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LAMIVUDINE LOADED MICROSPHERES FOR ORAL USE: DESIGN, DEVELOPMENT AND ESTABLISHMENT OF *INVIVO-INVITRO* CORRELATION

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The objectives of the present study were to select a formulation that has an ideal *in vitro* dissolution profile and to compare the sustaining/ controlling efficacy of the selected formulation with that of the commercial conventional tablet in order to establish a good degree of *in vitro–in vivo* correlation. Lamivudine (33%, 25% w/w)–loaded microspheres were prepared by a modified solvent evaporation method. The *in vitro* release profiles obtained in 0.01N HCl as dissolution medium. The microspheres were subjected to characterization for particle size, encapsulation efficiency, loose crystal study, stability study, *in vitro* release rate profile, release kinetics and *in vivo* study in New Zealand white rabbit species. A single-dose oral bioavailability study revealed significant differences in C_{max}, T_{max}, $T_{1/2}$, K_a, K_e, MRT, MDT and AUC between the conventional tablet and optimized microsphere dosage forms. Furthermore, linear relationship obtained between the percentages dissolved and absorbed suggests a means to predict *in vivo* absorption by measuring *in vitro* dissolution. One way ANOVA followed by turkey test was applied to verify the significance of the data. Thus F1 formulation showed the best *in vivo* performance exhibiting deliberate release, which correlates well with the *in vitro* release profile of 3TC from microsphere for better management of AIDS.

Keywords : Dissolution rate, *in vivo* parameters, *in vivo-in vitro* correlations, release kinetics.

INTRODUCTION

New drug delivery technologies are revolutionizing the drug discovery, development and creating R&D focused pharmaceutical industries to increase the momentum of global advancements. In this regard novel drug delivery systems (NDDS) have many benefits, which includes improved therapy by increasing the efficacy and duration of drug activity, increased patient compliance through decreased dosing frequency and convenient routes of administration and improved site specific delivery to reduce unwanted adverse effects[1]. Lamivudine is an active anti-retroviral drug belonging to non-nucleosides reverse transcriptase inhibitor. Lamivudine treatment has gained immense popularity in the AIDS treatment in the present era. Dosage and duration of lamivudine therapy should be individualized according to requirement and response of the patient. The daily-recommended dose is 150 mg b.i.d[2,3]. The oral administration of lamivudine exhibits side effects in GIT as well as in CNS. Thrombocytopenia, parasthesias, anorexia, nausea, abdominal cramps, depressive disorders, cough and skin rashes etc have been reported as possible adverse reactions[4]. Controlled release (CR) preparations helps to achieve maximum therapeutic effect with simultaneous minimization of adverse effects. Microparticulate drug delivery posses many advantages such as high bioavailability, rapid kinetic of absorption as well as avoidance of hepatic first pass effect and improvement of patient compliance[5, 6]. Absence of sufficient work in the direction of programmed delivery of lamivudine as indicated by literature survey ignited the urge of this research venture, which includes to select a formulation that has an ideal *in vitro* dissolution profile and to compare the sustaining/controlling efficacy of the selected formulation with that of the commercial conventional tablet in order to establish a good degree of *in vitro–in vivo* correlation[7].

MATERIALS AND METHODS

Materials

Lamivudine was received as a gift sample from GlaxoSmithKline Ltd., Mumbai, India. The polymers like Acrylacoat, L30D and S100 were obtained from Corel Pharma., Ahmadabad, India. All other chemicals and solvents used were of analytical grade and procured from an authorized dealer, USP XXI paddle type dissolution apparatus, FT-IR (Shimadzu IR spectrophotometer, Model 840, Japan), UV-Visible Spectrophotometer (Model-1700, Shimadzu, Japan) and HPLC (SP2, Shimadzu, Japan) etc. instruments were employed in the current study.

Preparation of Microspheres

Four lamivudine loaded microsphere formulations using two different acrylic polymers (Acrycoat S100 (poly [ethyl acrylate methyl methacrylate], Acrycoat L30D (poly [ethyl acrylate methyl methacrylate])) were prepared in this study by solvent evaporation method cited in reference[8]. Briefly, 1gm of polymer (S 100) was dissolved in 10 ml of methanol; specified amount of lamivudine was dissolved in to it. The resultant solution was stirred at 900 rpm for

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20 min in order to complete evaporation of the organic solvent. Then the microspheres were dried in room temperature for 12 hours and dried microspheres were collected. Drug to polymer ratios were 0.5 and 0.25 for F1 and F2 respectively. The same method was adopted for L30D polymer and the formulation ratio was narrated as F3 and F4.

Animal Experiments

Twenty adult male New Zealand white species rabbits weighing 1.6 - 1.9 Kg, were used for the study. The animals were housed in individual cages throughout the study period. The animals were kept fasted for overnight. The rabbits were provided with water adlibitum during fasting and throughout experiment. The rabbits were not anesthetized during or prior to the experiment and the formulations were administered with an oral cannula. All experimental protocols were reviewed and accepted by the Institutional Animal house Ethics Committee (IAEC) registration No: HPI/07/60/IAEC/0008 prior to the initiation of the experiment. The study design was a parallel design, in which each animal was received one formulation at a time. One group (4 animals) was fed with standard lamivudine tablet (Lamivir, Cipla) at a dose of 3 mg/kg.

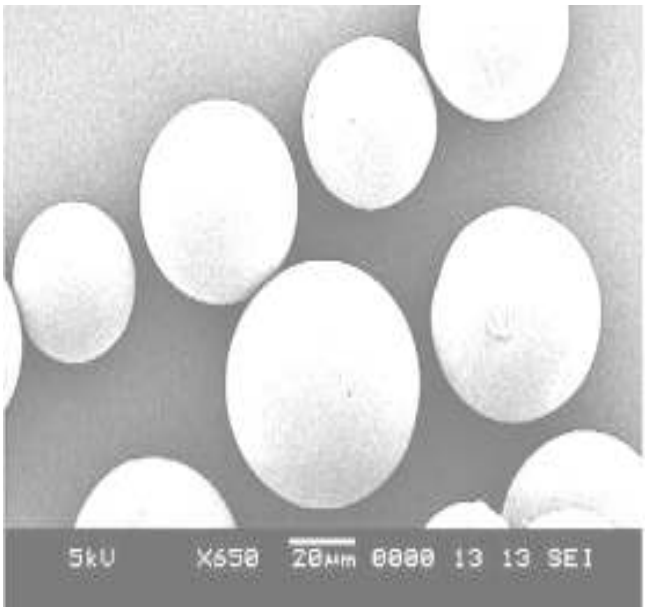

FIGURE 1. Scanning electron micrograph of prepared microspheres at higher resolution of X 650

Other four groups were administered with prepared lamivudine microspheres (F1, F2, F3 and F4). One animal was kept as control. Blood samples (3 ml) were collected from marginal ear vein using xylene into centrifuge tubes containing 0.4 ml sodium citrate 2.5 % solution from the control animal. The same method was followed for each group (both standard and test) at an interval of 30 min, 1 hour, 2, 4, 6, 8, 10 and 12th hours after dosing. Blood samples were centrifuged immediately at 2500 rpm for 5 min in cooling centrifuge machine to collect plasma. The plasma samples were stored in -20 °C until assayed. An undosed plasma sample was kept as blank sample at same condition[9-11].

Analytical methodology

About one ml plasma was mixed with 1.5 ml of Trichloro acetic acid (15 % w/v), shaken well for 3 min and centrifuged again at 4000 rpm for 15 min. The analysis of plasma samples were analyzed by HPLC method (SP2, Shimadzu, Japan). Exactly 1 ml of plasma was diluted to 25 ml with triple distilled water and 20 µl was injected at flow rate of 1ml/min using plasma water as blank. The concentration (µg/ml) was calculated[12].

CHARACTERIZATION OF MICROSPHERE

Particle size determination

The size of the prepared microspheres was measured by the optical microscopy method using a pre-calibrated stage micrometer. Particle size was calculated by using equation[13]

$$X_g = 10 \times [(n_i X \log X_i) / N]$$

X_g is geometric mean diameter, n_i is number of particle in range, X_i is the midpoint of range and N is the total number of particles. All the experimental units were analyzed in triplicate (n=3).

Scanning electron microscopy

Morphological characterization of the microsphere was done by taking scanning electron micrograph in (JEOL JSM Model 5200, Japan). Cross sectional view were obtained by cutting the microspheres with a razor blade. The samples were coated to 200A^0 thickness with gold-palladium using (Pelco model 3 sputter coater) prior to microscopy. A working distance of 20 nm, a tilt of 0° and accelerating voltage of 15 Kv were the operating parameters. Photographs were taken within a range of 50-500 magnifications.

Loose surface crystal study (LSC)

The prepared microspheres were evaluated by loose surface crystal study to observe the excess drug present on the surface of microspheres. From each batch, 100 mg of microspheres was shaken in 20 ml of double distilled water for 5 minute and then filtered through Whatman filter paper 41. The amount of drug present in filtrate was determined by using UV-Visible spectrophotometer

TABLE- 1 Evaluation Parameters of various microsphere formulations.

Formulation code	Yield (%) (X ± S.D)	Particle size (µm)(X ± S.D)	Drug Entrapment efficiency (%) (X ± S.D)	Loose crystal study (%) (X ± S.D)
F1	98.00 ± 0.017	23.22 ± 0.015	99.01 ± 0.12	18.21 ± 0.012
F2	96.00 ± 0.014	29.55 ± 0.021	95.36 ± 0.11	22.25 ± 0.011
F3	97.10 ± 0.019	27.89 ± 0.026	96.66 ± 0.22	18.45 ± 0.015
F4	95.56 ± 0.027	30.69 ± 0.019	97.01 ± 0.19	16.66 ± 0.022

All the results are represented as mean ± standard deviation (n=3). [Standard Error Mean (S.E.M) < 0.01]
F1 = drug: S-100 ratio (0.5), F2 = drug: S-100 ratio (0.25), F3 = drug: L30D ratio (0.5) , F4 = drug: L30D ratio (0.25)

TABLE- 2 *In Vitro*Drugrelease profile of different microsphere formulations.

Time (h)	Formulation Code F1	F2	F3	F4
1.	19.36	60.22	25.35	23.47
2.	15.73	70.47	21.30	22.63
3.	20.03	74.91	19.57	15.59
4.	17.94	72.09	21.27	21.31
5.	19.38	74.80	21.28	10.37
6.	24.02	72.09	18.41	10.89
7.	22.60	76.29	19.31	15.01
8.	24.71	72.93	21.06	18.83
9.	17.38	71.16	21.41	23.12

All release data are expressed in cumulative percentage drug release.
F1 = drug: S-100 ratio (0.5) F3 = drug: S-100 ratio (0.25)
F3 = drug: L30D ratio (0.5) F4 = drug: L30D ratio (0.25)

(Model-1700, Shimadzu, Japan) and calculated as a percentage of total drug content. Percentage of drug released with respect to entrapped drug in the sample was recorded.[14]

Determination of Drug entrapment efficiency

The microspheres were evaluated for percentage yield and

TABLE- 3 CorrelaTion coefficients according to different kinetic equations.

Kinetic Models	Regression co-efficient (r) F1	F2	F3	F4
Zero order	0.9992	0.9952	0.9891	0.9841
First order	0.7397	0.8282	0.8021	0.8724
Higuchi square root	0.9564	0.9931	0.9864	0.9932

Table values represents correlation coefficient (r) for linearity according to different kinetic equations used for describing the drug release from various formulations.
F1 = drug: S-100 ratio (0.5) F2 = drug: S-100 ratio (0.25)
F3 = drug: L30D ratio (0.5) F4 = drug: L30D ratio (0.25)

percent drug entrapment. The yield was calculated[13],

Percentage yield = $\frac{\text{Weight of microsphere recovered}}{\text{Weight (drug + polymer)}} \times 100$

Drug loaded microspheres (100 mg) were powdered and suspended in 100 ml methanol: water (1:99 v/v) solvent system. The resultant dispersion was kept for 20 min for complete mixing with continuous agitation and filtered through a 0.45 µm membrane filter. The drug content was determined using UV-Visible spectrophotometer at 270 nm (3) using a regression equation derived from the standard graph (r^2 = 0.9978). The drug entrapment efficiency (DEE) was calculated by the equation,

$$DEE = (Pc / Tc) \times 100$$

Pc is practical content, Tc is the theoretical content. All the formulations were analyzed in triplicate (n=3).

TABLE- 4 Stability profile of various formulations in different temperature.

Week	Temp. (° C)	Formulation Code			
		F1	F2	F3	F4
		Potency of formulations in percentage			
Initial	Room Temp.	99.24	99.42	99.56	99.88
1	(RT)				
	RT	99.20	99.01	99.66	99.78
	37 ± 1	98.53	98.89	99.05	98.55
	60 ± 1	98.23	98.88	99.00	96.36
2	RT	99.21	99.0	99.68	99.68
	37 ± 1	98.65	98.87	98.97	98.58
	60± 1	98.22	98.56	98.91	97.01
3	RT	98.92	98.97	98.99	98.90
	37 ± 1	98.56	98.79	98.87	98.53
	60± 1	98.12	98.50	98.82	97.21
4	RT	98.97	98.83	98.91	98.80
	37 ± 1	98.62	98.69	98.78	98.41
	60± 1	98.09	98.32	98.45	97.0

Verifying with one way ANOVA the data are found to be significant at 5 % level of significance (F= 3.395).

Determination of Stability of the microspheres

The formulations showing the best performance, with respect to *invitro* release, from each set of formulations, microspheres were stored at 4°C, room temperature and 45°C for a month. In an interval of every week, samples were withdrawn and were assayed by using UV-Visible spectrophotometer at 270 nm using distilled water as blank[15].

In vitro dissolution study

The USP rotating – paddle Dissolution Rate apparatus (Veego, Mumbai, India) was used to study drug release from the microspheres. The dissolution parameters [100 mg microsphere; 37 ± 2°C ; 50 rpm ; 900 ml of 0.01N HCl (n=3); coefficient of variation < 0.05] were maintained for all the four formulations. About 3 ml of aliquot samples were withdrawn at specified intervals and after suitable dilution were assayed by using UV-Visible spectro

TABLE- 5 Establishment of *In Vitro* in vivo correlation of F1 Microsphere formulation.

Degree of Correlation	*Invitro* data of F1	*Invivo* data of F1	Inference
% dissolved: Fraction absorbed	Y = 0.023x – 0.305 , 0.96	r^2 = 0.9680	Good degree of correlation
$T_{85\%}$ (h)	10.51	8.43	Sustaining action reported with good correlation
K V/s. AUC	Y = 1.767+3.431	r^2 = 0.829	well correlated
MDT V/s. MRT(hr)	5.787	6.169	Good degree of correlation

F1: Lamivudine loaded microspheres having drug: S-100 ratio of 0.5.

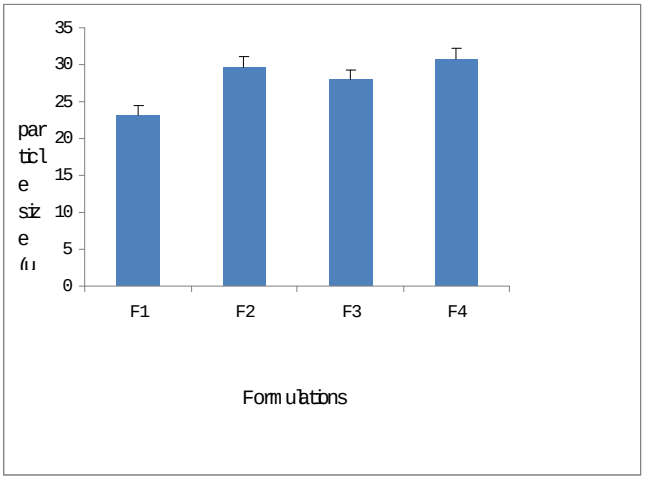

FIGURE 2. Particle size analysis of various microcapsules formulations. Each point represents mean ± standard deviation (n=3).
F1 = drug: S-100 ratio (0.5) F2 = drug: S-100 ratio (0.25)
F3 = drug: L30D ratio (0.5) F4 = drug: L30D ratio (0.25)

photometer at 270 nm. The data for percent drug release was fitted for zero order, first order and Higuchi matrix equation.[16-18]

Statistical analysis

All the data were verified with one way ANOVA followed by Turkeys test to determine its level of significance at 5 % level of significance.[19]

RESULT AND DISCUSSIONS

The microspheres thus obtained were found to be spherical as given in figure 1 and without aggregation. The mean geometric particle size was found in a range of 23 to 31 µm represented in Table -1. The particle size distribution of all the formulation was presented in Figure 2. Smaller sizes provide better absorption properties to the

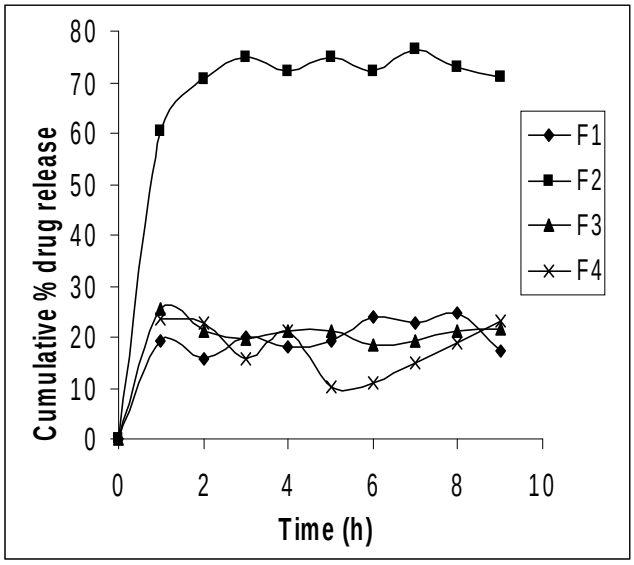

FIGURE 3. *In vitro* drug release profile of prepared lamivudine microsphere formulations.
F1 = drug: S-100 ratio (0.5) F2 = drug: S-100 ratio (0.25)
F3 = drug: L30D ratio (0.5) F4 = drug: L30D ratio (0.25)

formulation. The percentage yield of all the formulations was found to be satisfactory and each formulation demonstrated high drug entrapment efficiency (DEE), as summarized in Table-1, approving the manufacturing process. The F1 showed higher DEE among all the formulations. These loose surface crystal studies lend a hand to estimate the excess amount of drug attached on the surface of microspheres after a successful drug entrapment. The study was executed with various prepared formulations and the results were tabularized in Table 1. The *in vitro* drug release profiles for all the batches were condensed in Table-2. The F1 formulation shows slowest degree of release rate profile among all. The *in vitro* drug release profile was presented in Figure 3. To recognize the kinetics of drug release from microspheres, release data was analyzed according to different kinetic models. Table -3, explains the drug release from F1, F2, F3 formulations seems to obey zero order kinetics. The formulation F4 fit best in Higuchi square roots model. Statistical verification with one way ANOVA method attested the fact that the drug release data were found significant for F (50.0197) at 5 % level of significance ($p<0.05$). The stability studies of the prepared formulation were verified and recorded in Table-4. The mean lamivudine plasma concentration-time curves obtained after administration of the four investigated dosage forms in rabbit were represented in Figure 4. The calculated non-compartmental pharmacokinetic parameters were listed in Table-5. The

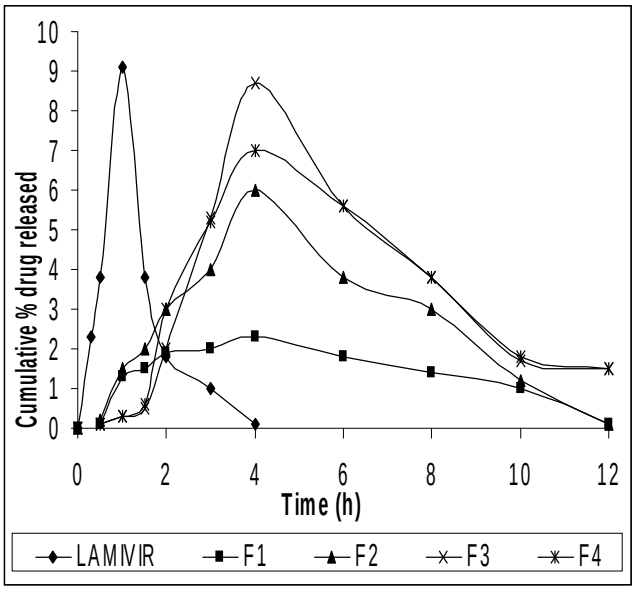

FIGURE 4. *In vivo* evaluation of the microsphere formulation and lamivir tablet.
F1= drug: S-100 ratio (0.5) F2 = drug: S-100 ratio (0.25)
F3 = drug: L30D ratio (0.5) F4 = drug: L30D ratio (0.25)

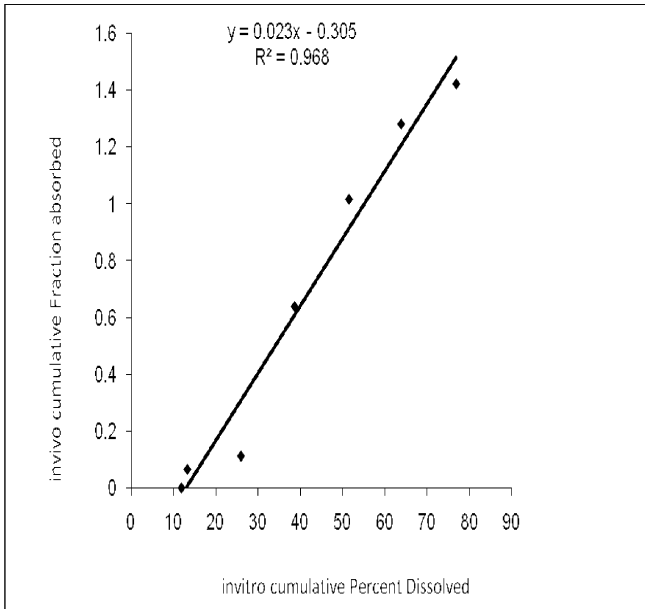

FIGURE 5. Establishment of *in vivo-in vitro* correlation of F1 microsphere formulation.

compartmental pharmacokinetic parameters of lamivudine observed that T_{max} value of all microsphere formulations were significantly longer and the C_{max} values were significantly lower than those of lamivir tablet as shown in figure 4. The K and MAT values of microspheres were significantly different from each other. The Figure-4, explained the plasma concentration after administration of the microsphere formulations (F1-F4) remain above

the minimum effective level for 10 hours where as in lamivir tablet the level was found very low 1.5 hours. The best correlation (Level A correlation) was obtained with the release profile in F1 formulation represented in Table-5 and figure 5. The results were verified with One way ANOVA followed by multiple range test (Scheff's test) and found to be significant at 5 % level of significance ($p<0.05$). As narrated in text the other parameters were evaluated and compared with *invitro* data in order to establish a good level of correlation. The result suggest that the developed *in vitro* model of F1 will allow for better prediction of the *in vivo* performance of 3TC microspheres and may be used for further development of oral sustained release 3TC formulations for effective management of AIDS.

CONCLUSION

A better control over 3TC plasma concentration profile was obtained after oral administration of Lamivudine loaded microspheres to rabbit (New Zealand white species) with respect to that of Lamivir tablet. The C_{max} was reduced, which may cause minimization of concentration dependent toxicity of Lamivudine. Furthermore, plasma concentration was maintained above the minimum effective concentration for longer time after administration of microsphere. Thus, F1 formulation showed the best *in vivo* performance exhibiting deliberate release, which correlates well with the *in vitro* release profile of 3TC from microsphere for better management of AIDS.

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