Stability and Enzymatic Studies with Omeprazole:Hydroxypropyl- β -Cyclodextrin

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Abstract

Omeprazole (OME) exhibits low stability to light, heat and humidity. In stress conditions OME stability should improve under inclusion complex form with hydroxypropyl- β -cyclodextrin (HP β CD). Stability of OME, its physical mixture (PM) with HP β CD and OME:HP β CD inclusion complex was assessed during 60 days. The inclusion complexes were prepared by kneading and freeze-drying techniques and characterized by differential scanning calorimetry (DSC) and Fourier transform infrared spectroscopy (FTIR). A molecular modelling was also held to predict the most probable tridimensional conformation of inclusion complex OME:HP β CD. The inhibitory activity of free and complexed OME on selected enzymes, namely, papain (protease model of the proton pump) and acetylcholinesterase (enzyme present in cholinergic neurons and also involved in Alzheimer's disease) was compared. The results obtained show that HP β CD do not protect against OME degradation, in any prepared powder, in the presence of light, heat and humidity. This may indicate that the reactive group of OME is not included in the HP β CD cavity. This fact is supported by molecular modelling data, which demonstrated that 2-pyridylmethyl group of OME is not included into the cyclodextrin cavity. In relation to enzymatic assays it was observed that free OME and OME in the binary systems showed identical inhibitory activity on papain and acetylcholinesterase, concluding that HP β CD do not affect OME activity on these two enzymes.

Keywords: omeprazole; hydroxypropyl- β -cyclodextrin; inclusion complex; stability; Enzymatic activity.

Introduction

Omeprazole (OME), which belongs to the family of substituted benzimidazoles (Fig. 1), is an inhibitor of the gastric proton pump H^+/K^+ -ATPase [1] but presents physical and chemical instability. In aqueous solution, its stability is dependent on pH; in acidic and neutral conditions it is rapidly degraded, showing greater stability in alkaline medium. In the solid state, the OME is degraded in presence of heat, light and humidity [2-4].

Cyclodextrins (CDs) constitute a new class of pharmaceutical excipients used to change the physico-chemical and biological undesirable properties of certain drugs. Due to its lipophilic cavity, CDs forms non-covalent inclusion complexes with poorly water-soluble drugs [5]. The possible benefits obtained from inclusion complexes include: increase of solubility of insoluble drugs; stabilization against oxidation, hydrolysis and photolysis reactions; reduction or elimination of unpleasant effects and others [6]. Hydroxypropyl- β -cyclodextrin (HP β CD) is a β -cyclodextrin derivative with a great interest for pharmaceutical formulation due to its higher solubility in aqueous solutions; very low toxicity and good complexing ability [7, 8]. Moreover, Loftsson *et al.* [9] published a study of the complexation of OME with HP β CD, in aqueous solution, reporting a solubility improvement of the drug (K_c 69 M^{-1}). Thus, knowing that the solubility of OME was increased, the aim of this study was to investigate whether or not, in the form of solid inclusion complexes, the OME would be more protected against stress conditions – light, heat and humidity – so less degradation would be protected against degradation under stress conditions (light, heat, humidity). The influence of HP β CD on the biological activity of OME on certain enzymes (papain and acetylcholinesterase), the inhibitory activity of free OME and OME of the inclusion complexes prepared, was evaluated by determination of drug concentration that inhibited 50% of the enzyme activity (IC_{50}). Papain is the enzyme normally chosen as an experimental model of enzyme kinetics of H^+/K^+ -ATPase due to its easily accessible. Both enzymes (papain and H^+/K^+ -ATPase) are cysteine proteases and, therefore, its catalytic mechanism is identical [10]. Acetylcholinesterase (AChE) is an enzyme present in cholinergic neurons and in the neuromuscular junctions that plays a key role on the control of nerve impulses. Its function is to catalyze the hydrolysis of the neurotransmitter acetylcholine to originate choline and acetate [11, 12]. The use of AChE inhibitors is a recent approach on the treatment of central nervous system degenerative diseases, particularly Alzheimer's disease, which consists on the replacement of acetylcholine by decreasing the degradation of the neurotransmitter, and is therefore a great improvement of the patients' life quality [13]. As the OME has the ability to cross the brain blood barrier [14] it was also intended to study the action of the drug on this enzyme in order to assess its efficiency as an AChE inhibitor.

Fig. 1 Structure of OME.

Materials

OME (M_r 345.4 g.mol^{-1}) and HP β CD (M_r $1387.15\text{ g.mol}^{-1}$) were kindly donated by MEDINFAR and by Roquette, respectively. Papain from papaya latex, 22.7 U/mg solid, 25.8 U/mg protein; acetylcholinesterase (AChE) type VI-S, extracted from electric eel 349 U/mg solid, 411 U/mg protein; Benzoyl-L-arginine-p-nitroanilide (L-BAPA); acetylthiocholine iodide (AChI); 5,5'-dithiobis [2-nitrobenzoic] acid (DTNB); dimethyl sulfoxide (DMSO); methanol HPLC-Grade (MeOH); 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) were all from Sigma. Sodium acetate from Riedel-de Haën.

Methods

Physical mixtures (PM)

The physical mixtures were prepared by mixing OME and HP β CD in 1:1 M molar (Fisher-Kendall Scientific Co., 12-811, USA) for 10 minutes.

Kneading (KN)

HP β CD (1.6210 g) was wetted by a basic aqueous solution (pH 10.3) ($\approx$ 50% w/w) in a ceramic mortar and then the required amount of OME (0.4040 g) was slowly added (1:1 M ratio) and kneaded for 30 min until a slurry was obtained.

Freeze-drying (FD)

HP β CD (0.8103 g) was dissolved in a basic aqueous solution (pH 10.3) and then the required amount of OME (0.2017 g) was added (1:1 M ratio) under stirring for 24 h. Furthermore, the resultant solution was frozen in liquid nitrogen and was lyophilized (Christ Alpha 1–4 freeze-dryer, B. Braun Biotech International, Germany).

Differential scanning calorimetry (DSC)

The thermal behaviour of the different powders were performed by DSC (TC 11, TA Processor, Mettler, Germany) using 4 mg samples in closed aluminum pans at a heating rate of 10 °C/min in 30 °C – 300 °C.

Infrared spectroscopy (IR)

Fourier transform-infrared spectroscopy (FT-IR) Infrared spectra were performed on a spectrophotometer (Nicolet spectrophotometer, model Impact 400, USA) using the KBr disk method and scanned from 4000 to 400 cm⁻¹.

Molecular simulation

The MOE program was used to construct the OME and HP β CD molecules. The HP β CD molecule was obtained from β CD structure, by adding the substitution hydroxypropyl groups. allowed (hydroxypropyl groups). The geometries of the HP β CD and OME molecules were optimized with a semi-empirical quantum method AM1 [15] using the program Gaussian03 (www.gaussian.com). Subsequently held the inclusion of the molecule of OME (previously optimized) in the cavity of HP β CD, previously constructed, and was performed a calculation of molecular docking in order to study the conformational space of the CD and get the most favourable conformation of the complex.

Stability Studies

Stability studies of free OME and PM, KN and FD powder samples were carried out in glass bottles sealed with rubber stopper and parafilm, in three different environmental conditions, 24 h per day (during 60 days):

- a) actinic light at to room temperature and humidity;
- b) 40 °C and 75% relative humidity (Vötsch, Industrietechnik, VC 2033);
- c) 4 °C in dark.

To assess the degradation of free OME (pure) and complexed OME (PM, KN and FD) aliquots were analyzed at 28th and 60th days by HPLC at a concentration of 20 μ g/mL (dissolved in MeOH). As reference, samples were analyzed by HPLC before the start of this test (time 0 days).

HPLC

The apparatus of HPLC (FinniganTM Surveyor[®] Plus Modular LC System) was equipped with a column Purospher[®] STAR RP-18 (Merck) with a UV detector set at 305 nm and the Xcalibur software. The following analytical conditions were used: injection volume of 25 μ L, flow 1.0 mL/min and mobile phase: methanol/ammonia-water (60/40, v/v) pH 8.4. The running time was 20 min.

HPLC

Enzymatic Studies

Kinetic experiments were performed for the enzyme papain and AChE, which were followed by UV-Vis spectroscopy in the presence and absence of OME and binary mixtures (PM, KN and FD). The control assay for each enzymatic reaction, corresponded, then, the absence of OME or binary mixture in the reaction medium and was considered 100% activity.

$$\% \text{ Inhibition} = 100 - \left(\frac{\text{enzim. rate sample}}{\text{enzim. rate control}} \right) \times 100 \quad \text{eq. 1}$$

where *enzim. rate sample* is the enzymatic rate, in absorbance per minute, of the reaction containing OME or binary mixture and *enzim. rate control* is the enzymatic rate, in absorbance per minute, of the control reaction (absence of OME).

Papain

The enzymatic activity of papain was quantified by adapting the method described by Zhao *et al.* [16]. Papain solution (0.2 mg/mL) in sodium acetate buffer 50 mM pH 4.8 (2.5 mM EDTA e 7.5 mM DTT) was prepared in order to create an acidic environment similar to the parietal cells in the stomach, at the proton pump. The papain solution prepared was activated by DTT for 2 h at room temperature. The L-BAPA (30 mM in DMSO) was the substrate for papain and was followed by the formation of product 4-nitroaniline at 410 nm. The absorbance at 410 nm was read at intervals of 1 min for 10 min. The graph of absorbance (*Abs*) versus time (*min*) was plotted and the the rate of reaction was obtained from the equation:

$$y = mx + b \quad \text{eq. 2}$$

where *y* corresponds to the absorbance, *m* to the rate of reaction and *x* to the time.

The OME concentrations were varied from 0.109 to 0.579 mM in order to determine the concentration that inhibits 50% of enzyme activity (IC_{50}). We used the concentration of OME who originated the IC_{50} in the sample free of OME for tests with binary mixtures and with free HP β CD. The assays were performed in triplicate.

Acetylcholinesterase (AChE)

The enzymatic activity of AChE was measured by adapting the method described by Falé *et al.* [17]. The substrate was acetylthiocholine iodide (AChI, 1 mM in distilled water). The product formed reacts with DTNB (3 mM in HEPES buffer 50 mM pH 8 with 50 mM NaCl and 20 mM MgCl₂·6H₂O), producing a colored compound, followed by reaction at 405 nm. The absorbance at 405 nm was read at intervals of 1 min for 5 min. The rate of the reaction was obtained by the same method that was used in papain.

The concentrations of free OME and OME in binary systems were varied from 0.015 mM to 0.434 mM in order to determine the concentration that inhibits 50% of enzyme activity (IC_{50}). Kinetic assays of AChE with free HP β CD with two different concentrations 0.506 and 0.203 mM were also performed.

The assays were performed in triplicate.

Statistical analysis

The significance of the IC_{50} values obtained on the inhibitory activity tests of OME and binary mixtures on AChE was tested by ANOVA (Analysis of Variance) F-Snedecor distribution with a range of 95%.

Results and Discussion

Differential scanning calorimetry (DSC)

The DSC thermogram of OME shows one characteristic sharp endothermic peak at around 156 °C, indicating the melting point of the drug, followed by an exothermic effect at 173 °C, relative to its thermal decomposition (Fig. 2a). The thermogram corresponding to HP β CD (Fig. 2b) shows a broad endothermic effect between 60 and 120 °C, attributed to the loss of water molecules in the cavity of the CD [18]. For the PM, both the characteristic peaks of the drug (melting peak) as the effect of HP β CD endothermic (dehydration) are present (Fig. 2c), without deviation of the OME melting point, demonstrating absence of inclusion complexes. The thermogram of the sample KN (Fig. 2d) shows an endothermic peak, corresponding to the OME melting point, but with low intensity and relatively broader than the pure drug, also suffering from a shift to a lower temperature. This indicates that some interaction between OME and HP β CD occurred. The disappearance of the OME melting point peak in FD sample suggests an incorporation of the drug in the HP β CD cavity (Fig. 2e).

Fig. 2 DSC thermograms of (a) OME, (b) HP β CD, (c) PM, (d) KN and (e) FD.

Infrared spectroscopy (IR)

The FTIR spectrum of OME (Fig. 3a) shows two characteristic bands at 1625.99 cm^{-1} , corresponding to stretching link vibration ($\text{C}=\text{C}-\text{N}$ and $\text{S}-\text{C}=\text{N}$), and at 1203.31 cm^{-1} relative to the $\text{Ar}-\text{C}-\text{OCH}_3$ vibration accompanied by resonance band at 1075 cm^{-1} (Fig. 3b) [4]. These two bands were used to analyze the interaction between OME and HP β CD.

None of the binary mixtures presented the formation of new bands, indicating that no covalent bond was established in the complexes formed [19].

The characteristic bands of OME and HP β CD are present on the IR spectrum of PM, indicating that there was no interaction between the CD cavity and the groups responsible for the relevant IR absorption (Fig. 3c).

Identical result was observed for the KN sample (Fig. 3d), although the band at 1203.31 cm^{-1} have less intensity than on the PM, reinforcing thus the idea of having partial interaction between the OME and CDs molecules, as mentioned above for the results obtained by DSC.

In FD sample it can be observed that the OME absorption band at 1625.99 cm^{-1} has disappeared, appearing a band at 1647.81 cm^{-1} (Fig. 3e), which is characteristic of HP β CD and corresponds to the elongation of the $\text{H}-\text{O}-\text{H}$ bonds [20]. The intensity of the band at 1203.31 cm^{-1} is also significantly decreased. These results suggest that restrictions on the vibration of $\text{C}=\text{C}-\text{N}$, $\text{S}-\text{C}=\text{N}$ and $\text{Ar}-\text{C}-\text{OCH}_3$ bonds due to its inclusion within the cavity of the CD probably occurred.

Fig. 3 IR spectra of (a) OME, (b) HP β CD, (c) PM, (d) KN and (e) FD.

Molecular simulation

The most probable tridimensional conformation of inclusion complex OME:HP β CD could be observed by molecular simulation (Fig. 4).

In the simulation model it is possible to observe that the benzimidazole ring of OME is included into the HP β CD cavity while the 2-pyridylmethyl group is outside the HP β CD cavity, featuring an energy of complexation of -68,22 kJ/mol.

Fig. 4 Molecular docking of OME with HP β CD molecule.

Stability Studies

Stability of both pure and complexed OME was evaluated under distinct environmental conditions such as light, heat and humidity.

At time 0 days, all samples (free OME, PM, KN and FD) were analyzed by HPLC. By the analysis of chromatograms it was found that different samples had no degradation products with the appearance of a well-defined peak, relative to OME in each sample, with retention time between 7.7 and 7.8 min (Fig. 5).

Fig. 5 HPLC chromatograms of OME in the different samples (PM, KN, FD) at 0 days.

Table 1 presents the results obtained for the degradation of OME in the various samples (free OME, PM, KN and FD) when exposed to different environmental conditions for 60 days and the Fig. 4 shows its physical aspect.

On Table 1 it can be seen that actinic light is the environmental condition which causes more degradation of OME. It is also possible to conclude that OME under inclusion complex is more degradable than pure OME in presence of actinic light and 40 °C with 75% RH. Unlike desired, complexation of OME with HP β CD did not increase drug stability to light, heat and humidity, instead led to an increase in its rate of decomposition. This can be attributed to the CD used (HP β CD). Different types of CD may lead to different modes of inclusion for the same molecule; depending on CD, the degradation of the drug can be accelerated, retarded or remain the same [21]. The fact of 2-pyridylmethyl group from OME might remain outside the cavity of the HP β CD, as shown by supporting data from molecular simulation, may accelerate the degradation of the drug through a reaction between 2-pyridylmethyl group and CD hydroxyl groups. There was also a change in the colour of the different samples over the 60 days, more clearly in binary systems (Fig. 6), which suggests a relationship between the degradation degree and the colour of the samples.

Thus, it seems not to be advantageous to prepare inclusion complexes OME:HP β CD for degradation protection despite this CD significantly increase the solubility of the drug [9].

Table 1 - Degradation (%) of free OME and OME present on different binary mixtures (physical mixtures - PM, complexes obtained by kneading - KN and the freeze-drying - FD methods) at different environmental conditions over 60 days.

% OME degradado												
Actinic light					40 °C, 75% humidity				4 °C in dark			
Time (days)	Pure OME	OME:HPβCD			Pure OME	OME:HPβCD			Pure OME	OME:HPβCD		
		PM	KN	FD		PM	KN	FD		PM	KN	FD
0	0	0	0	0	0	0	0	0	0	0	0	0
28	8.66	37.97	75.47	76.06	10.26	10.69	13.93	100.00	0	0	0	0
60	26.20	59.66	70.13	99.27	15.26	11.94	17.43	100.00	0	0	0	1.28

Fig. 6 Physical aspect of pure OME and FD sample.

Enzymatic Studies

Biological activity of pure OME and OME as inclusion complex was assessed by enzymatic assays using papain and AChE.

Papain

The IC_{50} value obtained for the pure OME was 0.425 mM. At this OME concentration the same inhibition of the papain was obtained for the samples PM, KN and FD (Fig. 7), concluding that the inhibitory activity of OME is not affected even in the presence of HP β CD. This could mean that the functional group of the drug, responsible for binding to the enzyme and, consequently, its inhibition, shall remain outside the CD cavity, enabling the reaction with papain and giving therefore rise to the same value of IC_{50} . An enzymatic assay of papain with free HP β CD (without OME) was also carried being observed that the enzyme activity is equal to the control assay. It was then concluded that this CD does not interfere with the activity of papain, and therefore, as expected, it is obtained similar enzyme activities between the various binary mixtures. Similar results were reported by Gubica *et al.* [22], where the presence of TM β CD ((2,3,6-tri-O-methyl)- β -cyclodextrin) did not affect the enzymatic reaction of tryptophan indole-lyase with the substrate L-tryptophan.

Fig. 7 Papain activity (%) in presence of pure and complexed OME. All samples had an OME concentration of 0.425 mM (n = 3).

Acetylcholinesterase

The inhibitory activity of OME on acetylcholinesterase had not yet been determined in any published study. OME concentrations were varied from 0.015 to 0.434 mM in order to determine the concentration that inhibits 50% of enzyme activity (IC_{50}). The IC_{50} value obtained for the pure OME was 0.224 ± 0.016 mM. This IC_{50} value differs from the IC_{50} of galantamine (1.07 ± 0.18 μ M) [23], an AChE inhibitor drug used to treat Alzheimer's disease [24]. However, the OME has a higher capacity of inhibit AChE compared to some natural compounds, particularly the monoterpenes, the major components of essential oils extracted from plants, flowers and fruits. 1.8-cineole and α -pinene are the most active monoterpenes with IC_{50} values of 0.670 mM and 0.630 mM, respectively. The difference in the inhibitory effect of the various compounds in relation to AChE is related to the enzyme binding site, which may occur directly in the active site or in peripheral binding site or both sites [25]. The OME is therefore a substance with a promising structure that could be tested as a lead molecule for the development of new compounds that are more effective in the inhibition of AChE. To infer whether HP β CD affects the inhibitory effect of OME on AChE activity, IC_{50} values of OME in the different binary systems (PM, KN and FD) were determined. The IC_{50} values were 0.255 ± 0.011 mM for PM, 0.216 ± 0.048 mM for KN and 0.279 ± 0.004 mM for FD (Fig. 8). The IC_{50} values are similar for the OME in the different binary systems and free OME (pure) as was proved through statistical analysis (ANOVA, F-test for a probability of $P = 0.05$), that these values are not significantly different ($F(3.78) < F_{crit}(4.07)$). This indicates that even when the OME is complexed with HP β CD, the functional group of the drug responsible for binding to the enzyme and, consequently, inhibition of the same, shall remain outside the cavity of the CD, allowing the reaction with AChE, giving therefore rise to the identical value of IC_{50} between free and complexed OME, as was the case with papain. An enzymatic assay of AChE with free HP β CD (without OME) was also carried out observing that the enzyme activity is equal to the control assay. Thus, it seems that, as in the assay with papain, the CD used does not affect the activity of AChE.

Fig 8. Acetylcholinesterase IC_{50} values (mM) for the different samples (free OME, PM, KN and FD).

Conclusion

The methods used to characterize the prepared inclusion complexes showed that there was interaction of the OME with the HP β CD cavity. However HP β CD didn't protect against OME degradation in the presence of light, heat and humidity, indicating that the reactive group of OME was not probably included into the CD cavity, as evidence by the data from molecular simulation. Enzymatic assays showed that free OME and OME in binary systems presented identical inhibitory activity on papain and acetylcholinesterase, concluding that HP β CD didn't affect OME activity on both enzymes. Moreover free HP β CD didn't affect neither papain nor acetylcholinesterase activity.

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References

1. Aihara T., Nakamura E., Amagase K., Tomita K.; Fujishita T., Furutani K., Okabe S.; Pharmacological control of gastric acid secretion for the treatment of acid-related peptic disease: past, present, and future; *Pharmacol. Ther.*; 98(1): 109-127; 2003.
2. Ruiz M., Reyes I., Parera A., Allardo V.; Determination of the stability of omeprazole by means of differential scanning calorimetry; *J. Therm. Anal.*; 51: 29-35; 1998.
3. Storpirtis S., Rodrigues D.; In vitro evaluation of dissolution properties and degradation products of omeprazole in enteric-coated pellets; *Drug Dev. Ind. Pharm.*; 24(11):1101-7; 1998
4. Arias M., Moyano J., Muñoz P., Ginés J., Justo A., Giordano F.; Study of Omeprazole- γ -cyclodextrin complexation in the solid state; *Drug Dev. Ind. Pharm.*; 26(3); 253-259; 2000.
5. Brewster M., Loftsson T.; Cyclodextrins as pharmaceutical solubilizers; *Advanced Drug Delivery Reviews* 59:645–666; 2007.
6. Hedges A.; Industrial applications of cyclodextrins; *Chem. Rev.*; 98: 2035-2044; 1998.
7. Marques H.; Cyclodextrins' derivatives. Absorption, toxicity, metabolism and fate; *Rev. Port. Farm.*; 44 (4): 147-156; 1994
8. Irie T., Uekama K.; Pharmaceutical Applications of cyclodextrins. III. Toxicological issues and safety evaluation; *J. Pharm. Sci.*; 86 (2): 147-162; 1997.
9. Loftsson T., Hreinsdóttir D., Másson M.; Evaluation of cyclodextrin solubilization of drugs; *Int. J. Pharm.*; 302 (1-2): 18-28; 2005.
10. Salas C., Goes M., Hernandez M., Lopes M.; Plant cysteine proteinases: Evaluation of the pharmacological activity; *Phytochem.*; 69: 2263–2269; 2008.
11. Ahmed M., Rocha J., Mazzanti C., Morsch A., Cargnelutti D., Corrêa M., Loro V., Morsch V., Schetinger M.; Malathion, carbofuran and paraquat inhibit *Bungarus sindanus* (krait) venom acetylcholinesterase and human serum butyrylcholinesterase in vitro; *Ecotoxicol.*; 16:363–369; 2007.
12. Abou-Donia, M.: Organophosphorus ester-induced chronic neurotoxicity. *Arch. Environ. Health* 58(8), 484–497 (2003)
13. Ibach B., Haen E.; Acetylcholinesterase inhibition in Alzheimer's disease; *Curr. Pharm. Des.*; 10: 231-251; 2004.
14. Cheng F., Ho Y., Hung L., Chen C., Tsai T.; Determination and pharmacokinetic profile of omeprazole in rat blood, brain and bile by microdialysis and high-performance liquid chromatography; *J. Chromat. A*, 949: 35–42; 2002.
15. Dewar M., Zoebisch E., Healy E.; AM1: A New General Purpose Quantum Mechanical Molecular Model; *J. Am. Chem. Soc.*; 107: 3902-09; 1985.
16. Zhao G., Zhou Z.; Vinyl Sulfonium as Novel Proteolytic Enzyme Inhibitor; *Bioorg. Medicinal Chem. Lett.*; 11: 2331–2335; 2001.
17. Falé P., Borges C., Madeira P., Ascensão L., Araújo M., Florêncio M., Serralheiro M.; Rosmarinic acid, scutellarein 4'-methyl ether 7-O-glucuronide and (16S)-coleon E are the main compounds responsible for the acetylcholinesterase and antioxidant activity in herbal tea of *Plectranthus barbatus* ("falso boldo"); *Food Chem.*; 114 (3): 798-805; 2009.
18. Mura P., Adragna E., Rabasco A., Moyano J., Pérez-Martínez J., Arias M., Ginés J.; Effects of the Host Cavity Size and the Preparation Method on the Physicochemical Properties of Ibuprofen-Cyclodextrin Systems; *Drug Dev. Ind. Pharm.*; 25(3): 279–287; 1999.
19. Hirlekar R., Kadam V.; Preparation and characterization of inclusion complexes of carvedilol with methyl- β -cyclodextrin; *J. Incl. Phenom. Macrocycl. Chem.*; 63:219–224; 2009.
20. Aleem O., Kuchekar B., Pore Y., Late S.; Effect of β -cyclodextrin and hydroxypropyl β -cyclodextrin complexation on physicochemical properties and antimicrobial activity of cefdinir; *J. Pharmaceut. Biomed. Anal.*; 47(3): 535–540; 2008.
21. Loftsson T., Brewster M.; Pharmaceutical applications of cyclodextrins. 1. Drug solubilization and stabilization; *J. Pharm. Sci.*; 85 (10): 1017-1025; 1996.
22. Gubica T., Winnicka E., Temeriusz A., Kańska M.; The influence of selected *O*-alkyl derivatives of cyclodextrins on the enzymatic decomposition of L-tryptophan by L-tryptophan indole-lyase; *Carbohydr. Res.*; 344 (3): 304-310; 2009.
23. López S., Bastida J., Viladomat F., Codina C.; Acetylcholinesterase inhibitory activity of some Amaryllidaceae alkaloids and Narcissus extracts; *Life Sci.*; 71: 2521–2529; 2002.
24. Marco L., Carreiras M.; Galanthamine, a Natural Product for the Treatment of Alzheimer's Disease; *Recent Patents CNS Drug Discov.*; 1: 105-111; 2006.
25. Houghton P., Ren Y., Howes M.; Acetylcholinesterase inhibitors from plants and fungi; *Nat. Prod. Rep.*; 23: 181–199; 2006.