v of chitosan) was added drop by drop (2mL/min) into W/O emulsion, and stirred for 2 h to solidify the microspheres. Then, a KCl solution (0.3 M) (50 mL) was added to the system, and the chitosan microspheres were separated in the aqueous phase. The oily phase of the mixture was slowly decanted and the chitosan microspheres were recovered by vacuum filtration (Agilent Technologies, Inc., Santa Clara, CA, membrane filters 0.45 μm). The chitosan microspheres were successively washed with Tween 40 (0.5% w/v), and KCl solution (0.3 M) until no more oil was detected by optical microscope evaluation. Chitosan microspheres were dried for 48 h at room temperature in a desiccator and stored for further experiments [4].

Particle size and morphology of chitosan microspheres

The size of the particles was measured by laser light diffraction using a Mastersize 2000 MU, (Malvern Instrument, UK). The samples were suspended in isobutyl alcohol and the volume-weight mean diameter ($d_{4,3}$) was measured.

The microstructure of the chitosan microspheres was determined through scanning electron microscopy technique (SEM, Quanta 250, Netherland), with voltage acceleration of 15 kV. In order to record SEM micrographs, the microspheres were fixed on aluminium stubs with double adhesive tape and vacuum coated with a fine layer of gold [7].

Determination of entrapment efficiency

The entrapment efficiency (EE%) of VANCO in chitosan microspheres was measured using, with some adjustments, the methods previously described [7, 16].

A suspension formed through the dispersion of 5 mg of microspheres into 20 mL of 0.1 N HCl was electromagnetically stirred for 1 h. The mixture was centrifuged at 5000 rpm for 30 min and filtered (0.2 μm filter paper, Whatman, UK). The VANCO concentration was determined spectrophotometrically at a wavelength of 280 nm using the UV-VIS spectrophotometer (Model Jasco 560, Germany).

The entrapment efficiency (EE%) is the fraction of the total amount of VANCO used for encapsulation (W_T) which is inside the particle (W_P) and was calculated using the following expression:

$$EE = \frac{W_p}{W_T} \cdot 100 \qquad (1)$$

Swelling studies

The swelling degree of microspheres (S_w) was determined by introducing 50 mg VANCO-loaded chitosan microspheres in a glass vial containing 50 mL simulated gastro-intestinal fluids (SGF pH 1.2 and SIF pH 7.5), at 37°C. The swelled microspheres were removed from the solution at predetermined intervals, dried on a filter paper and weighed. The following equation was used for computing the swelling degree:

$$SW = \frac{W_t - W_0}{W_0} \cdot 100 \qquad (2)$$

where: W_t is the mass of the swelled microspheres, weighted at different time intervals, and W_0 is the initial mass of dried microspheres.

In vitro release studies

The study of the *in vitro* release of VANCO from chitosan-TPP and chitosan-KOH microspheres was carried out using a previously described method [5].

The VANCO-loaded chitosan microspheres (25 mg) were added in an Erlenmeyer flask containing 50 mL SGF (pH 1.2), and SIF (pH 7.5) respectively. The suspension was slowly stirred at constant temperature (37ºC).

At specified time intervals, samples of 2 mL were withdrawn from the suspension, being immediately replaced with an equivalent volume of fresh media. The sink conditions were maintained at all time. The samples were filtered using 0.45 μm MF – Millipore membrane filter (Millipore Corporation, Bedford, USA). The VANCO concentration was determined spectrophotometrically at a wavelength of 280 nm using the UV-VIS spectrophotometer (Model Jasco 560, Germany). The blank sample was a phosphate buffered saline (PBS) solution. The amounts of VANCO released from the chitosan microspheres were calculated from the standard VANCO calibration curve. The experiments were carried out in triplicate.

The cumulative percentage of VANCO release (Q_t %) was calculated using eq (3):

$$Q = \sum_{t=0}^{t} \frac{M_t}{M_0} \cdot 100 \qquad (3)$$

where: Q is the cumulative release percentage, M_t is the cumulative amount of VANCO released at each sampling time and M_o is the initial mass of VANCO loaded in the sample.

In vitro antibacterial activity of VANCO-loaded chitosan microspheres

The antimicrobial activity of encapsulated VANCO in chitosan microspheres against *Streptococcus pyogenes* and Streptococcus faecalis was performed using the agar diffusion method [7, 8, 11]. The inoculums of these strains were prepared by inoculating the colonies from fresh cultures in Mueller Hinton Agar, tempered at 42ºC and then homogenized in sterile Petri dishes. After solidification, wells of 5 mm size made in the agar medium were loaded with 0.1 mg suspension of VANCO-loaded chitosan-TPP microspheres, 0.75 mg suspension of VANCO-loaded chitosan-KOH microspheres, a standard microcomprimat with VANCO; (32 μg) as control sample and VANCO -E test [8]. The plates inoculated were incubated at 37ºC for 24 h and at 35ºC. After incubation, the formation of a clear inhibition zone around the well indicated the antimicrobial activity. The inhibitory effect was assessed by measuring the diameter of the inhibition zone around the well.

Statistical analysis

Sigma Plot 11.0 for Windows XP was used for statistical analysis. Experiments were performed in triplicate and conventional statistical methods were used to calculate means and standard deviations. The size of chitosan microspheres, swelling ratio and release rate of VANCO from Chitosan-TPP microspheres and chitosan-KOH microspheres were statistically analysed by one-way ANOVA. The Student t-test was used for comparing the results and the differences were considered significant at the level of $p < 0.05$.

Results and Discussion

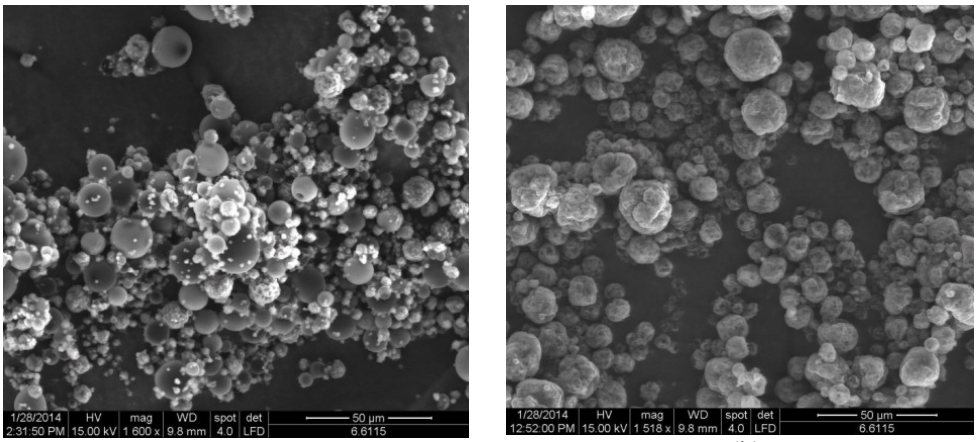
Preparation and morphological analysis of VANCO-loaded chitosan microspheres

The VANCO-loaded chitosan microspheres were prepared by emulsion/internal gelation method, using the sodium tripolyphosphate (TPP) as cross-linking agent and KOH as precipitation agent. TPP is a multivalent anion and its negative charges can form the electrostatic bonds with the positive charges of the protonated amino groups of chitosan.

Compared to TPP, KOH causes the precipitation of chitosan and the conversion of the aqueous droplets from W/O emulsion into solid microspheres.

The shape and surface morphology of VANCO-loaded chitosan microspheres were observed by scanning electron microscopy. The chitosan-KOH microspheres were spherical with a regular shape (Figure 1a), while the chitosan-TPP microspheres (Figure 1b) had a brain-like structure, resembling the structure of chitosan microcapsules obtained by Zou *et al.* [18].

Particle size analysis revealed that the size of the chitosan-KOH microspheres was in the range of 7.2 ± 0.8 μm to 25.3 ± 1.6 μm while the size of chitosan-TPP microspheres was in the range of 9.2 ± 1.3 μm to 28.7 ± 2.4 μm ($p < 0.05$).



(a) (b)

Figure 1.
SEM images of VANCO-loaded chitosan microspheres
a) chitosan-KOH microspheres; b) chitosan-KOH microspheres

Data presented in Table I show that the size of chitosan microspheres increased with the higher concentrations of TPP and with the increasing concentration of KOH ($p < 0.05$).

Table I

Characteristics of VANCO-loaded chitosan microspheres

Chitosan microspheres	TPP (% w/w)	KOH (% w/w)	Mean size (μm)	Entrapment efficiency EE%	Swelling degree (Sw%) after 4 h	
					SGF (pH 1.2)	SIF (pH 7.5)
CH: 3%						
CH-TPP (1)	5		9.1 ± 0.8	78.1 ± 2.7	73.7 ± 3.5	137.8 ± 3.5
CH-TPP (2)	10		9.8 ± 0.3	75.3 ± 1.8	84.2 ± 4.1	153.7 ± 4.1
CH-TPP (3)	15		12.3 ± 1.2	66.8 ± 3.4	83.7 ± 2.7	152.9 ± 2.8
CH: 3%						
CH-KOH (1)		2	6.9 ± 0.8	52.6 ± 3.1	87.9 ± 2.3	52.7 ± 1.2
CH-KOH (2)		4	7.4 ± 0.5	50.1 ± 1.3	98.6 ± 1.8	58.2 ± 1.7
CH-KOH (3)		6	8.2 ± 0.4	48.8 ± 1.7	97.8 ± 5.2	55.9 ± 2.2
CH-TPP	**10**					
CH 1%			9.2 ± 1.3	76.2 ± 3.1	79.8 ± 3.4	131.7 ± 3.8
CH 3%			9.8 ± 0.7	73.8 ± 1.2	84.2 ± 4.1	153.7 ± 4.6
CH 5%			28.7 ± 2.4	72.4 ± 0.9	83.7 ± 2.5	154.1 ± 3.4
CH-KOH	**4**					
CH 1%			7.2 ± 0.8	51.8 ± 1.5	94.6 ± 4.1	50.8 ± 1.4
CH 3%			7.4 ± 0.1	46.5 ± 1.7	98.6 ± 1.8	58.2 ± 3.5
CH 5%			25.3 ± 1.6	44.7 ± 2.3	97.8 ± 3.7	57.5 ± 2.9

CH: Chitosan; CH-TPP chitosan microspheres cross-linked with sodium tripolyphosphat; CH-KOH chitosan microspheres precipitated with KOH; SGF simulate gastric fluid; SIF simulate intestinal fluid; values are the means ±standard deviation of three replicate experiments.

Increasing of the chitosan content decreases the entrapment efficiency due to the large size of the W/O emulsions.

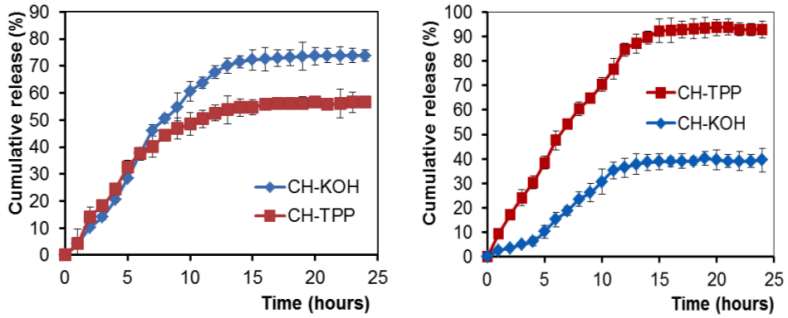
The entrapment efficiency of VANCO was in the range of 66.8 ± 3.7% to 78.1 ± 2.7% for chitosan-TPP microspheres while for chitosan-KOH microspheres, the entrapment efficiency was in the range of 48.8 ± 3.1% to 52.6 ± 2.3%. The entrapment efficiency decreases with increasing of TPP and KOH concentration.

The higher values of the encapsulation efficiency, in the case of the chitosan-TPP microspheres, are probably due to the gel structure obtained by the process of cross-linking chitosan.

Swelling analysis and releasing of VANCO from chitosan microspheres

The drug release from microspheres is affected by the degree of swelling, pH, ionic strength and temperature [3, 19]. In this paper we investigated the behaviour of VANCO- loaded chitosan microspheres into gastro intestinal fluids (SGF pH 1.2 and SIF pH 7.5).

The results show that the degree of swelling of chitosan-TPP microspheres in SGF was two times smaller than in the SIF ($p < 0.01$) (Table I). This increase of swelling ratio and release rate was due of the protonation of the amino groups in acid media which lead to a repulsion between the chains of the polycation [2, 19]. The release of VANCO is in accordance with the swelling degree of chitosan microspheres. In the SGF (pH 1.2) at 37ºC, after 15 h, the release rate of VANCO was of 47.5 ± 2.15% for chitosan-TPP microspheres and 72.23 ± 4.61% for chitosan-KOH microspheres (Figure 2a) while in SIF (pH 7.5) the release rate values of the VANCO for chitosan-TPP microspheres and chitosan-KOH microspheres were 85.78 ± 1.27% and 38.86 ± 2.11% respectively (Figure 2b). These values demonstrate that chitosan-TPP microspheres can be used for oral delivery of VANCO.



(a) (b)

Figure 2.

In vitro release profile of vancomycin from chitosan microspheres, a) into SGF (pH 1.2); b) into SIF (pH 7.5)

In vitro antibacterial activity of VANCO-loaded chitosan microspheres

VANCO is used parenterally mostly in infections due to staphylococci and enterococci resistant to β-lactams or in case of intolerance to these antibiotics [11]. In this paper we studied the possibility of oral VANCO delivery.

The disc-diffusion method to test VANCO against enterococci using agar plates incubated for 24 h was performed. The reference standards have been a VANCO aqueous solution (0.1%) and a standard microtablet with VANCO (32 µg). Also, an E-test® for VANCO was used.

Table II

Antimicrobial activity of VANCO -loaded chitosan microspheres

	Standard Microtablet VANCO (32µg)	VANCO aqueous solution (0.1%)	E-test® VANCO	VANCO-loaded Chitosan microspheres
Streptococcus pyogenes	23 ± 1.2	25 ± 2.4	0.19	22 ± 3.2[a] 23 ± 2.7[b]
Streptococcus faecalis	17 ± 0.7	16 ± 1.3	3	18 ± 1.4[a] 20 ± 4.2[b]

[a]Chitosan-TPP (2) microspheres; [b]Chitosan-KOH (2) microspheres; Data are given as mean of inhibition zone (mm) of three replicates

The results presented in the Table II showed that VANCO-loaded chitosan microspheres had an inhibitory effect on the tested enterococci with different degrees of inhibition. The E-test® values obtained for *Streptococcus pyogenes* and *Streptococcus faecalis* was in good agreement with The European Committee for Antimicrobial Susceptibility Testing-EUCAST [8].

Conclusions

In this work we studied the preparation of a new vancomycin oral delivery system. The aim of this work was the obtaining of chitosan microspheres loaded with vancomycin by emulsion/internal gelation method using the sodium tripolyphosphate as cross-linking agent and KOH as precipitation agent. The shape and size of the microspheres is affected by the content of chitosan, concentration of TPP and KOH.

The size of the chitosan microspheres decreased with chitosan content and increased with the higher concentrations of TPP and KOH.

The entrapment efficiency of chitosan-TPP microspheres was higher than chitosan-KOH microspheres, but the entrapment efficiency decreased with increasing of TPP and KOH concentration.

The behaviour study of VANCO- loaded chitosan microspheres into gastro- intestinal fluids (SGF pH 1.2 and SIF pH 7.5) showed that release rate of VANCO from chitosan-TPP microspheres is smaller into SGF and bigger into SIF in comparison with chitosan-KOH microspheres.

The tests showed that the antibacterial activity of VANCO-loaded chitosan microspheres had an inhibitory effect on *Streptococcus pyogenes* and *Streptococcus faecalis*.

Based on these results, it can be concluded that chitosan-TPP microspheres are protected against the acidity of gastric fluid without delivering substantial amounts of the loaded vancomycin.

References

1. Albu M.G., Ghica M.V., Spongious collagen-minocycline delivery systems. *Farmacia*, 2015; 63(1): 20-25.
2. Anal K.A., Stevens F. W., Chitosan–alginate multilayer beads for controlled release of ampicillin. *Intern. J. of Pharmaceutics*, 2005; 290: 45-54.
3. Cevher E., Orhan Z., Mülazimoğlu L., Sensoy D., Alper M., Yildiz A., Ozsoy Y., Characterization of biodegradable chitosan microspheres containing vancomycin and treatment of experimental osteomyelitis caused by methicillin-resistant *Staphylococcus aureus* with prepared microspheres. *Intern. J. of Pharmaceutics*, 2006; 317(2):127-35.
4. Cotirlet, M., Dima, Ş., Bahrim, G, Psychrotrophic *Streptomyces spp.* cells immobilization in alginate microspheres produced by emulsification-internal gelation. *J. of Microencapsulation*, 2013: doi 10.319/02652048.2013.808279.
5. Dima C., Pătraşcu L., Cantaragiu A., Alexe P., Dima S., The kinetics of the swelling process and the release mechanisms of *Coriandrum sativum* L essential oil from chitosan/alginate/inulin micro-capsules. *Food Chemistry*, 2015; 195: 39-48.
6. Dima S., Dima C., Iordăchescu G., Encapsulation of Functional Lipophilic Food and Drug Biocomponents. *Food Engineering Revue*, 2015; 7(4): 417-438.
7. Dima C., Cotarlet M, Alexe P., Dima S., Micro-encapsulation of essential oil of pimento [*Pimenta dioica* (L) Merr.] by chitosan/k-carrageenan complex coacervation method. *Innovative Food Science and Emerging Technologies*, 2014; 20: 203-211.
8. European committee on antimicrobial susceptibility testing, EUCAST, Antimicrobial wild type distributions of microorganisms. http://www.eucast.org/mic_distributions/.
9. Konstantinos Z.V., Konstantinos A. P., Konstantina P., Petros I. R., George S., Matthew E. F., Treatment failure and recurrence of Clostridium difficile infection following treatment with vancomycin or metronidazole: a systematic review of the evidence. Review. *International Journal of Antimicrobial Agents*, 2012; 40: 1-8.
10. Leclercq R., Cattoira, V., Bacteries a gram positif et glycopeptides. *Revue Francophone Des Laboratoires*, 2012; 445: 41-46.
11. Popa L., Ghica M.V., Dinu-Pîrvu C.E., Periodontal chitosan-gels designed for improved local intra-pocket drug delivery. *Farmacia*; 2013(61)2: 240-250.
12. Popescu M., Chiutu L., Mircioiu C., Dima S., Capsaicin microemulsions: preparation, characterization and *in vitro* release study. *Farmacia*, 2014; 62(1): 58-68.
13. Popescu M., Vialet R., Loundou A., Peyron F., Bues-Charbit M., Etude de la variabilite dans les reconstitutions de vancomycine intraveineuse en reanimation pediatrique. *Annales Francaises d'Anesthesie et de Reanimation*, 2011; (30): 726-729.
14. Reynolds P.E., Courvalin P., Vancomycin rezistence in enterococci due to synthesis of precursors terminating in D-alanyl-D-serine. *Antimicrobial Agents Chemotherapy*, 2005; 49: 21-25.
15. Shanmuganathan S., Shanumugasundaram N., Adhirajan N., Ramyaa Lakshmi T.S., Babu M., Preparation and characterization of chitosan micro-spheres for doxycycline delivery. *Carbohydrate Polymers*, 2008; 73: 201-211.
16. Swenson J.M, Anderson K.F, Lonsway D.R, Accuracy of commercial and reference susceptibility testing methods for detecting vancomycin-intermediate *Staphylococcus aureus*. *Journal of Clinical Microbiology*, 2009; 47(7): 2013-2017.
17. Tefas L.R., Muntean D.M., Vlase L., Porfire A.S., Achim M., Tomuță I., Quercetin-loaded liposomes: formulation optimization through a d-optimal experimental design. *Farmacia*, 2015; 63(1): 26-33.
18. Zou X., Zhao X., Ye L., Wang Q., Li H., Preparation and drug release behavior of pH-responsive bovine serum albumin-loaded chitosan microspheres. *Journal of Industrial and Engineering Chemistry*, 2015; 21: 1389-1397.