Alternative Possibilities to Asses a Phytohormone Release Rate from a Polyurethane Carrier

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Hormones are chemical substances which transmit a message as a signal within a physiological system between the place where they are secreted and the place of their receptors where they have the biological action. In the last decades the number of studies on phytohormones has increased continuously. In this study, polyurethane microstructures with and without brassinolide were synthesized in order to assess the release rate. Two different types of drug carriers were obtained (ether- and ester-based microstructures) and the evaluations were done by using two different methods: the measurement of microstructures' size and the determination of free brassinolide concentration. The results suggest that ester-based polyurethane microstructures degrade faster and the two methods present very similar results.

Keywords: brassinolide, drug carrier, DSC, polyurethane, UV-Vis, zetasizer

Traditional medicine heavily relies on the consumption of fruits and vegetables, which have been shown over the time that will ameliorate or cure many diseases. The roots and leaves of different plants are most frequently used in phytotherapies as alternative remedies [1]. Many studies on the aqueous or alcoholic plant-derived extracts with high contents of chemical compounds were published in the scientific literature in the last century. The role of these phytocompounds as food preservatives, pharmaceutical drugs with antimicrobial or antitumoral activity, was observed in these studies [2].

Many climate changes have occurred on Earth in the last hundred years, and plants were forced to modify their defense mechanisms to adapt to these changes. The phytohormones are the chemical substances which regulate the plants' ability to modify their growth and development [3]. The phytohormones are a wide class of chemicals which contain molecules with very different molecular weight and various essential metabolic pathways such as auxins, cytokinins, gibberellins, brasinosteroids, abscisic acid, ethylene, nitric oxide etc. [4]. These compounds appear in plants as various derivatives (complexes with amino acids and saccharides or degradation metabolites), and often present a similar biological activity with the free hormones [5].

The brassinosteroids are considered plant growth regulators belonging to the class of polyhydroxysteroids with an important role in the developments of plants [6]. The most active brassinosteroids is brassinolide, [(22R,23R,24S)-2a,3a,22,23-tetrahydroxy-24-methyl-homo-7-oxa-5a-cholestan-6-one] (fig. 1). It contains two vicinal hydroxyl groups (C2/C3), a lateral chain with other two hydroxyl groups with R configuration at C22/C23, a 24S methyl substitution, and a lactone group in C6/C7.

Oh and Clouse discovered the role of brassinolide as a cell division promoter, while Sasaki presented this compound as a generator of plants' buds; it was found its key role in the development of embryonic tissues too [7].

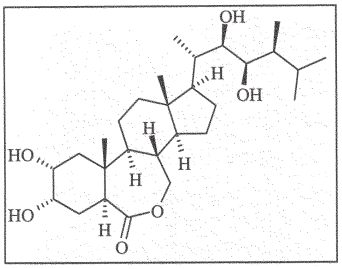

Fig. 1. Chemical structure of brassinolide

It is known that drug molecules must be dissolved in the body fluids to penetrate biological barriers and to be transported to their receptors. On the other hand, the drug concentration in relevant body fluids has a major impact on the therapeutic outcome; this concentration depends on two main parameters: its solubility in these fluids and its release from different drug carriers. The solubilization of drug molecules in an imposed solvent is often impossible due to its octanol-water partition coefficient and this is a situation when it is necessary to use a drug carrier [8]. The drug delivery systems are pharmaceutical formulations used to improve the properties of an active substance such as: the possibility to penetrate different membranes, the stability against strong acidic medium or UV degradation, the solubility, the targeted delivery etc. [9]. Nowadays, the knowledge from the border between nanosciece, nanotechnology, medicine, and pharmacy provides the opportunity to develop nano- and micro-structures used as a drug carrier with a controlled release which can adjust the potential toxicity of drugs. There were already synthesized nano- and micro-spheres, liposomes, micelle, carbon nanotubes, different nanofibres or organic dendrimers; among these, the polymeric nano- and micro-structures are considered the most promising drug delivery

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system used to improve the solubility of various types of hydrophobic compounds by physically entrapation [10].

Polyurethane nano- and micro-structures may be used as a drug carrier due to their easily adjustable sizes, their morphology and due to the simple way to modify the final properties by varying chemical composition and ratios [11]. There is a considerable interest in the use of drug delivery systems composed of biodegradable and biocompatible amphiphilic block copolymers in the administration of hydrophobic substances [10, 12]. Polyurethanes materials are used in many biomedical fields due to their biocompatibility and versatility; they are used in tissue engineering, obtaining of medical devices and as drug carriers [13]. The polyurethanes were discovered by Prof. Otto Bayer in 1937 and twenty years later first applications as biomaterials were developed when Pangman used polyurethanes as composite breast prostheses and Mandrino and Salvatore used them for *in situ* bone fixation [14].

The aim of this research was to obtain polyurethane microstructures with and without entrapped brassinolide and to characterize these samples.

Experimental part

Reagents

Hexamethylene-diisocyanate (HMDI), polyethylene glycol, M≈200 (PEG), the solvent (acetone) and surfactant (Tween®20) were obtained from Merck (Germany); ethylene glycol (EG) from Lach-Ner (Czech Rep.), 1,4-butanediol (BD) from Carl Roth GmbH (Germany), polycaprolactone diol, M≈530 (PCL) and brassinolide were purchased from Sigma-Aldrich. All reagents were maintained in the conditions indicated by the suppliers and there were used without any previous purification.

Obtaining of samples

The procedure used to obtain the polyurethanes microstructures was already described in the literature in our previous papers [15-19].

Step 1. At the beginning of the synthesis, the aqueous and the organic phase were prepared separately: the aqueous phase is a mixture of 0.7 mL EG, 0.8 mL BD, 0.5 mL PEG (or 0.55 g PCL), 1.3 mL Tween®20 and 15 mL distilled water (mixed at 400 rpm and heated at 40°C), while the organic phase contains 1.6 mL HMDI dissolved in 15 mL acetone (mixed at 400 rpm and heated at 25°C).

Step 2. The content of organic phase was rapidly poured into the aqueous phase under magnetic stirring at 800 rpm and 60°C. The stirring and heating of reaction chemicals were maintained at the same values for 4 h in order to assure the finishing of polyaddition process and the complete formation of polyurethane microstructures' walls.

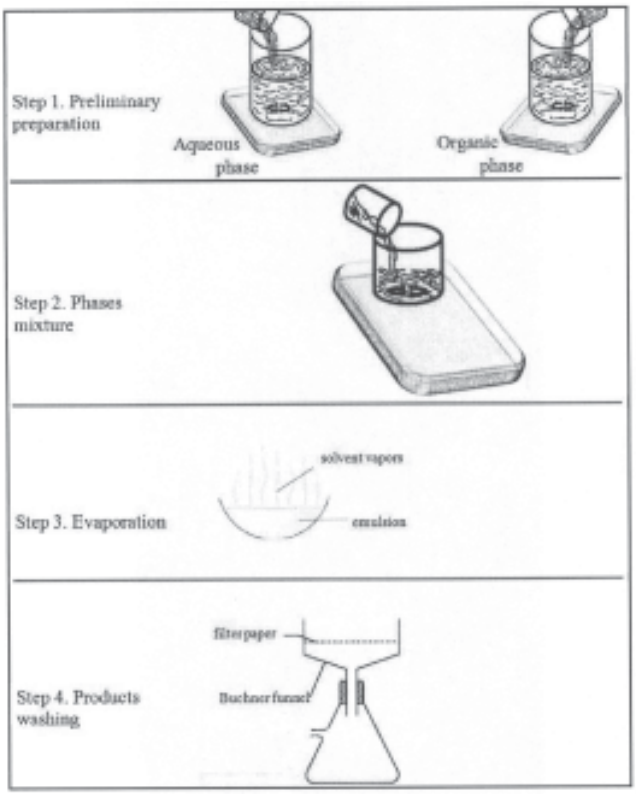

Fig. 2. Scheme of synthesis

Step 3. In the previous step there were obtained white emulsions which were stretched as thin layers on watch glasses and there were covered with Petri dishes' lids. The samples were dried using a PolEko SL115 laboratory oven with a heating program consisting of 8 h at 85°C and forced air convection daily.

Step 4. The obtained powders were repeatedly washed using a Buchner funnel and a mixture of water and acetone (1:9, v/v).

Four different samples were synthesized using the procedure described in figure 2; the raw materials used for the synthesis of every sample are presented in table 1.

Evaluation of samples' solubility and pH

The solubility of samples in different solvents (distilled water, methanol, ethanol, acetone, DMSO) was evaluated at 25°C. The *p*H values were evaluated in quadruplicate using a portable pH Meter Checker® (Hanna instruments, USA) and samples' aqueous solutions with the same concentration (0.9 mg/mL) at the same temperature (25°C).

Thermal behaviour and zetasizer characterization

The samples were characterized using Differential scanning calorimetry (DSC); the analysis was carried out

Sample code	Aqueous phase					Organic phase	Active substance
	EG	BD	PEG	PCL	Tween®20	HMDI	brassinolide
EPEt	x	x	x	-	x	x	-
BPEt	x	x	x	-	x	x	x
EPEs	x	x	-	x	x	x	-
BPEs	x	x	-	x	x	x	x

Table 1
THE RECIPE OF THE SYNTHESIS

Ion	Concentration, mmol/dm^3	
	Simulated Body Fluid	Human Blood Plasma
Na^+	142.0	142.0
K^+	5.0	5.0
Mg^{2+}	1.5	1.5
Ca^{2+}	2.5	2.5
Cl^-	147.8	103.0
HCO^{3-}	4.2	27.0
HPO_4^{2-}	1.0	1.0
SO_4^{2-}	0.5	0.5

Table 2
CHARACTERIZATION OF SIMULATED BODY FLUID [21]

with a Mettler-Toledo DSC1 instrument (Mettler-Toledo, Switzerland). Small portions of samples (between 4.1 and 4.3 mg) were placed in aluminum crucibles with pierced caps and were heated between 30-300°C in an oxidative atmosphere with a 10 degree/min heating speed. A reference material (empty aluminum crucible with pierced cap) simultaneously undergoes the same programmed time/temperature routine. The instrument was previously calibrated with the use of Zn and In standards.

The size and the stability of polyurethane microstructures were evaluated with a Cordouan Zetasizer instrument (Cordouan Technol., France) consisting of a Vasco Particle Size Analyzer and a Wallis Zetapotential Analyzer. There were set the following Vasco Particle Size Analyzer parameters: temperature (25°C), time interval (20 μs), and number of channels (400), laser power (85%), acquisition mode (continuous), and analysis mode (Pade-Laplace). The following Wallis Zetapotential Analyzer parameters were chosen: cuvette type (plastic, with a wavelength between 380 and 780 nm), temperature (25°C), laser power (90%), applied field (automatic), resolution (medium, 0.8 Hz), 3 measures/sequence, and Henry function (Smoluchowski).

Artificial degradation of samples

The literature describes many types of artificial medium used to simulate the natural degradation of samples inside the human body. T. Kokubo presented a solution called Simulated Body Fluid (SBF) in the early 90s [20] which have similar ions' concentrations with human plasma (table 2).

The preparation of Simulated Body Fluid was already described in detail in one of our previous paper [22]. The stock solution was divided into four flat-bottomed flasks, and an exact amount of every sample of polyurethane microstructures with and without brassinolide was inserted in this artificial degradation medium. There were extracted samples from every flat-bottomed flask every three days and these were analyzed with Vasco Particle Size Analyzer and a SI Analytics UViLine 9400 spectrophotometer in order to evaluate the release rate of brassinolide from polyurethane microstructures.

Results and discussions

Brassinolide has a chemical structure quite similar to the structure of cholesterol. This is the reason why this phytohormone has a very low solubility in water (around $5 \cdot 10^{-3}$ mg/mL) [23]. The solubility and the *p*H values of synthesized samples are presented in table 3.

The DSC analysis (fig. 3) shows an endothermic process while temperature is increasing between 50 and 110°C which can be associated to the dehydration and vaporization of solvents. This process presents a large enthalpy because of the evaporation of released water/acetone [24]. Inflections of BPet and BPEs curves around 250°C can be attributed to the melting point of brassinolide; there was not obtain a specific peak for the melting point probably due to the entrapation of brassinolide inside the polyurethane microstructures.

The size measurements of polyurethane microstructures were done using the same parameters of the instruments. The first measurement was done before the introduction of samples in the artificial degradation medium (SBF): the results of microstructures' sizes are shown in figure 4-7 and the stability of polyurethane microstructures are presented in figure 8 and 9 as mobility measured with Wallis Zetapotential Analyzer. It can be observed that initially

Parameter vs. Sample	EPEt	BPEt	EPEs	BPEs
pH	6.44±0.09	6.52±0.14	6.39±0.12	6.49±0.17
Solubility in water, mg/mL	0.95	0.91	0.98	0.96
Solubility in methanol, mg/mL	1.12	1.09	1.17	1.15
Solubility in ethanol, mg/mL	1.05	1.03	1.06	1.03
Solubility in acetone, mg/mL	1.17	1.16	1.18	1.19
Solubility in DMSO, mg/mL	1.21	1.19	1.20	1.15

Table 3
SOLUBILITY AND *p*H OF SAMPLES

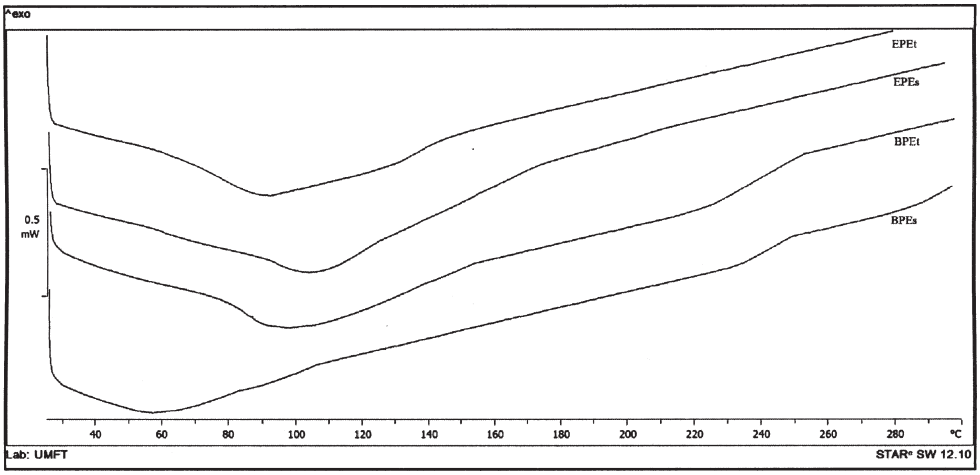

Fig. 3. DSC curves for the tested samples

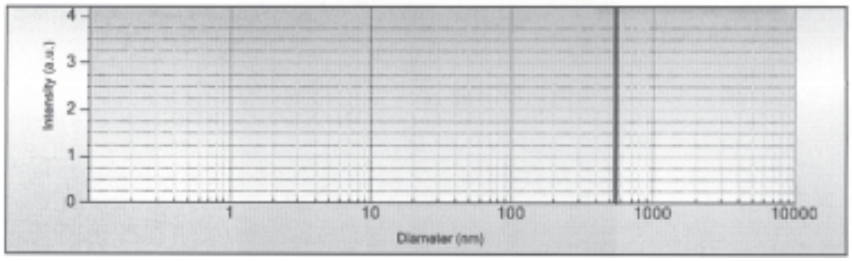

Fig. 4. Average sizes of polyurethane microstructures from sample EPEt

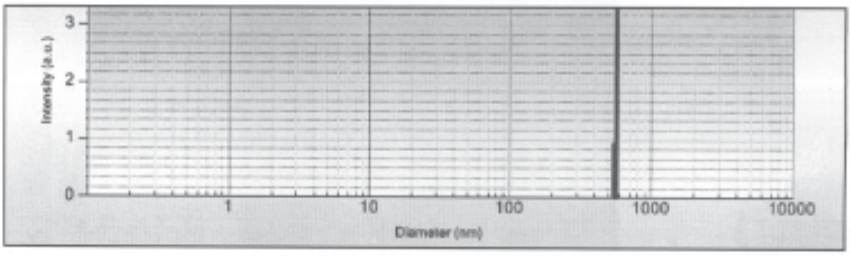

Fig. 5. Averages sizes of polyurethane microstructures from sample BPEt

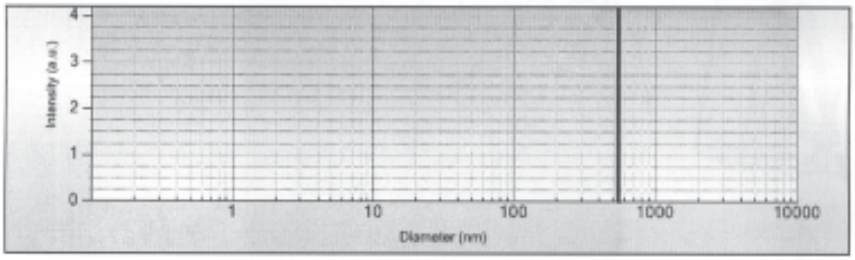

Fig. 6. Average sizes of polyurethane microstructures from sample EPEs

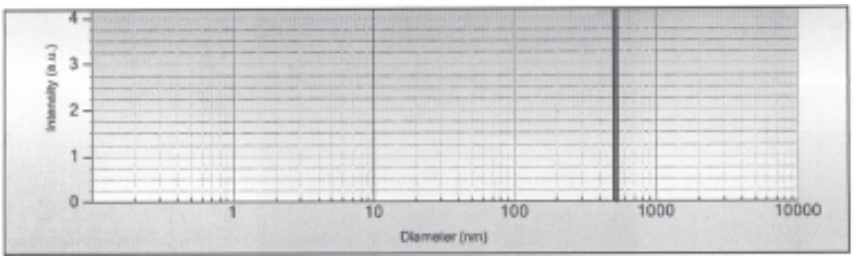

Fig. 7. Average sizes of polyurethane microstructures from sample BPEs

all samples contain relatively homogeneous polyurethane microstructures with diameters between 511 and 537 nm.

According to its guide, Wallis Zetapotential Analyzer primarily measures the electrophoretic mobility of charged nano- and micro-structures; the results are presented as Zeta potential values which are calculated from the values measured of the electrophoretic mobility based on the solvent properties and on the double layer model selected by the operator (Smoluchowski approximation, when Henry function equals 1 for aqueous solutions or Hückel approximation, when Henry function equals 1 for organic solutions) [25].

There were not observed important differences between the mobility of samples EPEt and EPEs (fig. 8). Both samples present a medium stability characterized by Zeta potential values between 22 and 29 mV. In the other case, samples BPEt and BPEs are very stable with Zeta potential values between 65 and 122 mV and it is interesting to note that samples with brassinolide are more stable than the samples containing empty polyurethane microstructures.

The first method used to evaluate the degradation rate of polyurethane microstructures is based on the observation of sizes' changes. Sizes of microstructures maintained in SBF were measured every three days and the evolution of average values is presented in figure 10.

The values of polyurethane microstructures' sizes were used to observe the release rate of brassinolide. The evolution curves were drawn using MS Excel and the same software was used to determine the slopes of these curves. Linear trendlines of every curve were drawn and the following equations were obtained: y = -7.0675x + 519.33

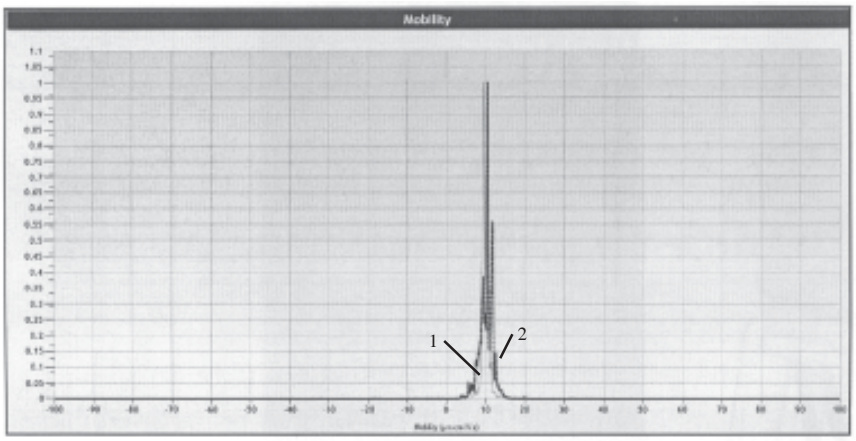

Fig. 8. The mobility of microstructures from samples EPEt (1) and EPEs (2)

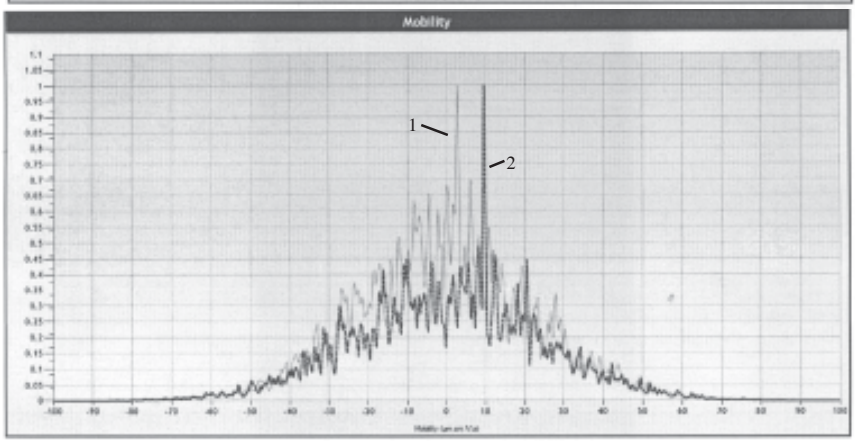

Fig. 9. The mobility of microstructures from samples BPEt (1) and BPEs (2)

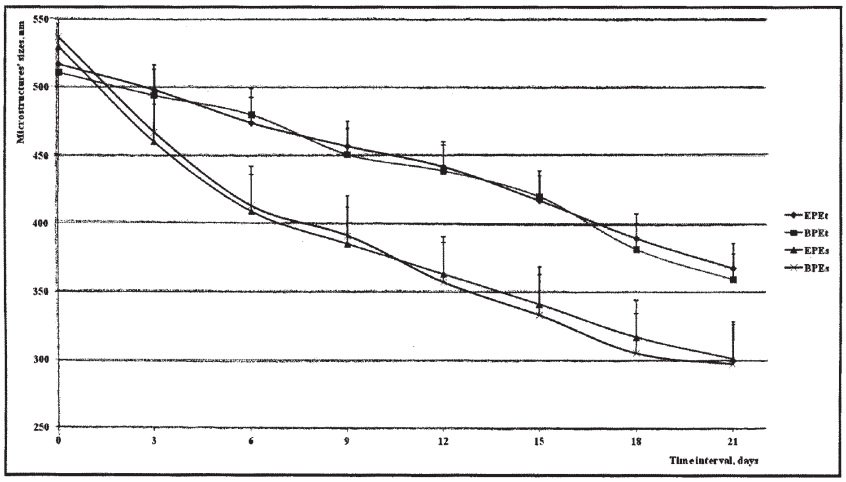

Fig. 10. Evolution of polyurethane microstructures' size

(EPEt sample), y = -7.2262x + 517.75 (BPEt sample), y = -10.095x + 494.25 (EPEs sample), and y = -10.968x + 502.67 (BPEs sample). It is easy to see that microstructures based on PCL (samples EPEs and BPEs) present a faster degradation than the microstructures based on PEG.

The second method which was used to assess the release rate of brassinolide is based on the concentration of this active substance in the SBF measured at every three days. 2 mL from every flat-bottomed flask was analyzed by UV-Vis and it was replaced with fresh SBF. Figure 11 and 12 present the UV-Vis spectrum of polyurethane microstructures without brassinolide; the recorded maximum absorption was at a wavelength $\lambda max=281$ nm.

Brassinolide presents a maximum absorption at 220 nm [26]. There were prepared standard solutions of brassinolide with different concentrations and there were recorded their absorbances in order to obtain the calibration curve. Based on this, the concentrations of free brassinolide were evaluate in the two flat-bottomed flasks (with BPEt and BPEs samples) at every three days and the evolution of average results is presented in figure 13.

The evolution of free brassinolide concentration is an increased curve with the following treendline: y = 0.8175x + 0.4167 (BPEt sample) and y = 1.2183x + 1.3333 (BPEs sample). Table 4 presents a comparison of slopes of degradation measured with Vasco Particle Size Analyzer and with SI Analytics UViLine 9400 spectrophotometer.

There were calculated the ratios between the slopes of ester/ether polyurethane microstructures with brassinolide in order to estimate a correlation between the two methods used in this study to assess the degradation rate. It was obtained a (-10.97/-7.23)=1.52 for the first method and a (1.22/0.82)=1.49 for the second method. The same ratio between the slopes of ester/ether polyurethane microstructures without brassinolide was: (-10.09/-7.07)=1.43.

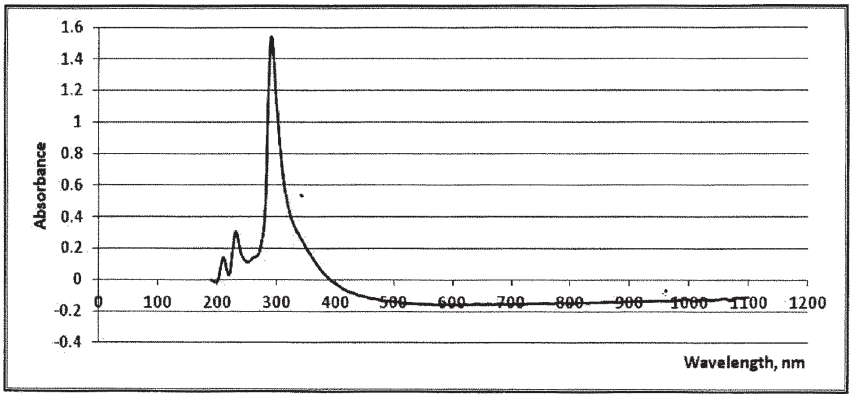

Fig. 11. UV-Vis spectrum of sample EPEt

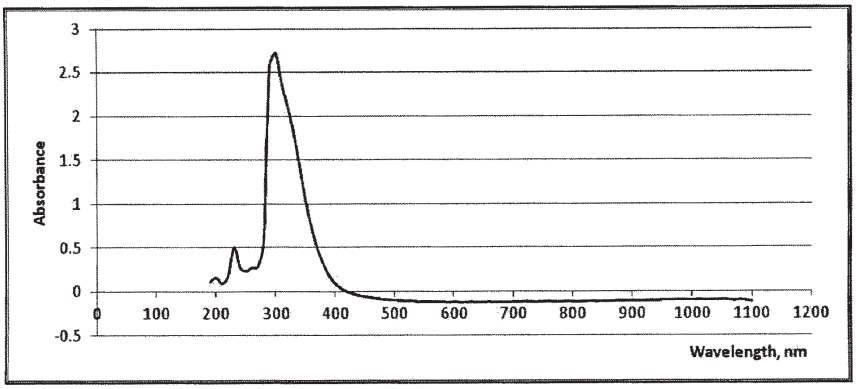

Fig. 12. UV-Vis spectrum of sample EPEs

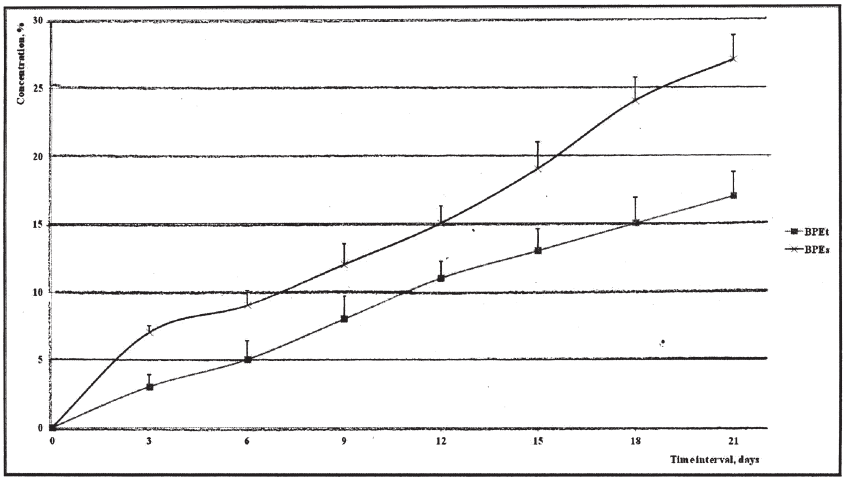

Fig. 13. Evolution of free brassinolide concentration

Sample	Slope by size measurement	Slope by concentration
EPEt	-7.07	-
BPEt	-7.23	0.82
EPEs	-10.09	-
BPEs	-10.97	1.22

Table 4
COMPARISON BETWEEN THE SLOPES OF DEGRADATION CURVES

Conclusions

In this study, polyurethane microstructures with and without brassinolide were synthesized in order to compare the degradation rate of the drug carrier. Microstructures with sizes between 511 and 537 nm were obtained, and they present a better solubility than the free brassinolide and average *p*H of aqueous solution between 6.4 and 6.5. The microstructures were maintained for 21 days in an artificial degradation medium (Simulated Body Fluid) and evaluations of microstructures' sizes and free brassinolide concentrations were done every three days. The results indicate that ester-based microstructures degrade themselves one and a half faster than ether-based microstructures due to the hydrolysis of ester groups. Both methods (the evaluation of microstructures' size on the one hand and the evaluation of free brassinolide concentration on the other hand) present very similar results which indicate that these methods can be used as procedures for evaluation of drug carriers' degradation rate.

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References

1. WATSON, R., PREEDY, V. (Eds.) Bioactive Food as Dietary Interventions for Arthritis and Related Inflammatory Diseases. Academic Press, Waltham (MA), 2013, p. 413.

2. D'SOUZA, D.H. Curr. Opin. Virol., **4**, 2014, p. 44.
3. FLOKOVÁ, K., TARKOWSKÁ, D., MIERSCH, O., STRNAD, M., WASTERNACK, C., NOVÁK, O. Phytochem., **105**, 2014, p. 147.
4. CUI, K., LIN, Y., ZHOU, X., LI, S., LIU, H., ZENG, F., ZHU, F., OUYANG, G., ZENG Z. Microchem. J., **121**, 2015, p. 25.
5. DIOPAN, V., ADAM, V., HAVEL, L., KIZEK, R. Molecules, **14**, 2009, p. 1825.
6. BISHOP, G.J., KONCZ, C. Plant Cell, **14**, 2002, p. S97.
7. CAI, Z., JING, X., TIAN, X., JIANG, J., LIU, F., WANG, X. S. Afr. J. Bot., **97**, 2015, p. 143.
8. WERMUTH, C.G., ALDOUS, D., RABOISSON, P., ROGNAN, D. (Eds.) The Practice of Medicinal Chemistry. 4th Ed. Academic Press, Waltham (MA), 2015, p. 699.
9. OHSIMA, H., MAKINO, K. (Eds.) Colloid and Interface Science in Pharmaceutical Research and Development. Elsevier, Amsterdam, 2014. p. 131.
10. ZHOU, L., LIANG, D., HE, X., LI, J., TAN, H., LI, J., FU, Q., GU, Q. Biomater., **33**, no. 9, 2012, p. 2734.
11. PIGNATELLO, R. (Ed.) Drug–Biomembrane Interaction Studies - The Application of Calorimetric Techniques. Woodhead Publishing Limited, Sawston, 2013, p. 335.
12. DING, M., HE, X., ZHOU, L., LI, J., TAN, H., FU, X., FU, Q. J. Control. Rel., **152**, Suppl. 1, 2011, p. e87.
13. KUMBAR, S., LAURENCIN, C., DENG, M. (Eds.) Natural and Synthetic Biomedical Polymers. Elsevier Inc., Amsterdam, 2014, p.123.
14. DAVIM, J.P. (Ed.) The design and manufacture of medical devices. Woodhead Publishing Limited, Sawston, 2012, p.115.
15. BORCAN, F., SOICA, C.M., GANTA, S., AMIJI, M.M., DEHELEAN, C.A., MUNTEANU, M.F. Chem. Cent. J., **6**, no. 87, 2012, p. 1.
16. BORCAN, F., SOICA, C.M., DEHELEAN, C.A., GANTA, S., AMIJI, M.M. Rev. Chim. (Bucharest), **63**, no. 11, 2012, p. 1164.
17. HEGHES, A., SOICA, C.M., ARDELEAN, S., AMBRUS, R., MUNTEAN, D., GALUSCAN, A., DRAGOS, D., IONESCU, D., BORCAN, F. Chem. Cent. J., **7**, no. 66, 2013, p. 1.
18. GALUSCAN, A., JUMANCA, D., BORCAN, F., SOICA, C.M., IONESCU, D., RUSU, L.C., CRAINICEANU, Z. Rev. Chim. (Bucharest), **65**, no. 2, 2014, p. 190.
19. JUMANCA, D., GALUSCAN, A., PODARIU, A.C., BORCAN, F., EARAR, K. Rev. Chim. (Bucharest), **65**, no. 12, 2014, p. 1473.
20. KOKUBO, T., KUSHITANI, H., SAKKA, S., KITSUGI, T., YAMAMURO, T. J. Biomed. Mater. Res., **24**, 1990, p. 721.
21. PERSAUD-SHARMA, D., BUDIANSKY, N. J. Biomim. Biomater. Tissue Eng., **18**, no. 1, 2013, p. 1.
22. ALBULESCU, R.C., BORCAN, F., PAUL, C., VELEA, I., PUIU, M. Int. Curr. Pharm. J., **3**, no. 6, 2014, p. 275.
23. MORERA-BOADO, C., ALONSO-BECERRA, E., GONZÁLEZ-JONTE, R., MONTERO-CABRERA, L.A., GARCÍA-DE-LA-VEGA, J.M. J. Molec. Graph. Modell., **27**, no. 5, 2009, p. 600.
24. GONG, Y. Pharmaceutical Solid Form Screening, Characterization,and Selection Enhancing Drug Bioavailability and Solubility [viewed on 17.07.2015]. http://www.slideshare.net/simoncurtis/pharmaceutical-solid-form-15594696.
25. ZETA POTENTIAL WALLIS [viewed on 17.07.2015]. http://www.cordouan-tech.com/en/ products/physical-chemistry-analysis/particles-characterization/zeta-potential-wallis.
26. GARBUZ, N.I., YANKOVSKAYA, G.S., KASHKAN, Zh.N., KANANOVICH, O.P., KOCHANKO, N.V. Chem. Nat. Comp., **28**, 1992, p. 63.

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