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QbD-Based Development and Evaluation of Time-dependent Chronopharmaceutical Drug Delivery System of Amoxicillin Trihydrate for Management of Bacterial Infection

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ABSTRACT

The present studies discuss about the quality by design (QbD)-based development and evaluation of chronomodulated release drug delivery system of amoxicillin trihydrate for management of bacterial infection. Initially, target product profile was defined and critical quality attributes were earmarked. Risk assessment study was performed for identifying the critical material attributes. Preformulation studies were carried out, and direct compression method was employed for the preparation of bilayer matrix tablets containing a delayed and a sustained release layer for preliminary optimization. Systematic formulation optimization was carried out using central composite design by selecting the concentration of Eudragit-L100 D55 and HPMCK4M. Mathematical modeling was performed and optimized compositions of the polymers were identified from the design space. Moreover, the prepared bilayer tablets were evaluated for various tablet properties including *in vitro* drug release study, release kinetics evaluation and characterized for FTIR, DSC, XRD, SEM studies, *in vitro* was-off test, antimicrobial assay and accelerated stability studies. In a nutshell, the present studies indicated the supremacy of designing a chronomodulated release bilayer tablet formulations of amoxicillin trihydrate for effective management of bacterial infections.

Keywords: Amoxicillin, Bilayer tablet, Dissolution, *In vitro* drug release, Antimicrobial assay.

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INTRODUCTION

Chronomodulated release drug delivery systems have recently gained increasing interest in the field of controlled release drug delivery systems, which releases drug in programmed pattern at appropriate time in a particular site and amount after a desired lag-time. Such systems are highly effective over other conventional drug delivery systems, owing to their

applicability for the diseases regulated by the circadian rhythm of body. [1-2]

Amoxicillin trihydrate (AMT) is a semi-synthetic broad spectrum β-lactam antibiotic used for the treatment of bacterial infections. It is primarily active against gram positive bacteria by inhibiting their cell wall synthesis. [3-4] It exhibit lower stability in gastric acid due to cleavage of C≡N bond of β-lactam ring which leads to loss of potency with reduced oral bioavailability. [5] Further, low half life (<1 h) with relatively high oral dosage regimen (250-300 mg b.i.d/t.i.d) requires the development of a novel once-a-day oral chronomodulated drug delivery systems of AMT for the management of bacterial infections. [6-7]

Bacterial infections are chronobiological diseases caused by *Streptococcus pyogenes, Streptococcus pneumonia*, whose progression depends upon the circadian rhythm of the body. The growth cycle of bacteria consists of four different phases such as lag phase, exponential phase, stationary phase and decline phase. Bacterial population is found to be higher in the reproductive phase specifically in the early day time, which is a chronobiological phenomenon. [8-9] Literatures reported that none of the conventional dosage forms (e.g., immediate, sustained or controlled release) dosage forms of AMT are fruitful for effective drug delivery application due to gastric degradation of drug in acidic pH, fluctuations in blood concentrations and lack of patient compliance. The chronomodulated release drug delivery systems are highly useful for the purpose, as they surmount all the challenges. Such systems have been reported in literature report for delivery of drugs like amoxicillin, clarithromycin in chronomodulated release-based formulations, which demonstrated significant improvement in the antimicrobial activity over the conventional immediate release formulations. [10-13]

Myriad formulation approaches have been used for the preparation of chronomodulated drug delivery systems *viz.* membrane erosion controlled matrix systems, pulsatile release capsular systems, osmotic systems, diffusion controlled systems, multiple unit pellet systems, etc. [14-15] Among these, the combined dissolution and diffusion controlled matrix systems are widely accepted owing to their ease of manufacturing, cost-economy and associated with less number of process variables. [16-20] The bilayer tablets are primarily prepared to provide drug release in different periods of time i.e., immediate followed by sustained release or delayed followed by sustained release, and also to provide drug release for two different drugs too. [21-26] Therefore, the present studies endeavor to provide an account on development of novel chronomodulated release bilayer matrix tablets of AMT, and evaluation of their *in vitro* performance and *ex vivo* antimicrobial activity. Besides, the prepared formulations will also provide enhanced stability of the drug in the gastric acidic environment along with time-dependent sustained release action for prolonged period of time. Figure 1 represents a pictorial depiction of mechanism of drug release from chronomodulated release bilayer matrix tablets as a function of time.

MATERIALS AND METHODS

AMT was obtained as a generous gift sample from M/s Ranbaxy Laboratories Ltd. (Gurgaon, India). Eudragit-L100 D55 and different viscocity grades of HPMC were also gifted by M/s Ranbaxy Laboratories Ltd., (Gurgaon, India). Aerosil-200, magnesium stearate and silicified microcrystalline cellulose (Prosolv-HD60) were obtained from M/s FMC Biopolymer (Mumbai, India). Deionized double-distilled water was used throughout the study (Milipore, Mumbai, India). All other chemicals and reagents used were of analytical grade. For chromatographic analysis HPLC grade methanol and dibasic potassium hydrogen phosphate has been used.

Defining target product profile (TPP)

As per the QbD-based approach of drug product development, the target product profile (TPP) was defined encompassing the summary of quality characteristics of the drug product for accomplishing the desired chronomodulated drug delivery for attaining maximal therapeutic benefits against bacterial infection. These particularly included the biopharmaceutical parameters of the drug and target drug delivery system.

Critical quality attributes

In order to meet the TPP, various patient-centric critical quality attributes (CQAs) pertaining to the quality of finished product were defined. CQAs include physiochemical, biological and microbiological attributes of the drug product.

Preformulation studies

Drug-excipients compatibility studies

The drug-excipients compatibility studies were carried out by preparing 1:1 physical mixture of the drug with individual excipients used for preparation of the time-dependent drug release systems amoxicillin trihydrate. The physical mixtures were stored in airtight containers at 4°C (control), 25°C (room temperature) and 40°C/65% RH (accelerated condition) up to 1, 2, 3 and 4 weeks. After the specified time period, the drug-excipients mixtures were evaluated for different physical observations like color change, odor, and physical state of the drug. Both FT-IR and DSC studies were performed to identify the possibility of chemical interactions between drug and excipients, if any.

Development of spectrophotometric analytical method

A double beam UV-VIS spectrophotometer (UV 3000+, M/s Labindia, Mumbai, India) equipped with holographic grating in Czerny-Turner mounting, high intensity tungsten, halogen and deuterium lamps with automatic changeover, and high sensitivity matched pair silicon photodiode detector was employed for analysis of the drug. Spectrophotometric absorbance of

the samples were measured using a 10 mm quartz cell with the spectral bandwidth fixed at 1 nm and data analysis was performed using UV-WIN software ver. 5.2.0. The value of absorbance, used to calculate the concentration of amoxicillin trihydrate, was scanned in the range of 200 to 400 nm to observe the absorption maxima (λ_{max}).

Development of chronomodulated release bilayer tablets

The pre-optimized chronomodulated release tablet formulations containing delayed and sustained release layers were individually prepared and evaluated.

Preparation of delayed release layer

The delayed release granules were prepared by wet granulation technique. An accurately weighed quantity of amoxicillin trihydrate was taken and passed through BSS #40 mesh sieve. Subsequently, the dry powder mass was transferred into a clean glass mortar. The binder solution was prepared by dissolving the enteric coated delayed release polymer (i.e., Eudragit-L100 D55) in acetone. The solution was then used for performing granulation of powder drug placed in mortar. The granulation was performed by hand mixing and wet mass was passed through the sieve (#BSS18). The obtained granules were dried in hot air oven at 40°C for 30 min and subjected to lubrication with the help of magnesium stearate. The prepared granules were further used while compression of bilayer tablets. Tablet 2 summarizes the formulation composition of delayed release layer tablets.

Preparation of sustained release layer

The sustained release granules of amoxicillin trihydrate were prepared by dry blending of drug with sustained release polymer (i.e., HPMC K4M, K15M, K100M) and diluent (i.e., Prosolv-HD60) in a clean and dry polyethylene bag and subjected to blending for 15 minutes with the help of hand. The blend was then subjected to lubrication by adding Aerosil 200 and mixing for 10 minutes. Subsequently, magnesium stearate was added and mixing was continued for another 5 minutes. Tablet 3 summarizes the formulation composition of delayed release layer tablets.

Compression of bilayer tablets

The tablet compression was performed by filling both the layers of bilayer tablet into the die cavity one after another into a 20 × 9 mm capsular punch in a rotary tablet compression machine (Minipress D-8, Cadmach, India). The compression was performed in a single bilayer tablet compression machine at a fixed compression load of 15 kg/cm^2.

Table 1: Drug-excipient compatibility study data at different time intervals

Excipients	Drug	Vial with punctured rubber closure/cap at 25°C (room temperature)				Sealed vial kept at 40°C/65% RH			
		1 week	2 week	3 week	4 week	1 week	2 week	3 week	4 week
Eud-L100 D55	1:1	No change	No change	No change	No change	No change	No change	No change	No change
MCC-PH101	1:1	No change	No change	No change	No change	No change	No change	No change	No change
Mg. Stearate	1:1	No change	No change	No change	No change	No change	No change	No change	No change
HPMCK4	1:1	No change	No change	No change	No change	No change	No change	No change	No change
HPMCK15	1:1	No change	No change	No change	No change	No change	No change	No change	No change
HPMCK100	1:1	No change	No change	No change	No change	No change	No change	No change	No change
Prosolv-HD60	1:1	No change	No change	No change	No change	No change	No change	No change	No change
Colloidal silicon dioxide	1:1	No change	No change	No change	No change	No change	No change	No change	No change

Table 2: Formulation composition of delayed release tablets of AMT

Ingredients (mg)	F1	F2	F3	F4	F5	F6
Amoxicillin	145	145	145	145	145	145
Eud-L100 D55	50	70	90	50	80	90
MCC-PH101	45	25	5	45	15	5
Mg. Stearate	10	10	10	10	10	10
Total	250	250	250	250	250	250

Table 3: Formulation composition of sustained release matrix tablets of AMT

Ingredients (mg)	F1	F2	F3	F4	F5	F6	F7	F8	F9
Amoxicillin	755	755	755	755	755	755	755	755	755
HPMCK4	150	200	220	0	0	0	0	0	0
HPMCK15	0	0	0	150	200	220	0	0	0
HPMCK100	0	0	0	0	0	0	150	200	220
Prosolv-HD60	90	40	20	90	40	20	90	40	20
Aerosil	10	10	10	10	10	10	10	10	10
Mg. Stearate	8	8	8	8	8	8	8	8	8
Total	1013	1013	1013	1013	1013	1013	1013	1013	1013

Evaluation of delayed and sustained release layers

The micromeritic properties of prepared delayed and sustained release granules were evaluated for %LOD (Loss on drying), angle of repose, bulk density and tapped density, Carr's compressibility index and Hausner's ratio.

Evaluation of delayed release tablets

The tablets prepared from delayed release granules were evaluated for hardness, thickness, friability, weight variation and drug content. The *in vitro* drug release from different delayed release tablet formulations were performed initially in 0.1N HCl (pH 1.2) for 2 h followed by phosphate buffer (pH 7.4) up to 6 h (n=3) in USP Type-I apparatus (Electrolab, Mumbai, India) at 100 rpm and 37 ± 0.5°C temperature. At different time intervals the aliquots (5 mL) were withdrawn and replaced with fresh media maintained at 37 ± 0.5°C. The samples were filtered by membrane filter, suitably diluted and absorbance was measured spectrophotometrically at 273 nm. The percentage drug release was calculated and graph was plotted between cumulative percentage drug release *versus* time.

Evaluation of sustained release tablets

The prepared sustained release tablet formulations were evaluated for hardness, thickness, friability, weight variation and drug content as per USPXXI specifications. [27] For determination of drug content, an accurately weighed quantity of powder was taken and suitably dissolved in phosphate buffer (pH 7.4), appropriate dilutions were made and analyzed spectrophotometrically at 273 nm to calculate the percentage drug content. The acceptance criteria of all these tests were purely based on the USPXXI specifications. The sustained release tablets were also evaluated for the *in vitro* drug release studies, drug release kinetic evaluation, water uptake, swelling and erosion studies, and SEM.

Risk assessment study

The risk assessment studies were carried out to identify the critical material attributes of bilayer tablet formulation. Ishikawa fish-bone diagram was constructed to establish the potential cause-effect relationship among the product and process variables. Prioritization studies were carried out for selecting the critical material attributes by constructing Risk Estimation Matrix (REM), where factors were assigned with low, medium and high risk scores. Only high risk factors were taken further for systematic optimization study.

Systematic optimization of bilayer tablets using design of experiments

The systematic optimization of the delayed and sustained release layers of bilayer tablet formulation was carried out using a response surface design on the factors influencing the responses. A two-factor and three-levels containing central composite design was selected for optimization study. A total of 13 trial formulations were prepared and evaluated for *in vitro* drug release in dissolution media (0.1N HCl and pH 7.4 phosphate buffer) as the response variables. Table 4 illustrates the design matrix as per the central composite design with two factors (i.e., Eudragit L100 D55 and HPMC K4M) studied at three different levels such as low (-1), medium (0) and high (+1). All the characterization studies were performed in triplicate for accuracy of the observations.

Table 4: Design matrix indicating trial formulations of chronomodulated release drug release system as per the central composite design

Trials	Type of points	Factor 1 (Eudragit L100 D55)	Factor 2 (HPMC K4M)
1	Factorial	150	150
2	Axial	50	225
3	Factorial	50	150
4	Center	100	225
5	Center	100	225
6	Center	100	225
7	Center	100	225
8	Axial	100	150
9	Center	100	225
10	Axial	100	300
11	Axial	150	225
12	Factorial	150	300
13	Factorial	50	300

Optimization data analysis and search for optimum formulation

The optimization data analysis was carried out by evaluating the response variables. Subsequently, mathematical modeling and fitting of data was performed by multiple linear regression analysis (MLRA). The appropriateness of model was evaluated using parameters like model p-value, coefficient of correlation (R) and lack of fit analysis. The response surface mapping was carried out using 3D and 2D-plots for critical understanding of the factor-response relationship. At the end, the optimum formulation was identified with the help of numerical optimization and desirability function, where target values of the responses were provided to meet the desired objectives. Moreover, the graphical optimization was also performed for locating the optimum formulation within the design space. Validation study of the selected DoE model was performed by selecting six confirmatory check-point formulations, where the observed and predicted values of the responses were compared with the help of linear correlation plots. Also, the residual graphs were plotted to calculate the percent prediction error between observed values and residuals.

Characterization of optimized bilayer tablets

In vitro drug release study

The *in vitro* drug release from the prepared sustained release tablet formulations of amoxicillin tablets were carried out using USP Type-I dissolution apparatus (Electrolab, Mumbai, India) at 100 rpm and 37±0.5°C. The dissolution was carried out in phosphate buffer (pH 7.4) up to 16 h in replicates (n=3) and percentage drug release at different time intervals were measured spectrophotometrically at 273 nm. The graph was then

plotted between cumulative percentage drug release *versus* time.

Drug release kinetics modeling

The *in vitro* drug release data obtained from dissolution studies were subjected to mathematical modelling employing zero-order (Eq. 1), first-order kinetic model (Eq. 2), Higuchi square root model (Eq. 3) [28] and Hixon-crowell model (Eq. 4). [29]

$$Q = k_0 t \quad \ldots\ldots\ldots\ldots (1)$$

Where, Q is the amount of drug released at time t, and k_0 is the release rate constant,

$$\ln(100 - Q) = \ln 100 - k_1 t \quad \ldots\ldots\ldots\ldots (2)$$

Where, k_1 is the release rate constant

$$Q = k_2 t_{1/2} \quad \ldots\ldots\ldots\ldots (3)$$

Where, k_2 is the diffusion rate constant

$$W_0^{1/3} - W_t^{1/3} = Kt \quad \ldots\ldots\ldots\ldots (4)$$

Where, W_0 and W_t= Initial amount of drug present in the matrix and amount of drug release at time t, K= release rate constant

Evaluation of drug release mechanism

In order to predict the mechanism of drug release from the prepared sustained release matrix tablets, Korsmeyer-Peppa's equation (5) was applied as follows:

$$\frac{M_t}{M_\infty} = Kt^n \quad \ldots\ldots\ldots\ldots (5)$$

Where, M_t/M_∞ is the fractional solute release, t is the release time, K is the kinetic constant which is the characteristic of the drug/polymer system, and n is an exponent that characterizes the mechanism of drug release. For cylindrical matrix tablets (n<0.5), the drug release follows quasi-Fickian diffusion mechanism, n=0.5 follows drug release by Fickian diffusion, 0.5 < n <1 then the drug release by anomalous diffusion, n=1 indicates Case-II Transport or typical zero-order release and n>1 indicates non-Fickian super-case II transport mechanism. [30-31]

Water uptake studies

The % water uptake by sustained release tablets were determined by placing the tablets in a beaker containing 100 mL of pH 7.4 phosphate buffer placed on a horizontal shaker at 37°C temperature. At predetermined time intervals, tablets were removed from the medium, carefully bloated on tissue paper to remove surface water and weighed. [32] The % water uptake was calculated using equation (6) as follows:

$$\%Water\ uptake = \frac{W_t - Wo}{Wo} \times 100 \quad \ldots\ldots\ldots\ldots (6)$$

Where, Wo = Initial weight of the tablet, W_t = Weight of the tablet at time "t"

Swelling and erosion studies

Swelling and erosion studies were performed on the selected sustained release tablet formulations of AMT to understand the influence of swelling and erosion behaviour of the hydrophilic polymers. It also determines the effect of polymer concentration and viscosity on swelling and erosion mechanism. [33-34] The prepared tablets were put into the dissolution apparatus under the standard set of conditions as specified in the *in vitro* drug release studies using pH 7.4 phosphate buffers. At different time intervals, swollen and hydrated tablets were removed from the medium using a small basket, and carefully soaked on tissue paper and weight of swollen matrix was taken. Swollen tablets were dried in a vacuum oven at 45°C up to 3 h for drying to a constant weight. Finally, % swelling and % erosion were calculated using the equation (7) and (8) as follows:

$$\%Swelling = \frac{S}{R} \times 100 \quad \ldots\ldots\ldots\ldots (7)$$

$$\%Erosion = \frac{(T - R)}{T} \times 100 \quad \ldots\ldots\ldots\ldots (8)$$

Where, T = Initial weight of the matrix at time "0", S = Weight of the swollen matrix at time "t", R = Weight of the dry eroded matrix at time "t"

Scanning electron microscopy (SEM)

Surface morphology of the selected sustained release matrix tablet formulation before and after dissolution were analyzed by SEM. The sustained release tablets after dissolution at different time intervals (0, 3, 6, 9 h) were soaked on a tissue paper and dried under vacuum oven at 45°C up to 6 h. The dried tablets were subjected to gold coating and mounted on brass stubs using carbon paste and kept in a dessicator for one week. A working distance of 39 mm was maintained, and the acceleration voltage of 10kv was used with secondary electron image as the detector. Surface morphology of tablets were observed using the electron microscope (LEO 1550VP, Carl Zeiss-Leica Ltd., USA) under suitable magnification. [35]

Evaluation of optimized chronomodulated release bilayer tablets

Optimized delayed and sustained release layers of tablet formulation were finally compressed into bilayer tablets. As per the previously reported procedure, the delayed and sustained granules release granules were weighed. Initially, the sustained release layer was filled in to the die cavity of rotary tablet punching machine and subjected to pre-compression. Then the delayed release granules were filled into the die cavity, and finally both the layers were compressed into a single bilayer tablet using punch size of 20 × 9 mm. The optimized tablets were subsequently subjected to different evaluation techniques.

General evaluation studies

The optimized bilayer tablets were evaluated for hardness, thickness, weight variation, friability study.

In vitro drug release study

In vitro drug release study was carried on the optimized bilayer tablet in two steps. Initially, the dissolution was performed in 0.1N HCl (pH 1.2) for 2 h

and phosphate buffer (pH 7.4) solution for another 6h in a USP Type-I apparatus at 100 rpm and 37 ± 0.5°C temperature. At specified time intervals, the aliquot samples (5 ml) were withdrawn, followed by replacement with equal volume of fresh dissolution media. Analogous procedure was also used for dissolution study of conventional marketed preparation. The dissolution data analysis was carried out and the graph was plotted between cumulative percent drug release *versus* time.

Antimicrobial assay

The antimicrobial assay of the optimized bilayer tablets was performed by using agar plate diffusion method. The zone of inhibition (ZOI) and MIC were calculated to evaluate the efficacy of the prepared bilayer tablet formulation vis-à-vis conventional marketed preparation. [36-37] The different dilutions of pure drug amoxicillin trihydrate (standard) were prepared in pH 7.4 phosphate buffer with concentrations ranging from 1-250µg/mL. The prepared bilayer tablets (test) and conventional marketed immediate release tablet preparation (Amoxil®, Dabur, India) of amoxicillin trihydrate were subjected to dissolution in pH 7.4 phosphate buffer using the same method as mentioned earlier. The aliquots collected from dissolution study at different time intervals were filtered through 0.45µm nylon filter and carefully transferred into the wells prepared on solidified agar plate in petridishes inoculated with test organisms such as *Staphylococcus aureus*-ATCC29213 (gram positive cocci) and *E. coli*-ATCC25922 (gram negative bacilli). [38] The petridishes were kept in an incubator at controlled temperature (25°C) condition. After 24 h incubation, the ZOI for prepared bilayer tablets and marketed preparation were measured (in mm) and compared with standard dilution of antibiotic. [39] On the basis of ZOIs, the MIC was calculated with respect to the amount of drug release at each specified time interval responsible to reduce the viable growth of microorganisms.

Accelerated stability studies

The optimized bilayer tablets were also subjected to accelerated stability study as per ICH-guidelines by suitable packaging in plastic polyethylene bottles up to 6 months. At different time periods of 1, 2, 3 and 6 months, the formulations were observed for colour, hardness, %friability, weight variation, drug content and *in vitro* drug release. For estimation of shelf-life, the bilayer tablets in HDPE bottles were stored at 30 ± 0.5°C, 40 ± 0.5°C and 50 ± 0.5°C temperature up to a period of three months. Samples were withdrawn after specified time intervals (0, 1, 2, 3 and 6 months), concentration and log concentration of amoxicillin trihydrate remained was calculated. Order of reaction in which drug degradation occur was estimated. The reaction rate constant (K) for the degradation was measured from slope of lines at each elevated temperature using equation (9), and an Arrhenius plot was constructed (i.e., plot of log K at various elevated temperatures against the reciprocal of absolute temperature). From the plot, K value at 25°C was determined and used for prediction of shelf-life by substituting in equation (10).

$$Slope = \frac{-K}{2.303} \quad \text{..... (9)}$$

$$t_{90} = \frac{0.1052}{K_{25}} \quad \text{..... (10)}$$

Table 5: Target product profile (TPP) of chronomodulated release drug delivery systems of amoxicillin trihydrate

TPP Elements	Target	Justification
Dosage design type	Chronomodulated release	Helps in maintaining the therapeutic effect of drug for prolonged periods of time by maintaining optimal drug release at predefined time intervals.
Dosage form type	Bilayer matrix tablet	Selection of a bilayer tablet with a delayed and a sustained release layer provides time-dependent drug release characteristic for reducing the bacterial population growth as a chronokinetic phenomenon
Route of administration	Oral	Oral route is recommended for delivery of amoxicillin trihydrate and available marketed formulations are also meant for oral intake only.
Dosage strength	775 mg	It is the unit dose of amoxicillin trihydrate needs to be deliver for once-a-daily administration
Packaging	PVC blister	The tablet formulations can easily be delivered by packing in PVC blister for patient compliance, portability and manufacturing ease
Stability	At least 24 months at room temperature	To maintain therapeutic potential of the drug during storage period

RESULTS AND DISCUSSION

Defining target product profile (TPP)

Table 5 summarizes the target product profile of chronomodulated release drug delivery systems of amoxicillin trihydrate. As per the QbD principles, a summary of the quality characteristic of the designed dosage form has been provided.

Critical quality attributes (CQAs)

Based on the TPP requirements, the CQAs were earmarked. These include the key patient-centric attributes which have direct influence on therapeutic performance of the drug. In the present case, *in vitro* drug release performance of the designed formulations at different time points like 2, 4, 8 hours were selected as the CQAs.

Drug-excipient compatibility studies

Solid state characterization using FT-IR and DSC studies revealed lack of any significant interaction between drug and excipients. FT-IR spectra of drug-excipient mixture stored at different temperature conditions were compared with spectrum of pure drug revealed no change in characteristic peaks of the drug

in all solid admixtures are shown in Figure 1, which indicated absence of change in the peak area of the drug. Similarly, no significant change in the endothermic melting peak of drug-excipient mixture under DSC further supported lack of interactions (Figure 2).

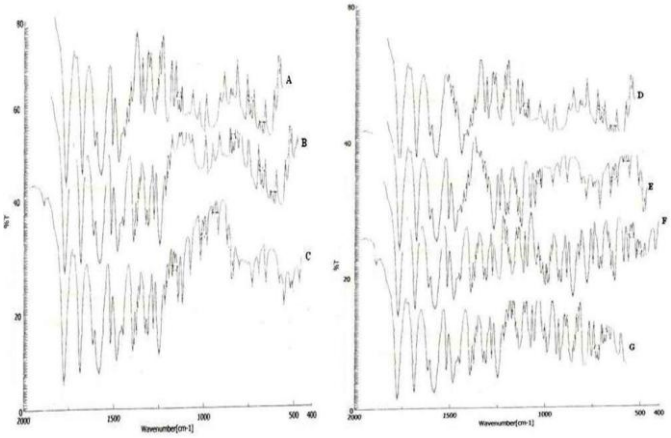


Fig. 1: Drug-excipient compatibility studies by FT-IR spectra, (A) Pure drug; (B) Drug+HPMCK4M; (C) Drug+Eudragit-L100D55

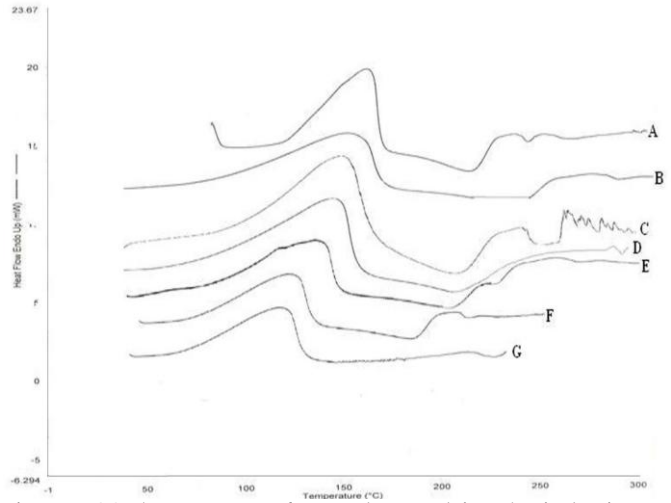


Fig. 2: DSC thermograms of pure drug and its physical mixture with polymer materials, (A) Pure drug (AMT); (B) Drug+HPMCK4M; (C) Drug+Eudragit L100 D55; (D) Drug+Prosolv HD60; (E) Drug+Aerosil 200, (F) Dug+Magnesium Stearate; (G) Composite mixture of drug with all the excipients

Evaluation of delayed & sustained release granules

Evaluation of delayed release granules

Micromeritic characterization

The %LOD of all batches of delayed release granules were found to be less than 13% due to the hydrated nature of the drug, represented that the prepared granules were dried. All other micromeritic properties like angle of repose between (25-30%), Carr's index between (13-22%) and Hausner's ratio (<1.3) for both delayed and sustained release granules indicated good flow property and compressibility characteristics. Table 6 provides the micromeritic characterization data of delayed release granules.

Technological characterization

Various technological characterization of delayed release tablets including hardness, thickness, friability, weight variation and drug contents are shown in Table 7. Results showed that all formulations passed the USP limits for various quality control tests. The hardness challenge test showed that tablets prepared with Eudragit-L100 D55 (F1-F6) were found to be quite harder at normal compression pressure because granulation with Eudragit is rubbery in nature, which provides high hardness after compression owing to negligible elastic deformation. [40]

Table 6: Granulometry data of sized delayed release granules

Formulation code	Loss on drying (%)	Bulk density (g/cc)	Bulk density (g/cc)	Hausner's ratio	Carr,s index
F1	12.5	0.58	0.74	1.28	21.62
F2	12.2	0.63	0.79	1.25	20.25
F3	12.7	0.65	0.78	1.20	16.67
F4	12.8	0.68	0.81	1.19	16.05
F5	12.6	0.69	0.82	1.19	15.85
F6	12.9	0.71	0.83	1.17	14.46

Table 7: Technological characterization of delayed release tablets of AMT (Mean ± S.D.; n=6)

Formulation code	Thickness (mm)	Hardness (kg/cm²)	Weight variation (mg)	Drug content (%)
F1	2.1 ± 0.02	1.5 ± 0.02	250 ± 0.01	100.11 ± 0.04
F2	2.3 ± 0.06	1.7 ± 0.01	252 ± 0.06	99.88 ± 0.06
F3	2.5 ± 0.04	1.7 ± 0.00	249 ± 0.04	100.28 ± 0.02
F4	2.0 ± 0.00	2.0 ± 0.08	250 ± 0.02	99.86 ± 0.08
F5	2.2 ± 0.03	2.2 ± 0.06	246 ± 0.03	102.49 ± 0.12
F6	2.1 ± 0.02	2.1 ± 0.02	252 ± 0.02	100.8 ± 0.02

In vitro drug release and lag-time evaluation

In vitro drug release from different delayed release tablets formulations (F1-F6) containing varying concentration of pH dependent delayed release polymer observed drug release with variable lag-time. Formulation F5 showed desired lag time up to 3h in SGF (pH 1.2), with burst release after changing the pH to 7.4 in phosphate buffer. Hence, it was finally selected for preparation of bilayer tablets. Figure 3 portrays the acid-resistant test for the delayed release tablet formulations.

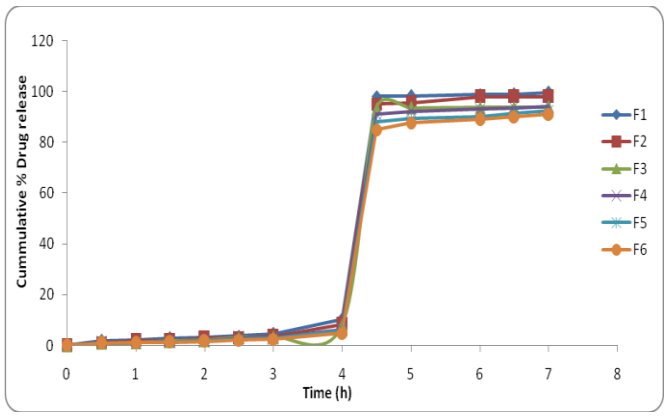


Fig. 3: *In vitro* drug release profile of different delayed release tablet formulations

Evaluation of sustained release tablets

The prepared sustained release tablets showed good physical appearance with hardness, thickness, friability, weight variation and drug content with in the acceptable pharmacopoeial limits as enlisted under Table 7. Results indicated that all batches of prepared tablet formulations were met the USPXXI specifications

with thickness <5%, hardness 13 kg/cm², friability <1% and weight variation within ±10. Drug content uniformity was within 98.9 ± 0.35 to 102.4 ± 0.16%, respectively.

Table 7: Technological characterization of sustained release tablets of AMT (Mean ± S.D, n=6)

Formulation code	Thickness (mm)	Hardness (kg/cm²)	Friability (%)	Weight variation (mg)	Drug content
F1	5.2 ± 0.02	13.4 ± 0.06	0.90	901 ± 0.06	100 ± 0.04
F2	5.1 ± 0.04	13.2 ± 0.04	0.61	904 ± 0.08	98.91 ± 0.14
F3	5.3 ± 0.07	12.9 ± 0.03	0.54	901 ± 0.03	100.5 ± 0.07
F4	5.0 ± 0.00	13.4 ± 0.06	0.55	904 ± 0.07	101.9 ± 0.06
F5	5.2 ± 0.06	13.2 ± 0.08	0.65	898 ± 0.12	101 ± 0.06
F6	5.4 ± 0.08	13.3 ± 0.09	0.52	900 ± 0.07	98.91 ± 0.35
F7	5.6 ± 0.10	12.4 ± 0.12	0.64	905 ± 0.07	99.4 ± 0.12
F8	5.2 ± 0.02	13.6 ± 0.09	0.72	884 ± 0.10	100 ± 0.03
F9	4.9 ± 0.08	13.1 ± 010	0.41	902 ± 0.12	102 ± 0.16

Water uptake studies

The water uptake studies revealed that formulation F7 showed high water uptake of 49.7% within 8 h due to high viscosity and particle size, which helped in first polymer hydration and formation of more viscous gel layer to provide sustained action.

Swelling and erosion studies

The swelling and erosion studies revealed that there was a significant increase in percentage swelling for formulations containing identical concentration (50 mg) of different viscosity grades of HPMCs such as F1 (HPMCK4M), F4 (HPMCK15) and F7 (HPMCK100). The results showed higher swelling with formulations containing higher viscosity grades of HPMC which is in the order of F7 (558%) > F4 (485%) > F1 (401%), while percentage erosion was predominantly higher in low viscosity grade polymers which is in order of F1 (95%) > F4 (91%) > F7 (85%), respectively. It was due to the presence of high viscosity grade polymer, which has faster water absorption capacity and tend to swell more rapidly compared to low viscosity grade polymer. [41] Apart from these, increase in viscosity increases the net water uptake by the polymer to attain pesudoplastic non-newtonian flow, which leads to higher swelling and lower erosion rate. Thus, high viscosity grade HPMCs exhibit more gel strength, longer diffusional path length and lower diffusion coefficient of drug release from the interior part of the matrix than low viscosity grades. [42]

In vitro drug release studies

In vitro drug release studies of the prepared sustained release tablets signified that HPMC based formulations have good sustained action. Figure 12 illustrates the *in vitro* drug release profile of the tablet formulations prepared using sustained release granules. Several factors such as nature of polymer, concentration of polymer, compression force applied, water uptake capacity, swelling and erosion tend to affect the drug release behaviour. [43] Figure 4(a) depicts *in vitro* drug release profile of the formulation F1-F3 containing HPMCK4M as rate-controlling polymer in the concentration ranging between 50 to 70 mg. The formulations showed good sustained release action up to 16 h, with initial drug release in the first three hour varied from 25 to 27%, respectively. Figure 4(b-c) represents the comparative *in vitro* drug release profile of formulation F4-F6 and F7-F9. These formulations containing HPMCK15 and HPMCK100 in the concentration of 50 to 70 mg, also showed good sustained action up to 20 h and 24 h, respectively. The drug released in first three hour varied from 19 to 25 % in HPMCK15 and 16 to 25% with HPMCK100. This confirmed that the drug release was decreased both by the increased concentration and viscosity of the polymer and the formulations containing lower viscosity grades of HPMC showed faster drug release vis-à-vis higher viscosity grade polymers. It has been reported that increase in the polymer concentration increases the gel strength, while increase in viscosity increases swelling tendency and formation of gel layer with longer diffusional path length. [44-45] Hence, the formulations prepared with HPMCK100 showed higher sustained action as compared to formulations containing HPMCK15 and HPMCK4M. [46]

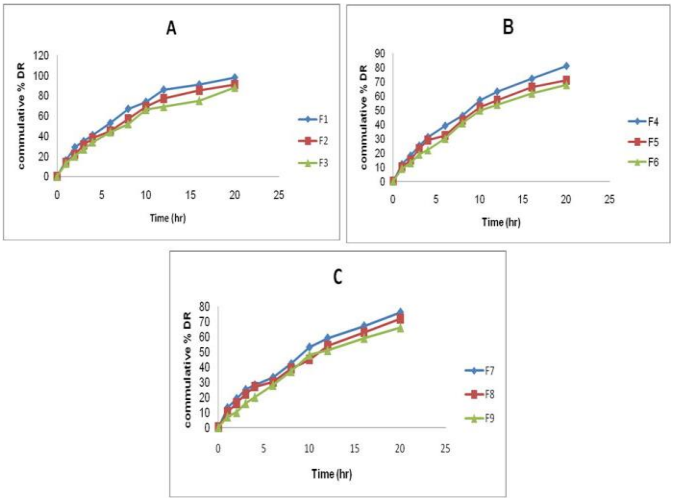


Fig. 4: Comparative *in vitro* drug release profiles of different batches of sustained release tablets, (A) F1-F3 with HPMCK4M (B) F4-F6 with HPMCK15 (C) F7-F9 with HPMCK100

Systematic optimization of time-dependent release bilayer tablets

The systematic optimization of bilayer tablet formulation was performed using experimental design. Figure 5 to 8 illustrates the 3D-response surface plots and 2D-contour plots for the *in vitro* drug release profile observed from the bilayer tablet formulation. All the plots indicated presence of significant interaction among the factors on the response variables such as *in*

vitro drug release at 2 h, 4 h and 8 h. Figure 5 indicated significant decrease in dissolution of the drug with increase in the levels of Eudragit L100 D55, which helps in gastric protection of the drug. Figure 6 also indicated significant decrease in release of the drug after 4hr of dissolution upon increase in the levels of Eudragit L100 D55. On the other hand, increase in the levels of HPMC K4M showed a sharp positive influence on drug release profile. Figure 7 shows that increase in the levels of Eudragit L100 D55 exhibited increase in dissolution profile in 8hr, while increase in the levels of HPMC K4M showed positive influence on the drug release profile.

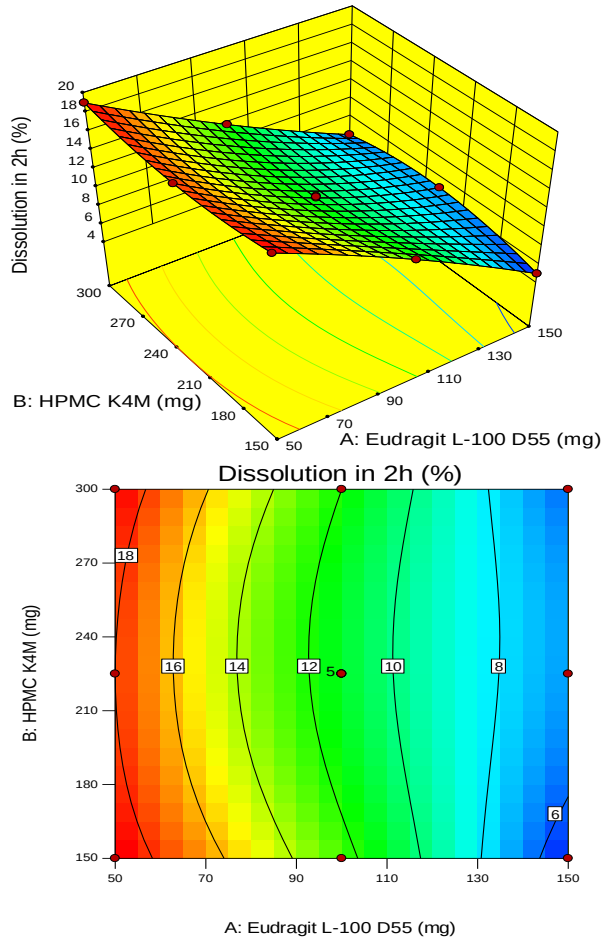
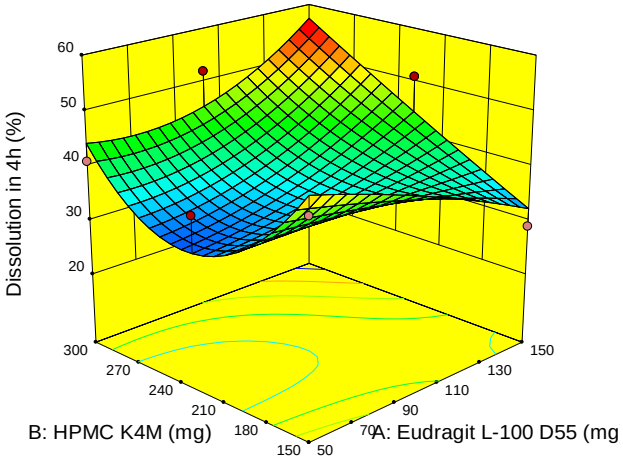


Fig. 5: 3D-response surface and 2D-contour plots depicting the influence of Eudragit L100 D55 and HPMC K4M on percent drug release after 2 hours from bilayer tablet formulation



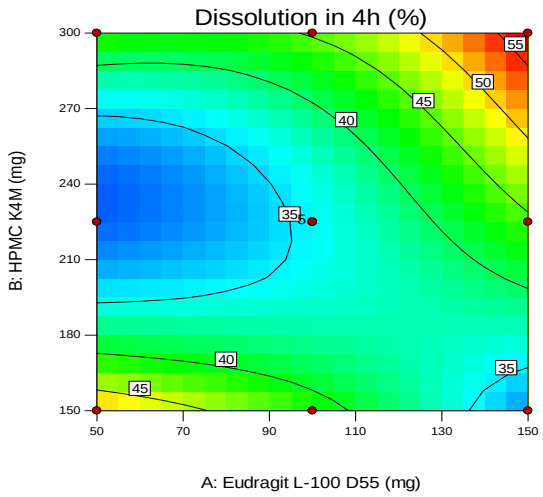


Fig. 6: 3D-response surface and 2D-contour plots depicting the influence of Eudragit L100 D55 and HPMC K4M on percent drug release after 4 hours from bilayer tablet formulation

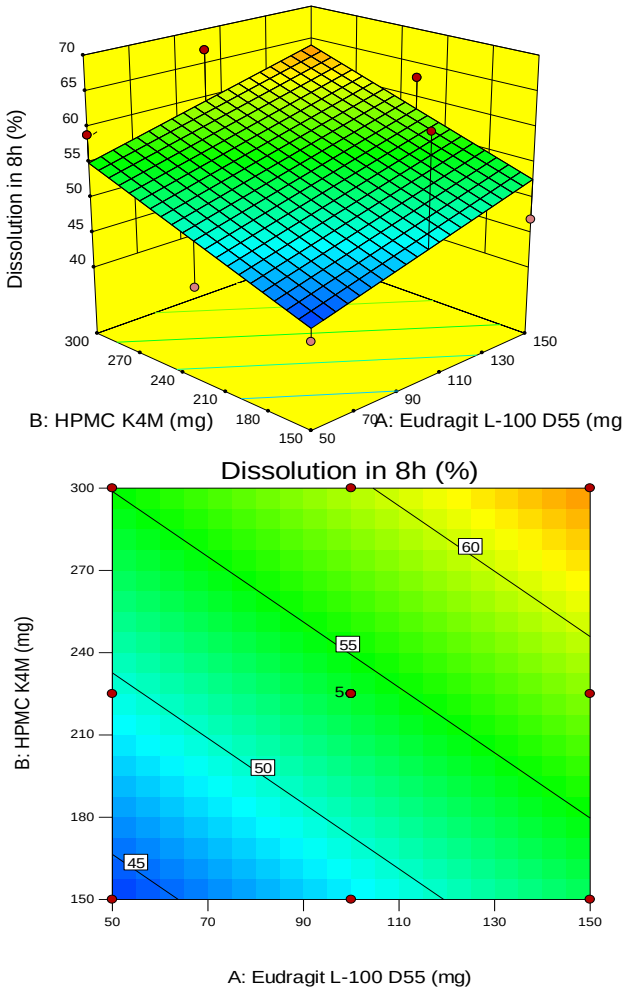


Fig. 7: 3D-response surface and 2D-contour plots depicting the influence of Eudragit L100 D55 and HPMC K4M on percent drug release after 8 hours from bilayer tablet formulation

Search for the selection of optimized formulation

The optimized time-dependent release bilayer tablet formulation was identified by numerical optimization by selecting the desired ranges for the response variables as shown in Table 8. Further, the optimized bilayer tablet formulation was demarcated in the design space overlay plot shown in Figure 8.

Table 8: Constraints of the responses selected for numerical optimization

Constraints			
Name	**Goal**	**Lower Limit**	**Upper Limit**
A:Eudragit L-100 D55	is in range	50	150
B:HPMC K4M	is in range	150	300
Dissolution in 2 h	is in range	5	19
Dissolution in 4 h	is in range	29	54
Dissolution in 8 h	is in range	42	67
Dissolution in 16 h	is in range	67	84
Dissolution in 24 h	is in range	83	93

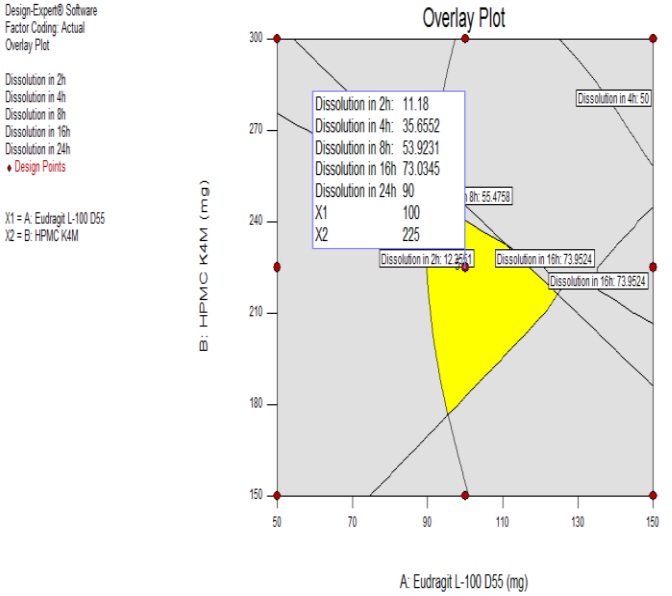


Fig. 8: Overlay plot depicting yellow color region design space and flagged point as the optimized bilayer tablet formulation

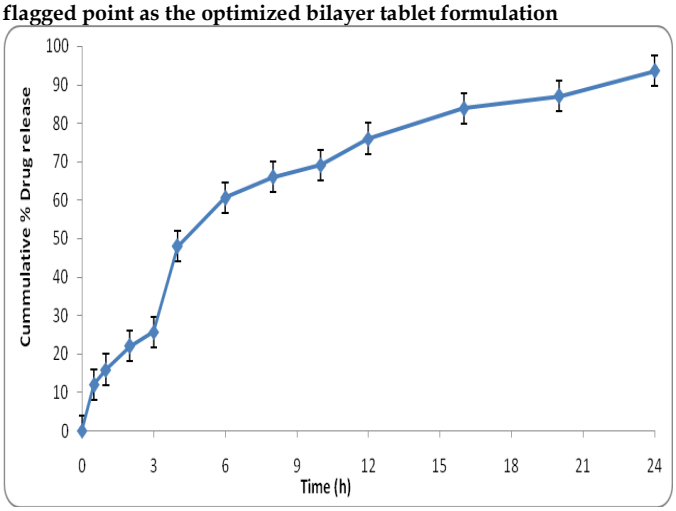


Fig. 9: *In vitro* drug release profile of bilayer tablets

Characterization of optimized bilayer tablets

In vitro drug release studies

The *in vitro* drug release profile of the bilayer tablet formulation indicated time-dependent drug release behaviour with a combination of delayed and sustained release action (Figure 9). The optimized formulation showed drug release profile analogous to that of the drug release profile predicted by experimental design. This indicated high degree of closeness among the results and ratified excellent prognostic ability. The bilayer tablet exhibited a characteristic drug release between 3 to 4 hours in pulse form, followed by a sustained drug release profile up to 16 hours.

Evaluation of drug release kinetic

In vitro drug release data for the optimized bilayer formulation was fitted to various kinetic models which indicated higher values of coefficient of correlation (r^2) with Higuchi's square root model as 0.951. The "n" value was found to be 0.419, indicated drug release by quasi-Fickian diffusion (n<0.5), where the drug release is controlled by combined action of diffusion along with polymer chain relaxation process.

Scanning electron microscopy (SEM)

SEM images of the optimized bilayer tablet formulation after dissolution at different time intervals (0, 3 & 6 hours) are portrayed in Figure 10 to 12. The surface morphology showed that formation of small pores on the matrix surface. This indicated that drug release was initially by diffusion mechanism and supported the Higuchi's square root model, followed by drug release predominantly due to erosion phenomena. [47]

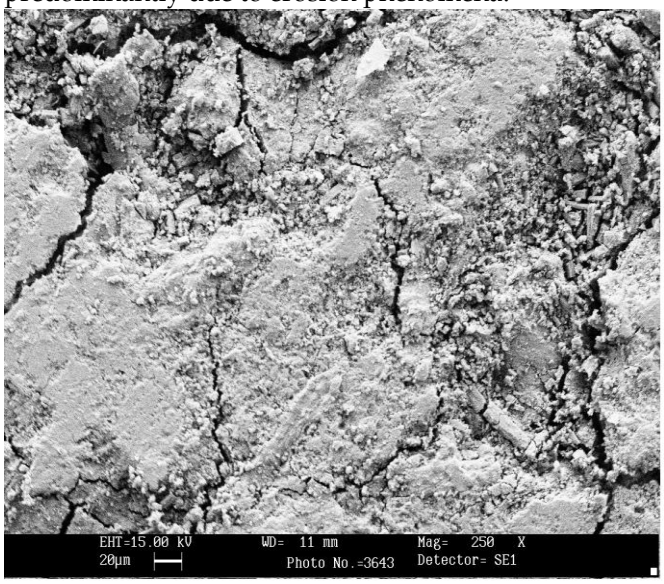


Fig. 10: Control tablet at initial 0 hour time point

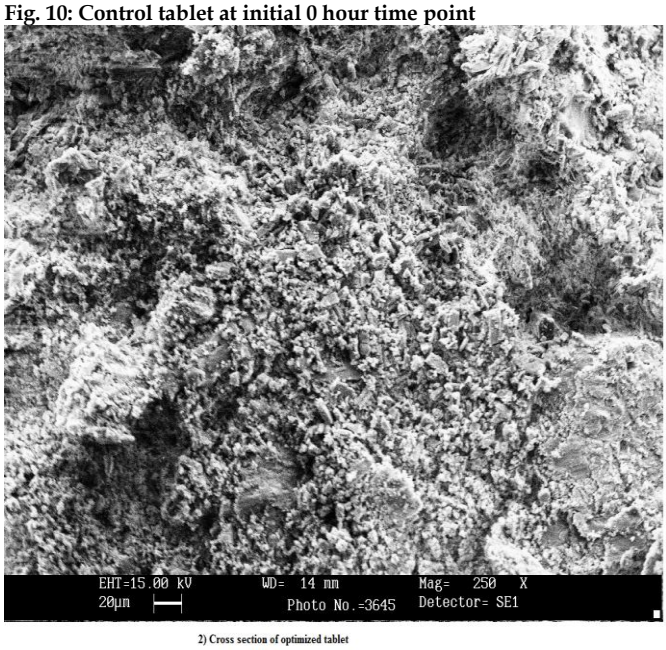
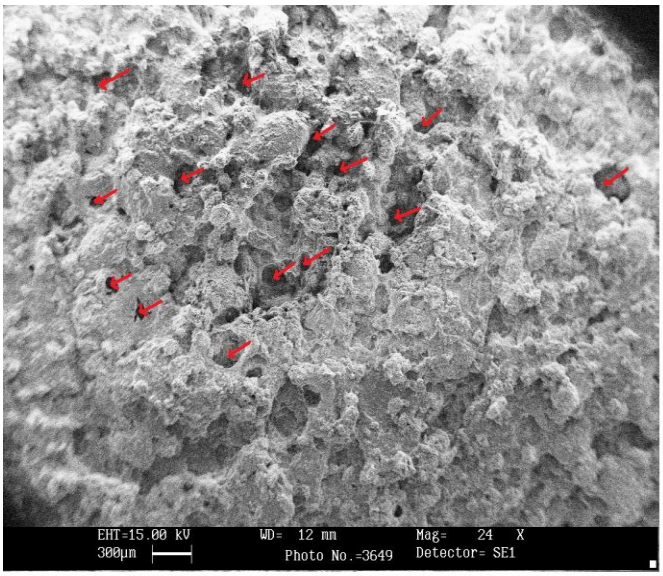
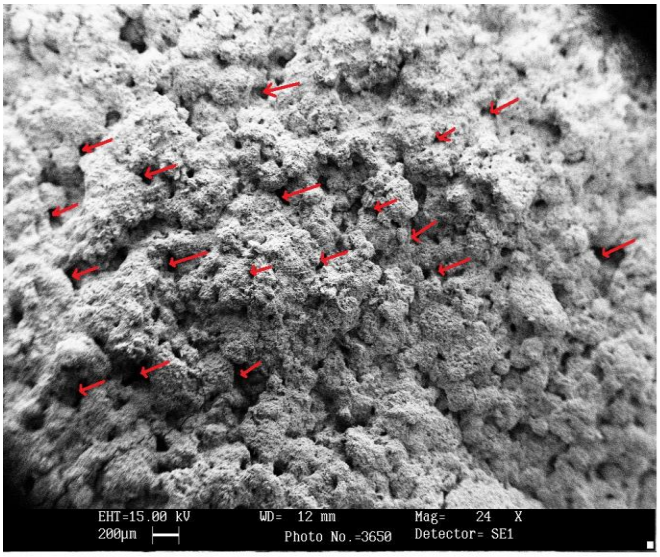


Fig. 11: Cross section of tablet at initial 0 hour time point



3) Suface morphology of matrix tablet after dissolution for 3 hr showing pores

Fig. 12: Tablet surface showing pore formation after 3 hours of dissolution



4) Surface morphology of matrix tablet after dissolution for 6hr showing pores

Fig. 13: Tablet surface showing pores formation after 6 hours of dissolution

Antimicrobial assay

Table 9 and 10 depicts the ZOI of standard dilutions of pure antibiotic, prepared bilayer tablets and marketed tablet formulation. It has been observed that as per the designed drug release profiles, there was a significant decrease in ZOI of the bilayer tablet formulation at 3 hr of dissolution with value 19.3 mm and 22.0 mm for G. positive cocci and G. negative bacilli, which matched with the ZOI of pure drug with dilution at 2µg/mL. On the contrary, the marketed formulation showed ZOI value of 29.3 mm and 29.7 mm, which were matched with the ZOI of pure drug with dilution at 5µg/mL. This indicated that bilayer tablet formulation has lower value of MIC vis-à-vis the marketed formulation in both gram positive as well as gram negative micro organisms. [10, 13] Moreover, the prepared formulation indicated higher efficacy of chronomodulated release bilayer tablet formulation over the conventional marketed product. [48-49]

Table 10: Antibiotic sensitivity of pure drug at standard dilution against

Conc. (µg/ml)	ZOI (in mm ± S.D.) for Gram positive Cocci	ZOI (in mm ± S.D.) for Gram negative Bacilli
0	0 ± 0.00	0 ± 0.00
1	0 ± 0.00	0 ± 0.00
2	19.6 ± 2.5	19.0 ± 2.6
5	23.2 ± 1.6	23.0 ± 2.2
10	29.2 ± 2.0	30.3 ± 1.4
15	34.2 ± 1.7	35.0 ± 2.0
20	41.5 ± 1.4	39.4 ± 1.3
50	48.4 ± 2.6	44.6 ± 2.6
100	50.2 ± 1.4	46.8 ± 1.6
200	51.4 ± 1.7	51.2 ± 1.7
250	58.3 ± 1.9	56.3 ± 2.2

Table 11: Antibiotic sensitivity of time-dependent release tablet formulation after dissolution *vs* Marketed formulation product

Dissolution Time (h)	Optimized Bilayer tablets		Marketed product (Amoxil)	
	ZOI (in mm ± S.D.) for Gram positive Cocci	ZOI (in mm ± S.D.) for Gram negative Bacilli	ZOI (in mm ± S.D.) for Gram positive Cocci	ZOI (in mm ± S.D.) for Gram negative Bacilli
0	0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00
0.5	8.6 ± 2.07	11.2 ± 3.10	12.3 ± 2.07	17.2 ± 2.10
1	10.7 ± 1.53	14.3 ± 2.33	19.7 ± 3.51	22.3 ± 1.09
2	13.7 ± 3.06	18.3 ± 3.53	22.7 ± 3.06	26.2 ± 1.80
3	19.3 ± 3.91	22.0 ± 1.00	29.3 ± 3.93	29.7 ± 2.90
4	33.2 ± 2.02	32.7 ± 1.38	32.4. ± 2.00	33.9 ± 1.05
5	37.7 ± 2.59	35.7 ± 3.08	38.7 ± 2.92	37.3 ± 2.10
6	41.4 ± 1.03	38.7 ± 4.53	41.7 ± 1.53	41.1 ± 2.01
8	45.1 ± 4.01	41.7 ± 3.06	43.7 ± 2.01	43.7 ± 2.32
12	46.1 ± 2.04	44.0 ± 1.03	45.7 ± 1.05	46.4 ± 1.07

Accelerated stability studies

The accelerated stability studies revealed that there was no significant change in the various physical characterization parameters like color, hardness, friability, weight variation and assay of optimized bilayer tablet formulation. The dissolution profile revealed that there was no change in the *in vitro* drug release behaviour.

The present studies successfully embarked upon formulation of once-a-daily optimized chronomodulated release bilayer matrix tablet formulations of AMT. The developed formulation showed satisfactory drug release profile to maintain the concentration of drug throughout the day which helps in reducing the MIC value of the drug. The drug release profiles from the formulations were successfully fitted to mathematical modeling for predicting the drug release kinetics. The drug releases from the sustained layer followed Higuchi model owing to drug release predominantly by diffusion and surface erosion phenomenon. The microbiological studies confirmed the decrease in MIC and enhanced antimicrobial activity owing to gastric protection of the drug and programmed site-specific drug release. The promising outcomes of present studies on AMT can be extrapolated successfully to other antimicrobial agents acting on the diseases whose progression depend on the circadian rhythm of body.

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