Original Article

Liposomal oxytetracycline and doxycycline: studies on enhancement of encapsulation efficiency

Marianna Budai[1,2,*], Patricia Chapela[2], Livia Budai[3], Melinda E. Wales[4], Ilona Petrikovics[2], Andreas Zimmer[5], Pal Gróf[6], Imre Klebovich[1], Maria Szilasi[7]

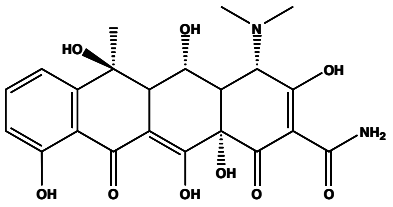
[1] *Semmelweis University, Department of Pharmaceutics, Budapest, Hungary;*
[2] *Sam Houston State University, Department of Chemistry, Huntsville, TX, USA;*
[3] *Department of Mass Spectrometry, Chemical Research Centre of the Hungarian Academy of Sciences, Budapest, Hungary;*
[4] *Texas A&M University, Department of Biochemistry and Biophysics, College Station, TX, USA;*
[5] *Karl-Franzens University, Department of Pharmaceutical Technology, Graz, Austria;*
[6] *Semmelweis University, Institute of Biophysics and Radiation Biology, Budapest, Hungary;*
[7] *University of Debrecen, Medical and Health Science Center, Department of Pulmonology, Debrecen, Hungary.*

ABSTRACT: Liposomal encapsulations of oxytetracycline (OTC) and doxycycline (DC) with various lipid compositions and hydrating solutions have been studied in order to develop a new liposomal formulation to treat bacterial infections. Encapsulation efficiencies as a function of pH (pH 4.0-8.0) in ionic (phosphate buffers) and non-ionic (mannitol or glucose) hydrating solutions with various lipid compositions (lecithin or α-L-dipalmitoyl-phosphatidylcholine, with or without cholesterol) were determined and compared to the character of lipid vesicles. Based on our encapsulation efficiency studies and on the drug stability considerations it can be concluded that for OTC/DC encapsulation the use of non-ionic solutions is the most promising.

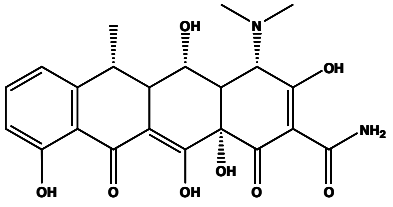
Keywords: Liposomal antibiotics, Oxytetracycline, Doxycycline, Encapsulation efficiency, Nano-delivery systems

1. Introduction

Oxytetracycline (OTC) and doxycycline (DC) are bacteriostatic agents exhibiting broad spectra of activity against many different aerobic and anaerobic bacteria (Figure 1). Despite of their broad antibacterial spectra, resistance of many bacterial groups has been reported with both of them (*1*). Furthermore, under abnormal conditions (heat, pH, humidity), tetracyclines undergo reversible epimerisation at positions C4 and C6 (*2-5*). The degradation products have very low antibiotic activity; in addition, some of them can be toxic. OTC is commonly used against external bacterial infections of the eye, such as keratoconjunctivitis, neonatal conjunctivitis, ocular rosacea or trachoma (*6-10*). In the ophthalmologic practice the so-called "oxytetracycline-eye-drop" is a frequently prescribed drug, prepared in pharmacies *ex tempore* with a short half life of only 3 days (*11*). The half-life of OTC in water was found to be 34 hours (*12*), thus hindering the safe use of the antibiotic preparations (*13*). To overcome the problems of bacterial resistance and chemical instability, liposomal formulations are being developed. The advantages of liposome entrapped drugs have been well documented (*14*). Liposomal carriers of encapsulated



Oxytetracycline



Doxycycline

Figure 1. Chemical structures of oxytetracycline and doxycycline.

**To whom correspondence should be addressed:*
Dr. Marianna Budai, Semmelweis University, Department of Pharmaceutics, Hőgyes E. u. 7., Budapest, H-1092, Hungary;
e-mail: bmariann@gyok.sote.hu

OTC or DC also offer the potential for greater penetration and prolonged release of the delivered tetracycline derivatives. In addition, lipid vesicles may provide advantages of chemical stability, similarly as observed for the reverse micelles (*12*). Improvement in OTC stability can be attained with reverse micellar systems. Dissolving OTC in reverse micelles, its half-life increases to 2,402 hours (*12*). There are no data on liposomal OTC formulations; however, encapsulation rates with some selected lipid compositions for DC have been examined (*15*). It was found that encapsulation efficiency for DC was higher with all examined types of liposomes than for tetracycline. The highest encapsulation rates for DC were achieved using cationic or neutral liposomes (*15*). Although pH is an important factor from the aspect of OTC/DC stability, in the literature there are no data reporting on the effect of pH or the type of hydrating solution on the encapsulation efficiency of tetracycline derivatives.

Present studies report results on the encapsulation efficiency of two tetracycline derivatives – OTC and DC – in liposomes with various lipid compositions including lecithin (LEC), α-L-dipalmitoyl-phosphatidylcholine (DPPC) and cholesterol (CHOL). The effects of pH (pH 4.0-8.0), liposomal cholesterol content and type of hydrating medium (phosphate buffer, glucose or mannitol solution) on the encapsulation rate of OTC and DC are also discussed. Encapsulation efficiencies for OTC and DC are compared to each other.

2. Materials and Methods

2.1. *Preparation of liposomes*

Multilamellar vesicles (MLVs) were prepared using the thin-film hydration method. Two milligrams of α-L-dipalmitoyl-phosphatidylcholine (DPPC) or lecithin (LEC) alone, or with cholesterol (CHOL) (70/30, mol/mol) were dissolved in absolute ethanol. The mixture was dried to thin-film under nitrogen stream, and any remaining solvent was removed from the lipid film in vacuum. The samples were stored in a desiccator overnight. Thin lipid films were hydrated above the main-transition temperature of DPPC, at ~50°C. One mL of the solution of oxytetracycline hydrochloride (OTC) (MP Biomedicals Inc.) or doxycycline hydrochloride (DC) (MP Biomedicals Inc.) at a concentration of 0.1354 mg/mL was used for thin film hydration. The final lipid concentration was 2 mg/mL. The tested hydrating solutions, such as isotonic phosphate buffers (pH 6.0, 7.0, and 8.0) or glucose solutions (GLU) (5% m/m; pH 4.0, 6.0, 7.0, and 8.0) or mannitol solutions (MAN) (5% m/m; pH 4.0, 6.0, 7.0, and 8.0) were always freshly prepared. The isotonic phosphate buffer was prepared from Na_2HPO_4 and KH_2PO_4 and NaCl (Mallinckrodt Baker Inc.). The osmolarity of the solutions were measured by Osmomat 030-D osmometer (Medizintechnik Matel GmbH., Austria). The pH values of the GLU and MAN solutions were adjusted with diluted solutions of NaOH or HCl. The lipid/drug molar ratio for liposomes encapsulating OTC or DC was approximately 10:1. Control liposomes were hydrated with the appropriate solution without drug molecules. All lipid and cholesterol components were used without purification. All reagents were purchased from Sigma-Aldrich (St Louis, MO, USA) unless otherwise indicated.

2.2. *Measurement of encapsulation efficiency*

Freshly prepared OTC-, or DC-liposomes (400 μL) were centrifuged with a Galaxy 16 DH eppendorf centrifuge (2 × 10 min, 13,000 × g) through Nanosep 10K Omega Centrifugal Filter Devices (PALL Life Science Inc.) with a cut-off value of 10 kDa. The concentrations of OTC or DC in the filtrate, -representing the amounts of OTC or DC not encapsulated within the liposomes-, were determined by spectrophotometry (Genesys 10UV, Thermo Electron Corporation, NY, USA). (Wavelenghs: 354 nm for solutions with a pH between 4.0 and 6.0; 361 nm for solutions with a pH of 7.0; and 369 nm for solutions with a pH of 8.0 for OTC and 347 nm for solutions with a pH between 4.0 and 6.0; 348 nm for solutions with a pH of 7.0 and 8.0 for DC). The absorbance values for hydrating solutions containing OTC or DC (0.1354 mg/mL) served as 100% for the encapsulation efficiency determinations. Each liposomal samples were measured at least in 3 replicates, and mean and standard deviation (S.D.) values were calculated. For data analysis statistical *t*-test were used with a significance level of 0.05.

2.3. *Zeta-potential measurements*

The zeta-potentials of liposomes were determined at a lipid concentration of 0.5 mg/mL. Samples were diluted with the appropriate freshly filtrated (0.2 μm pore size, Corning, Corning Incorporated, Germany) solution before the measurement. The measurements were carried out with a Zetasizer 3000 HSA (Malvern Instruments, UK).

3. Results and Discussion

According to the special requirements for the ophthalmic and parenteral formulations, our liposomal dosage forms satisfy the criteria of osmolarity and pH as previously described (*16*). The pH of the GLU and MAN solutions were adjusted to the required value (pH 4.0, 6.0, 7.0, and 8.0) after addition of OTC or DC. The presence of lipids does not have a significant impact on the pH value of the hydrating solution. The osmolarity of the samples hydrated with phosphate buffer (pH 6.0, 7.0, and 8.0) were approximately 310 mmol/L.

The GLU and MAN solutions are widely used and accepted in pharmaceutical formulations and both are well tolerated by the patients. The concentration of the antibiotics, OTC or DC, in our liposomal formulations (0.1354 mg/mL), was within the recommended range used in the antimicrobial therapy. For the sake of comparison, the marketed product ("Doxycycline for Injection USP" supplied as a sterile lyophilized powder) must be diluted with the given solvent(s) in order to provide a final DC concentration between 0.1 to 1.0 mg/mL (*17*).

Although for intravenous applications small unilamellar vesicles (SUVs) (diameter < 100 nm) are recommended, for these studies only multilamellar vesicles (MLVs) were prepared and evaluated. Our preliminary experiments with MLVs and SUVs showed only a slight (1-5%) difference in the encapsulation rates for the same drug molecule under identical conditions. Thus, on the basis of the data determined for MLVs one can have expectations how to formulate SUVs for the purpose of intravenous applications with relative high encapsulation efficiency.

There was no pH-dependent difference in the encapsulation efficiencies for OTC in phosphate buffer in both liposomal types (LEC, DPPC) when determined at pH 6.0 and 7.0 (Table 1). However, at pH 8.0 the encapsulation efficiencies were significantly different ($p < 0.05$), from that measured at lower pH values (pH 6.0 and 7.0) for the same liposomal composition. This finding is consistent with the reported pK_a values of 3.6, 7.5, and 9.4 for OTC (*18*). On the basis of these pK_a values, OTC molecules are positively charged at pH values below 3.6, they are present in zwitterionic form in the pH range of 3.6 to 7.5, and they bear one negative charge in the pH range of 7.5 to 9.4, while they have two negative charges at higher pH values than 9.4. At the pH-values examined in our study OTC is in zwitterionic form at pH 6.0 and 7.0, while it bears a negative charge at pH 8.0. The encapsulation efficiency seems to depend on the molecular form of the OTC. Interestingly, LEC encapsulates more, while DPPC less from the negative form of OTC than from the zwitterionic OTC molecules. It is important to remark, that according to our surface potential measurements the surface charge of OTC-free LEC and OTC-free DPPC liposomes in phosphate buffer is neutral or slightly positive (~0-4 mV). The value of the surface potential measured does not significantly depend on the pH value of the hydrating phosphate buffer (*19*). Thus, the difference observed between the encapsulation rates at higher (pH 8.0) and lower pH-values (pH 6.0 and 7.0) may be explained with the different membrane rigidities observed for LEC and DPPC. The membrane rigidity is an important factor: according to our measuring method, the term and value of the encapsulation efficiency reflect not only the amount of drug enclosed into the inner aqueous volume of liposomal vesicles but also the amount of drug molecules that is more or less immersed into the liposomal bilayer(s) or weakly bounded to it. Excluding the special molecular interactions between lipids and OTC/DC, the amount of encapsulated OTC/DC would be proportional to the internal volume of the MLVs. According to our earlier electron paramagnetic resonance (EPR) spectroscopy measurements with MLVs (lipid concentration of 2 mg/mL), the inner/encapsulated volume of liposomes is approximately 2% of the total sample volume (*19*). The encapsulation efficiency data for the OTC- and DC-liposomes (data varying between 7.8% and 52.5%) (Tables 1-4) proved the accumulation of OTC/DC in the liposomes: a) in the lipid bilayer, b) attached to the liposomal surface with weak molecular interactions or c) in the inner aqueous volume.

The presence of CHOL has only a slight impact on the encapsulation rate determined for OTC containing LEC liposomes (Table 1). The results with the mixed liposomes (DPPC/CHOL 70/30 and LEC/CHOL 70/30) can be explained by the recognized feature of CHOL making the fluid bilayers more rigid and the rigid membranes (such as DPPC) more fluid. It is expected that the presence of CHOL in DPPC in 30% (mol/mol) concentration – at room temperature that is below the phase transition temperature of DPPC – makes the DPPC bilayer more fluid, similarly to the fluidity of LEC liposomes with unsaturated chains. Therefore, the addition of CHOL to DPPC results in a liposomal membrane that is more similar to the LEC liposomal membrane from the aspect of membrane fluidity and encapsulation efficiency, too.

For DC in phosphate buffers LEC showed higher encapsulation efficiencies than DPPC – at each pH value examined (Table 2). It can be supposed that the fluid structure of LEC allows DC to immerse (deeper) to the liposomal membrane.

Table 1. Encapsulation efficiency values for OTC in phosphate buffers with various lipid composition and various pH values (pH 6.0, 7.0, and 8.0)

pH	OTC encapsulation efficiency (%) (mean ± S.D.)			
	LEC	DPPC	LEC/CHOL 70/30	DPPC/CHOL 70/30
6.0	14.94 ± 3.34	23.23 ± 1.19	12.40 ± 2.25	8.68 ± 4.52
7.0	14.46 ± 2.10	24.10 ± 2.67	13.27 ± 0.50	7.77 ± 5.15
8.0	20.12 ± 0.42	12.97 ± 2.48	21.26 ± 1.71	16.83 ± 3.29

Table 2. Encapsulation efficiency values for DC in phosphate buffers with various lipid composition and various pH values (pH 6.0, 7.0, and 8.0)

pH	DC encapsulation efficiency (%) (mean ± S.D.)	
	LEC	DPPC
6.0	24.30 ± 2.12	15.98 ± 2.64
7.0	17.88 ± 3.16	16.18 ± 2.63
8.0	31.96 ± 3.07	16.29 ± 1.53

Table 3. Encapsulation efficiency values for OTC in glucose 5% m/m (GLU) and mannitol 5% m/m (MAN) solutions into LEC and DPPC liposomes at various pH values (pH 4.0, 6.0, 7.0, and 8.0)

pH	OTC encapsulation efficiency (%) (mean ± S.D.)			
	LEC GLU	DPPC GLU	LEC MAN	DPPC MAN
4.0	11.38 ± 1.12	20.99 ± 2.98	16.27 ± 0.10	24.09 ± 0.65
6.0	13.78 ± 5.88	16.98 ± 1.44	10.45 ± 4.32	10.45 ± 4.32
7.0	10.23 ± 1.62	8.06 ± 0.36	16.19 ± 3.44	17.94 ± 2.76
8.0	38.18 ± 3.31	38.72 ± 0.78	15.43 ± 1.54	12.99 ± 5.05

Table 4. Encapsulation efficiency values for DC in glucose 5% m/m (GLU) and mannitol 5% m/m (MAN) solutions into LEC and DPPC liposomes at various pH values (pH 4.0, 6.0, 7.0, and 8.0)

pH	OTC encapsulation efficiency (%) (mean ± S.D.)			
	LEC GLU	DPPC GLU	LEC MAN	DPPC MAN
4.0	46.16 ± 2.06	23.85 ± 6.17	52.18 ± 0.38	44.54 ± 1.06
6.0	42.10 ± 2.10	24.28 ± 0.30	48.72 ± 3.81	39.47 ± 7.27
7.0	38.21 ± 6.89	28.31 ± 4.79	38.20 ± 8.27	30.36 ± 1.92
8.0	42.84 ± 1.14	21.20 ± 3.95	44.83 ± 2.93	52.47 ± 6.45

OTC and DC differ in their chemical structures only by a hydroxyl group (Figure 1), thus OTC possesses more hydrophilic character than DC. According to our earlier experience, small changes in the chemical structure (*e.g.* the introduction of a methoxy group, similarly to the introduction of a hydroxyl group in our case) can result in pronounced alterations in the molecular interactions between the drug and liposomal membranes, thus influencing the encapsulation rates (*20*). It can give explanation to the fact, that DC can be more successfully encapsulated into LEC liposomes at each examined pH value. Similarly, as observed for OTC, in LEC at pH 8.0 the encapsulation efficiency for DC is higher than at pH 6.0 and 7.0. It is in coincidence with the pK_a values of 3.4, 7.7, and 9.3 determined for DC (*21*).

Evaluating the effects of the hydrating buffers on the encapsulation efficiency it can be concluded, that the use of non-ionic hydrating solutions (GLU or MAN) results in higher encapsulation efficiencies than the use of ionic phosphate buffers. In phosphate buffer the highest encapsulation efficiencies of 24.10 ± 2.67% and 31.96 ± 3.07%, while in the non-ionic solutions the encapsulation efficiency values of 38.72 ± 0.78% and 52.47 ± 6.45% for OTC and DC were achieved, respectively (Tables 1-4). It was also observed that employing the non-ionic hydrating solutions, (GLU or MAN), instead of the ionic phosphate buffer can lead to a significant increase in the OTC/DC encapsulation efficiency. When GLU or MAN is used instead of phosphate buffer, the encapsulation efficiency can be enhanced by approximately two to three times in case of the LEC and DPPC liposomes (Tables 1-4). The ions of the phosphate buffer and the non-dissociated molecules of the non-ionic hydrating solutions (*e.g.* GLU or MAN) behave in different ways in the environment of liposomal membranes, thus influencing the molecular interactions between drugs and lipids in a different manner. While the charged ions can "cover" and "mask" the original charge/surface potential of liposomal membranes, the GLU or MAN molecules do not have a significant impact on it. The ionic hydrating solutions do not allow to manifest the original surface charge of the bilayers and can influence the molecular interactions between the drug and lipid molecules. However, in GLU and MAN the original surface charge of the MLVs – through weak electrostatic interactions – can have a contribution to the relative high encapsulation efficiency for OTC/DC (*19*).

When comparing the encapsulation rates for OTC and DC in non-ionic hydrating solutions, it can be stated that DC can be encapsulated more effectively than OTC (Tables 3 and 4). The observation is possibly due to the difference in the chemical structures of the two antibiotics (Figure 1) as explained above.

On the basis of the results it can be stated that LEC encapsulates more – or at least not significantly less- DC than does DPPC at all pH values examined, and in all kinds of hydrating solutions used (phosphate buffer, GLU or MAN) (Tables 2 and 4). When considering the LEC liposomes, a wide variety of fatty acid chains (saturated and unsaturated, with various chain lengths) can ensure the encapsulation of DC molecules, leading to higher encapsulation rates. These data demonstrate that the liposomal encapsulation of DC requires LEC as the optimal lipid constituent.

When designing the parameters for encapsulating the drugs OTC/DC, care must be taken to choose the right pH value. It is known that the tetracyclines (such as OTC and DC) are more stable in the acidic pH range than at higher pH values (*2-5*). Thus a lower (acidic) pH seems to be the optimal choice. Taking both aspects (high encapsulation efficiency and higher drug stability at lower pH) into consideration, the liposomal compositions denoted with shadow in Tables 1-4 are recommended for the preparation of an optimal OTC/DC containing liposomal dosage form.

4. Conclusion

Based on our encapsulation efficiency studies and on the drug stability considerations it can be concluded that for OTC/DC encapsulation the use of non-ionic solutions (*e.g.* GLU) is the most promising. However, the use of GLU or MAN for hydration may increase the possibility of microbiological contamination of the liposomal preparations during storage. Therefore, it is necessary to lyophilize the liposomal samples for storage, and to rehydrate the samples before their therapeutic use. In consistence with the different

membrane rigidity values for LEC *vs*. DPPC the rate of the drug release for OTC and DC is also expected to be different in case of LEC and DPPC (*22*,*23*).

Acknowledgements

These studies were supported in part by research funds from the National Institute of Health (Grant #: 5 U01 NS058035-02) and the Robert A. Welch Foundation (x-0011) at Sam Houston State University.

References

1. Tally FT, Ellestad GA, Testa RT. Glycocyclines: a new generation of tetracyclines. J Antimicrob Chemother. 1995; 35:449-452.
2. Adriana MD, Michael KS. The kinetic of oxytetracycline degradation in deionized water under varying temperature, pH, light, substrate, and organic matter. J Aquat Anim Health. 2000; 12:246-253.
3. Injak R, Djordjevic-Milic V, Srdjenovic B. Thermostability testing and degradation profiles of doxycycline in bulk, tablets, and capsules by HPLC. J Chrom Sci. 2007; 45:623-628.
4. Liang Y, Denton MB, Bates RB. Stability studies of tetracycline in methanol solution. J Chromatogr A. 1998; 827:45-55.
5. Mitscher LA. The chemistry of the tetracycline antibiotics; Medical Research Series, Vol. 9. Marcel Dekker, New York, NY, USA, 1978.
6. Bartholomew RS, Reid BJ, Cheesbrough MJ, Macdonald M, Galloway NR. Oxytetracycline in the treatment of ocular rosacea: a double-blind trial. Br J Ophthalmol. 1982; 66:386-388.
7. Brussieux J, Boisivon A, Théron HP, Faidherbe C, Machado N, Michelon B. Prevention of neonatal conjunctivitis. A comparative clinical and bacteriologic study of 2 eyedrops: silver nitrate and oxytetracycline chlorhydrate. Ann Pediatr. 1991; 38:637-641.
8. Guzey M, Aslan G, Ozardali I, Basar E, Satici A, Karadede S. Three-day course of oral azithromycin *vs* topical oxytetracycline/polymyxin in treatment of active endemic trachoma. Jpn J Ophthalmol. 2000; 44:387-391.
9. Eastmann TG, George LW, Hird DW, Thurmond MC. Combined parenteral and oral administration of oxytetracycline for control of infectious bovine keratoconjunctivitis. J Am Vet Assoc. 1998; 212:560-563.
10. Ward DA, Clark ES. Ocular pharmacology. Vet Clin North Am Food Anim Pract. 1991; 7:779-791.
11. Izer K, Török I, Pintér-Magyar G. Stability of oxytetracycline in eye-drops, prepared in pharmacies. Acta Pharm Hung. 1994; 64:63-66.
12. Sah H. Degradation patterns of tetracycline antibiotics in reverse micelles and water. Biomed Chromatogr. 2006; 20:1142-1149.
13. Okerman L, Van Hende J, De Zutter L. Stability of frozen stock solutions of beta-lactam antibiotics, cephalosporins, tetracyclines and quinolones used in antibiotic residue screening and antibiotic suspectibility testing. Anal Chim Acta. 2007; 586:284-288.
14. Pinto-Alphandary H, Andremont A, Couvreur P. Targeted delivery of antibiotics using liposomes and nanoparticles: research and applications. Int J Antimicrob Agents. 2000; 13:155-168.
15. Sangaré L, Morisset R, Omri A, Ravaoarinoro M. Incorporation rates, stabilities, cytotoxicities and release of liposomal tetracycline and doxycycline in human serum. J Antimicrob Chemother. 1998; 42:831-834.
16. Völker-Dieben HJ, Kok-Van Alphen CC, Hollander CC, Kruit PJ, Batenburg K. The palliative treatment of the dry eye. Documental Ophthalmol. 1987; 67:221-227.
17. Bedford Laboratories. Doxycycline for injection. 2004; USP, DIV-DCY-P03.
18. Tavares MFM, McGuffin VL. Separation and characterization of tetracycline antibiotics by capillary electrophoresis. J Chromatogr A. 1994; 686:129-142.
19. Budai M, Szabó Z, Zimmer A, Szögyi M, Gróf P. Studies on molecular interactions between nalidixic acid and liposomes. Int J Pharm. 2004; 279:67-79.
20. Budai M, Szabó Z, Szogyi M, Gróf P. Molecular interactions between DPPC and morphine derivatives: a DSC and EPR study. Int J Pharm. 2003; 250:239-250.
21. Sun XX, Aboul-Enein HY. Internal solid contact sensor for the determination of doxycycline hydrochloride in pharmaceutical formulation. Talanta. 2002; 58:387-396.
22. Anderson M, Omri A. The effect of different lipid components on the *in vitro* stability and release kinetics of liposome formulations. Drug Deliv. 2004; 11:33-39.
23. Budai L, Hajdú M, Budai M, Gróf P, Béni S, Noszál B, Klebovich I, Antal I. Gels and liposomes in optimized ocular drug delivery: Studies on ciprofloxacin formulations. Int J Pharm. 2007; 343:34-40.

(*Received January 23, 2009; Accepted February 9, 2009*)