

FORMULATION AND EVALUATION OF OSELTAMIVIR PHOSPHATE CAPSULES

P. PANDIAN, K. KANNAN*, M. MANIKANDAN, R. MANAVALAN

Department of Pharmacy, Annamalai University, Annamalai Nagar 608002, Tamil Nadu. *Email: egkkannan@yahoo.co.in

Received: 04 Aug 2012, Revised and Accepted: 11 Sep 2012

ABSTRACT

Osetamivir is an antiviral drug, a neuraminidase inhibitor used in the treatment and prophylaxis of both influenza A and influenza B. The objective of this research was to formulate and evaluate the Osetamivir phosphate capsules. The Osetamivir capsules were prepared by using various excipients namely, Pregelatinised Starch, Croscarmellose sodium, Povidone K-30, Talc and Sodium stearyl fumerate. Drug excipients compatibility studies were conducted to select the most appropriate excipients. Based on the preliminary studies, various formulation trials were carried out with different concentrations of disintegrants and lubricants. The formulation was carried out by both dry granulation and wet granulation technique. The capsule filling was done by manual capsule filling machine. Among all the formulation, formulation F5 showed satisfactory results with various physicochemical evaluation parameters like disintegration time, dissolution profile and assay when compared with that of the marketed product. The *in vitro* release profiles of drug could be best expressed by Korsmeyer - peppas equation as the plots showed high linearity ($R^2 = 0.975$). The stability studies show that the capsules were found to be stable. The present study was to develop a pharmaceutically equivalent, low cost, quality improved and stable formulation of Osetamivir Phosphate capsules.

Keywords: Osetamivir phosphate, Capsules, Anti viral drug, Dry granulation.

INTRODUCTION

Oral route of drug administration is perhaps the most appealing route for the delivery of drugs^{1,2}. The capsule is probably the most versatile of all dosage forms. The word 'capsule' in the English language is derived from the Latin word 'capsula', which means a small box or container³. The administration of liquid and solid drugs enclosed in soft and hard gelatin capsules is one of the most frequently utilized dosage forms. Capsules are solid dosage form in which medicinal agents and/or inert substances are enclosed in a small shell.

Osetamivir phosphate is a white crystalline solid with the chemical name (3R,4R,5S)-4-acetylamino-5-amino-3-(1-ethylpropoxy)-1-cyclohexene-1-carboxylic acid, ethyl ester, phosphate (1:1). The chemical formula is $C_{16}H_{28}N_2O_4$ (free base)⁴. The molecular weight is 312.4 for Osetamivir free base and 410.4 for Osetamivir phosphate salt and its chemical structure given in figure 1.

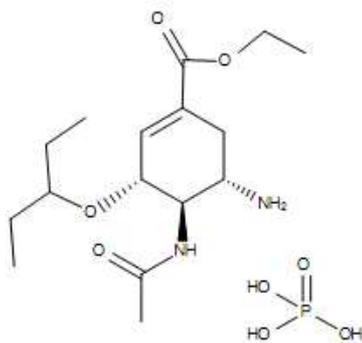


Fig. 1: It shows the structure of Osetamivir phosphate

Osetamivir Phosphate (OP) is an ester prodrug which is the first orally available inhibitor of influenza virus neuraminidase, an enzyme involved in the release of new virus particles from infected cells^{5,6}. It is used in the treatment and prophylaxis of both influenza A and influenza B⁷. Osetamivir is an antiviral drug, slows the spread of influenza (flu) virus between cells in the body by stopping the virus from chemically cutting ties with its host cell^{8,9}. The prodrug Osetamivir is itself not virally effective, however, once in the liver, it is converted by natural chemical processes, hydrolysed hepatically to its active metabolite, the free carboxylate of Osetamivir (GS4071)⁹.

The present investigation applied a systemic approach to formulate and evaluate a pharmaceutically equivalent, low cost, quality improved and stable formulation of Osetamivir Phosphate capsule dosage form.

MATERIALS AND METHODS

Osetamivir Phosphate was procured as gift sample from Cipla Pharmaceuticals Ltd (India). Pregelatinised Starch (Colorcorn Asia Pvt Ltd), Povidone K-30, Croscarmellose sodium and Talc (Signat Chemical Corporation) and Sodium stearyl fumerate (J.R.S Pharma) were commercially procured and used for this study.

Drug Excipients Compatibility Studies

Drug-excipients compatibility studies lay the foundation for designing a chemically stable formulation for clinical and commercial development. Drug excipients compatibility studies are conducted to select the most appropriate excipients. Active ingredient was mixed with all excipients in binary ratio and small portion of this mixed powder was placed in clean and dry vial in stability chamber at $40^\circ\text{C} \pm 2^\circ\text{C} / 75\% \pm 5\% \text{RH}$ and $25^\circ\text{C} \pm 2^\circ\text{C} / 60\% \pm 5\% \text{RH}$ for 4 weeks.

Table 1: Table shows the formulation composition of Osetamivir phosphate granules

Ingredients (mg)	Formulation Code				
	F1	F2	F3	F4	F5
Osetamivir phosphate	98.5	98.5	98.5	98.5	98.5
Microcrystalline cellulose(101)	40	38	-	-	-
Pregelatinised starch	-	-	49	48.4	45.5
Croscarmellose sodium	7.5	7.5	7.5	8.5	10
Povodone K-30	8	10	3	3	3
Sodium stearyl fumerate	3	3	1.5	1.5	1.5
Talc	3	3	1.5	1.5	1.5
Purified water	q.s	q.s	-	-	-

Manufacturing process of Oseltamivir Phosphate granules

Oseltamivir Phosphate granules were prepared by employing various excipients as mentioned in the Table 1. Weighed quantity of Oseltamivir phosphate, Pregelatinized starch, crosscarmellose sodium, povidone K-30 were sifted through #30 mesh and mixed for 10 mins in hexagonal blender. Sodium stearyl fumarate and talc sifted through #40 mesh were loaded to the blender along with the above sifted materials and mixed well for 2 mins in slow speed. The finally obtained dry blends are filled into size 2 capsules by manual capsule filling machine.

Characterization of Blend ^{10,11,12,13}

The blend was evaluated for their characteristics parameters such as Bulk density, Tapped density, Carr's index, Hausner's ratio and Angle of repose.

Bulk Density ¹⁰⁻¹³

Bulk density is determined by pouring the blend into a graduated cylinder via a large funnel and measuring the weight and volume obtained by the blend without tapping and calculated using the following formula

$$\text{Bulk Density} = \frac{\text{Weight of the Blend}}{\text{Bulk Volume of the Blend}}$$

Tapped Density ^{10,11,12,13}

Tapped density is determined by placing a graduated cylinder containing known mass of blends on a mechanical tapped apparatus, which is operated for a fixed number of taps until the powder bed volume has reached a minimum volume. Using the weight of the

drug in the cylinder and minimum volume, the tapped density was computed using the following formula

$$\text{Tapped Density} = \frac{\text{Weight of Blend}}{\text{Tapped Volume of Blends}}$$

Carr's Index ^{10,11,12,13}

Carr's Index was measured using the values of the bulk density and tapped density. The following equation is used to find the Carr's index

$$\text{CI} = \frac{(\text{TD} - \text{BD})}{\text{BD}} \times 100$$

Where, TD= Tapped Density, BD= Bulk Density

Angle of Repose ^{10,11,12,13}

Weighed quantity of granules was passed through a funnel kept at a height of 2cm from the base. The powder is passed till it forms heap and touches the tip of funnel. The radius was measured and angle of repose was calculated using the following formula

$$\tan \theta = \frac{h}{r}$$

Where h = Height of the heap, R = Radius of the heap.

Hausner's Ratio ^{10,11,12,13}

Hausner's ratio was determined by the ratio between the tapped density to that of the bulk density.

$$\text{Hausner's ratio} = \frac{\text{TD}}{\text{BD}}$$

Where, TD = Tapped Density, BD= Bulk Density

Table 2: Table shows Carr's Index, Hausner's Ratio, Angle of repose with corresponding Flow characters.

Type of flow	Carr's index	Angle of repose (degrees)	Hausner's ratio
Excellent	≤10	25-30	1.00-1.11
Good	11-15	31-35	1.12-1.18
Fair	16-20	36-40	1.19-1.25
Passable	21-25	41-45	1.26-1.34
Poor	26-31	46-55	1.35-1.45
Very poor	32-37	56-65	1.46-1.59
Very Very poor	>38	>66	>1.60

Evaluation of capsules

Weight variation test ¹⁴

Ten capsules were individually weighed and the contents were removed. The emptied capsules were individually weighed and the net weight of the contents was calculated by subtraction and the percent weight variation was calculated by using the following formula

$$\text{Weight variation} = \frac{(\text{Weight of capsule} - \text{Average weight}) \times 100}{\text{Average weight of capsules}}$$

Weight variation should not be more than 7.5 %.

Lock length ¹⁴

Ten individual capsules were taken from formulation trial batch and lock length was measured manually by using vernier calipers and average of ten capsules was noted.

Disintegration ¹⁵

The capsules were placed in the basket rack assembly, which is repeatedly immersed 30 times per minute into a thermostatically controlled fluid at 37°C. To fully satisfy the test, the capsules should disintegrate completely into a soft mass having no palpably firm core without any fragments of the gelatin shell. If one or two capsules fail, the test should be repeated on additional of 12 capsules. Then, not fewer than 16 of the total 18 capsules tested should disintegrate completely.

Dissolution studies ^{16,17}

Dissolution is a process by which the disintegrated solid solute converted into solution. The test determines the time required for a definite percentage of the drug in capsules to dissolve under specified conditions.

The release of Oseltamivir phosphate was determined using a dissolution apparatus of USP Type II (paddle) at 50 rpm. 900ml of 0.1N hydrochloric solution acid was used as the dissolution medium and were maintained at the temperature of 37.5±0.5°C. A sinker was used to avoid capsule flotation ¹⁴. The samples were drawn at 5, 10, 15 30 and 45 mins and equal amount of fresh medium were replaced to maintain the sink conditions. Samples withdrawn were analyzed to determine the percentage of drug released.

Kinetics of drugs release ^{2,18,19}

Kinetics of drug release is studied by plotting the data obtained from *in vitro* release in various kinetics models.

Zero Order Kinetics

The graph was plotted as cumulative % drug release Vs Time where the drug release rate is independent of its concentration ²⁰.

$$C = K_0t$$

Where, K_0 = Zero order rate constant expressed in units of concentration/time

t = Time in hours.

First order Kinetic model

The graph was plotted as log cumulative % of drug remaining Vs Time, where release rate is concentration dependent ²¹

$$\text{Log } C = \log C_0 - Kt / 2.3030$$

Where, C₀ = Initial concentration of drug

K = First order constant

t = Time in hours.

Higuchi kinetics

Higuchi describes the release of drugs from insoluble matrix as a square root of time dependent process based on Fickian diffusion. The graph was plotted as cumulative % drug released Vs square root of time ²².

$$Q = Kt^{1/2}$$

Where, K = Constant reflection design variable system

t^{1/2} = Time in hours.

Hence, drug release rate is proportional to the reciprocal of square root of time. If the plot yields a straight line, and the slope is one then the particular dosage form is considered to follow Higuchi kinetics of drug release.

Hixson-crowell erosion equation

It describes the drug release with changes in the surface area and the diameter of particles the data were plotted using the Hixson and crowell rate equation. The graph was plotted by cube root of % drug remaining in matrix Vs time ²³.

$$Q_0^{1/3} - Q_t^{1/3} = KHC t$$

Where, Q_t = Amount of drug released in time t

Q₀ = Initial amount of drug in tablet.

KHC = Rate constant for Hixson crowell rate equation

Korsmeyer-Peppas equation

To find out the mechanism of drug release, it was further plotted in peppas equation as log cumulative % of drug released Vs log time ^{24, 25}.

$$M_t / M_\infty = Kt^n$$

$$\text{Log } M_t / M_\infty = \log K + n \log t$$

Where, M_t / M_∞ = Fraction of drug released at time t

K = Kinetic rate constant

t = Release time

n = Diffusion exponent indicative of the mechanism drug release.

This model is used to analyze the release of pharmaceutical polymeric dosage forms when the release mechanism is not known or more than one type of release phenomenon was involved. The n value could be obtained from slope of the plot of log cumulative % of drug released Vs log Time.

Table 3: Table shows Diffusion exponent and solute release mechanism for cylindrical shape

Diffusion exponent (n)	Overall solute diffusion mechanism
0.45	Fickian diffusion
0.45 < n < 0.89	Anomalous (non-fickian) diffusion
0.89	Case-II transport
n > 0.89	Super case-II transport

Stability Studies²

Stability of the drug has been defined as the ability of particular formulations, in a specific container, to remain within its physical, chemical, therapeutic and toxicological specification. The purpose of stability testing is to provide evidence on how the quality of a drug substance or drug product varies with time under the influence of a variety of environmental factors, such as temperature, humidity etc.

The storage conditions for stability studies were accelerated condition (40±2°C / 75±5%RH) and Long term condition (25±2°C / 60±5%RH). The capsules were packed as 30's count in HDPE containers, induction sealed with adsorbent cotton ²⁶⁻²⁹.

RESULTS AND DISCUSSION**Drug - excipients compatibility studies**

The physical compatibility test and assay test between the drug and capsule components was carried out at 40°C ± 2°C / 75% ± 5% RH and 25°C ± 2°C / 60% ± 5% RH for 4 weeks. The mixture does not show any visible change, thus indicating drug and other capsule components do not have any physical compatibility. Hence, there are no interactions between the drug, polymers and other excipients used in the capsules.

Evaluation of capsules**Weight variation**

The average weight of capsules within each formulation was found to be uniform. This indicates uniform filling of powder blend during capsule filling. Not more than two of the individual weights deviated from the average weight by more than 7.5% and none deviated by more than twice that percentage, which provided good weight uniformity.

Table 4: Table shows the weight variation, locking length and disintegration time of formulations

Formulation	Weight Variation (mg)	Locking Length (mm)	Disintegration Time (Mins)
F1	210	17.6	20
F2	218.4	18	17
F3	223.2	18	15
F4	223.9	18	14
F5	225.1	18	11

Table 5: Table shows the *In vitro* dissolution profile of Oseltamivir phosphate capsules (F1 to F5)

S. No.	Time (Mins)	Formulation Trials				
		F1	F2	F3	F4	F5
1	05	65.4	65.4	68.2	70.2	78.6
2	10	76.3	74.9	76.0	82.5	96.4
3	20	82.8	80.1	86.8	87.4	98.2
4	30	84.5	82.6	92.2	94.7	99.7

DISCUSSION

Formulation F1 was carried out by using Microcrystalline cellulose (101) as diluents and povidone K 30 as binder through wet granulation process, but the required fill weight of the capsule was not achieved in this trial.

In formulation F2, the capsule fill weight was achieved by altering the concentration of diluents and binder but the assay and dissolution values were found to be very low.

The manufacturing technique was changed in formulation F3, to dry granulation instead of wet granulation technique. Pregelatinized

starch was used as diluents instead of microcrystalline cellulose. Dissolution and Assay values obtained in this technique were found to be better when compared to that of the wet granulation technique, but does not match with the marketed product.

In formulation F4, the percentage of the croscarmellose sodium was increased. Dissolution and Assay values were greater than the previous formulation but not match with that of marketed product.

Finally, the percentage of the croscarmellose sodium was again increased in formulation F5. In this formulation, Dissolution and Assay values were greater than the previous formulation and shows better results when compared to marketed product.

Release kinetics study for formulation F5

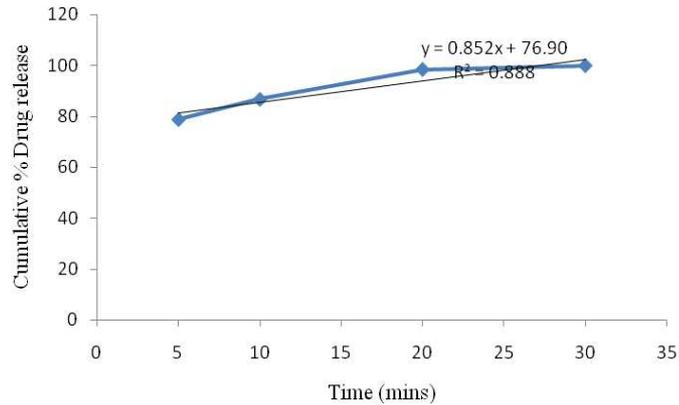


Fig. 2: It shows the Zero order release model of Oseltamivir phosphate formulation (F - 5)

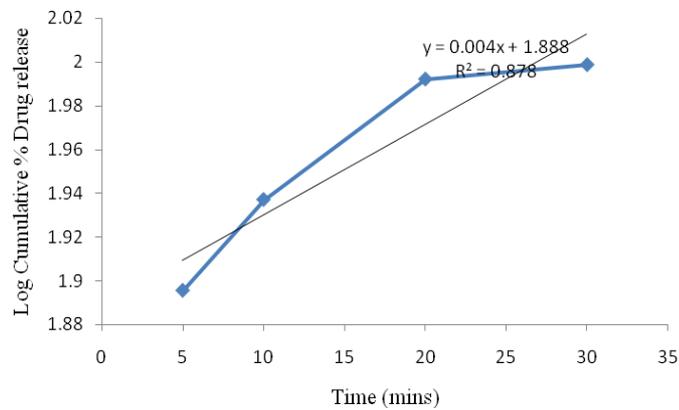


Fig. 3: It shows the First order release model of Oseltamivir phosphate formulation (F - 5)

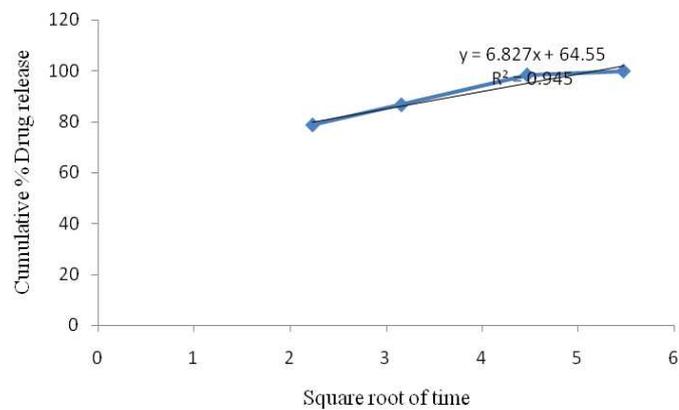


Fig. 4: It shows the Higuchi release model of Oseltamivir phosphate formulation (F - 5)

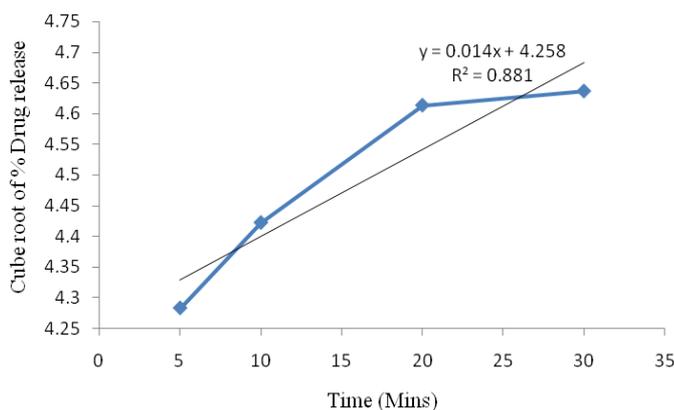


Fig. 5: It shows the Hixson-Crowell cube root plots of Oseltamivir phosphate formulation (F - 5)

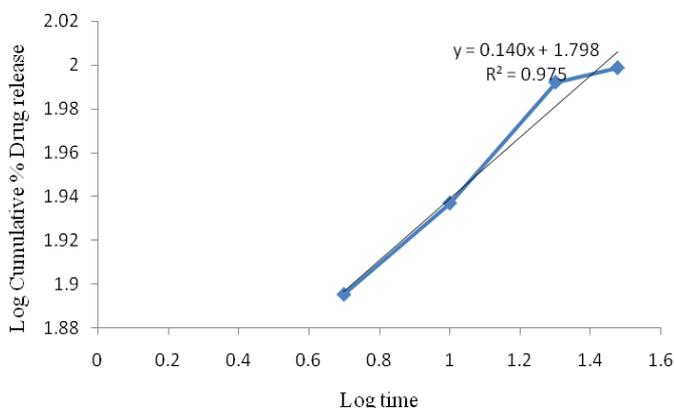


Fig. 6: It shows the Korsmeyer - Peppas plots of Oseltamivir phosphate formulation (F - 5)

Table 6: Table shows the stability studies for F5 Oseltamivir phosphate capsules.

Test	Stability Results					
	40±2°C / 75±5%RH			25±2°C / 60±5%RH		
	1 st month	2 nd month	3 rd month	1 st month	2 nd month	3 rd month
Description	*	*	*	*	*	*
Moisture content (% W/W)	2.50±0.03	3.23±0.02	4.38±0.03	2.81±0.01	3.45±0.04	4.13±0.02
Disintegration time (Mins)	11±1	12±2	10±1	10±3	11±2	9±1
Assay (%)	101.90±1.03	100.60±1.97	99.09±2.01	102.2±1.12	102.6±2.32	99.10±1.45
Dissolution	98.9±0.98	103.6±2.65	98.2±1.92	105.6±1.34	102.3±2.53	99.5±1.45

* Carmel/pink coloured size 2 capsules containing white to off white granular powder

Stability Studies

Stability studies were conducted for the optimized formulation F5. The stability study was performed at 40±2°C / 75±5%RH and 25±2°C / 60±5%RH for a specific time period. The capsules were analyzed for appearance, water content, drug content and *in vitro* drug release. The overall results showed that the formulation is stable at above mentioned storage conditions shown in table 6.

CONCLUSION

The objective of the present research work was to formulate and evaluate the Oseltamivir phosphate capsules. Oseltamivir phosphate is widely used as Anti-viral agent. They are formulated as Oseltamivir phosphate capsules which show better patient acceptability and compliance.

Dry granulation process was the preferred technology for the preparation of Oseltamivir phosphate capsule. Based on the preliminary studies, various formulation trials (F1-F5) were carried out with different concentrations of disintegrants, diluents. From the

various formulations it was concluded that the formulation batch of F5 was finalized as the optimized formula. The drug release kinetics of the optimized formulation correspond best to Korsmeyer-peppas model and the drug release mechanism as per n value of Korsmeyer - peppas is fickian diffusion.

Formulation F5 showed satisfactory results with various physicochemical evaluation parameters like Disintegration time, Dissolution profile, Assay when compared with that of the marketed product. The stability studies, indicates that the formulated capsules were found to be stable. Hence, it is finally concluded that, Oseltamivir phosphate capsules are pharmaceutically equivalent, low cost, quality improved and stable formulation.

REFERENCES

1. Nagaraju R, Rajesh kaza. Formulation and Evaluation of Bilayer Sustained Release Tablets of Sulbutamol and Theophylline. Int J Pharmaceut Sci and Nanotech 2009; 2 (3): 638-946.
2. Kannan K, Manikandan M, Periyasamy G, Manavalan R. Design, Development and Evaluation of Metoprolol Succinate and

- Hydrochlorothiazide Bilayer Tablets. *J Pharm Sci and Res* 2012; 4 (3): 1827 – 1835.
3. Podczeczek F, Jones BE. In: Jones BE. *Pharmaceutical Capsules 2nd ed.* London: Pharmaceutical Press; 2004. p 1, 2, 9, 17, 23, 91, 242, 449.
 4. Ashish Ashok Thatte, Pramila T. Simple Extractive Colorimetric Determination Of Oseltamivir Phosphate by Ion-Pair Complexation Method In Bulk And Capsule Dosage Form. *Int J Res in Pharmaceut and Biomed Sci* 2011; 2 (2): 543-547.
 5. De Jong D, Tran T, Truong K, Vo H, Smith J, Nguyen C, Bach C, Phan Q. Oseltamivir resistance during treatment of influenza A (H5N1) infection. *The New Eng J of Med* 2005; 353 (25): 2667–2672.
 6. Tappenden P, Jackson R, Cooper K, Rees A, Simpson E, Read R, Nicholson K. Amantadine, oseltamivir and zanamivir for the prophylaxis of influenza (including a review of existing guidance no. 67): a systematic review and economic evaluation. *Health Technol Assess.* 2009; 13: 11.
 7. Hsieh HP, Hsu JTA. Strategies of development of antiviral agents directed against influenza virus replication. *Current Pharm Design.* 2007; 13: 3531-3542.
 8. Li W, Escarpe PA, Eisenberg EJ, Cundy KC, Sweet C, Jakerman KJ, Merson J, Lew W, Williams M, Zhang L, Kim CU, Dischofberger N, Chen MS, Mendel DB. Identification of GS 4104 as an Orally Bioavailable Prodrug of the Influenza Virus Neuraminidase Inhibitor GS 4071. *Antimicrobial Agents and Chemo.* 1998; 42: 647-653.
 9. Burch J, Corbett M, Stock C. Prescription of anti-influenza drugs for healthy adults: a systematic review and meta-analysis. *Lancet Infect Dis* 2009; 9 (9): 537–45.
 10. Banker GS, Anderson NR. Tablets. In: Lachman L, Lieberman HA, Kanig JL. *The theory and practice of industrial pharmacy.* 3rd ed. New Delhi: CBS publishers and distributors: 2009. P. 315.
 11. Pragnesh patel, Anupkumar Roy, Vinod kumar SM, Martand kulkarni. Formulation and evaluation of colon targeted tablets of ornidazole for the treatment of Amoebiasis. *Int J Drug Dev and Research* 2011; 3 (1): 52-61.
 12. Manikandan M, Kannan K, Thirumurugu S, Manavalan R. Design and Evaluation of Amlodipine Besilate and Atorvastatin Calcium Tablets. *Res J Pharm Bio Chem Sci* 2012; 3 (1): 425-434.
 13. Sweta K. Singh, Vijay Sharma, Kamla Pathak. Formulation and evaluation of taste masked rapid release tablets of Sumatriptan Succinate. *Int J Pharm Pharm Sci*, 2012; 4(2): 168-174.
 14. Anand Kumar M, Lakshmi PK, Balasubramaniam J. Formulation Development and In-vitro Evaluation of Tamsulosin Hcl Extended Release Pellets. *Int J Pharm Tech Research* 2011; 3 (2): 968 – 979.
 15. Savan R. Vachhani, Jatin J. Patel, Dipen Patel, Prajapati ST, Patel CN. Formulation and in-vitro evaluation of floating capsules of Loratadine. *J Chem Pharm Res*, 2010; 2(2): 57-64.
 16. Banker GS, Anderson NR. Tablets. In: Lachman L, Lieberman HA, Kanig JL. *The theory and practice of industrial pharmacy.* 3rd ed. New Delhi: CBS publishers and distributors: 2009. p. 316
 17. *Indian Pharmacopoeia.* Ministry of Health and Family Welfare. 4th ed. New Delhi: Controller of publication; 1996. A-80- A-84, p. 735-736.
 18. Harris shoab M, Jaweria tazeen, Hamid A. Merchant, Rabia ismail yousuf. Evaluation of drug release kinetics from ibuprofen matrix tablets using HPMC. *Pak J Pharm Sci*, 2006; 19 (2): 119-124.
 19. Viveksarathi K, Rajarajan R, Kannan K, Manavalan R. Dosage form design and evaluation of eperisone hydrochloride matrix film coated extended release tablets. *Int J Pharm Pharm Sci*, 2012; 4(2): 575-581.
 20. Hadjiioannou TP, Christian GD, Koupparis MA, Macheras PE. *Quantitative Calculations in Pharmaceutical Practice and Research*, New York: VCH Publishers Inc; 1993. p. 345-348.
 21. Uday S Rangole, Kawtikwar PS, Sakarkar DM. Formulation and In- Vitro Evaluation of Rapidly Disintegrating Tablets Using Hydrochlorothiazide as a Model Drug. *Research J Pharma and Tech* 2008; 1(4): 349-352.
 22. Higuchi T. Mechanism of sustained action medication. Theoretical analysis of rate of release of solid drugs dispersed in solid matrices. *J Pharm Sci*, 1963; 52: 1145-1149.
 23. Hixson AW and Crowell JH. Dependence of reaction velocity upon surface and agitation (I) theoretical consideration. *Ind Eng Chem*, 1931; 23: 923-931.
 24. Korsmeyer RW, Gurny R, Doelker E, Buri P and Peppas. NA Mechanisms of solute release from porous hydrophilic polymers. *Int J Pharm*, 1983; 15: 25-35.
 25. Korsmeyer RW, Lustig SR and Peppas NA. Solute and penetrant diffusion in swellable polymers. I. Mathematical modeling. *J Polym Sci Polym Phys Ed*, 1986a; 24: 395-408.
 26. Swamy PA, Areefulla SH, Shrisand SB, Gandra S, Prashanth B. Orodispersible tablets of meloxicam using superdisintegrant blends for improved efficiency. *Ind J Pharm Sci*, 2007; 69(6): 836-840.
 27. Malke S, Shidhaye S, Kadam VJ. Formulation and evaluation of oxcabazepine fast dissolving tablets, *Ind J Pharm Sci*, 2007; 69(2): 211-214.
 28. Patel MM, Patel DM. Fast dissolving valdecoxib tablets containing solid dispersion of valdecoxib, *Ind J Pharm Sci* 2006; 68 (2): 222-226.
 29. Vineet Bhardwaj, Mayank Bansal, Sharma PK. Formulation and Evaluation of Fast Dissolving Tablets of Amlodipine Besylate Using Different Super Disintegrants and Camphor as Sublimating Agent. *American-Eura J Sci Research* 2010; 5 (4): 264-269.