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Article

Antimicrobial activity assessment of time-dependent release bilayer tablets of amoxicillin trihydrate

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The aim of present study was the assessment of antimicrobial activity of prepared time-dependent release bilayer tablets of amoxicillin trihydrate and *in vitro* evaluation of drug release by antimicrobial assay using agar plate diffusion method. The bilayer tablets comprised of a delayed and sustained release layer. Direct compression method was used for the preparation of bilayer tablets containing Eudragit-L100 D55 as delayed release polymer, and HPMCK4M and HPMCK15 as sustained release polymers. The prepared bilayer tablets containing amoxicillin trihydrate were evaluated for hardness, thickness, friability, weight variation and drug content. Further, *in vitro* drug release was assessed by antimicrobial assay using *S. aureus* and *E. coli* as test microorganisms. The aliquot samples of *in vitro* drug release study were found to be effective against both microorganisms for 16 hours due to sustained action. The *in vitro* drug release study and antimicrobial assay showed that bilayer tablets have sustained release profile of drug delivery with time-dependent burst release after a lag-time of 2 hours. The lower MIC value (2 µg/mL) of prepared bilayer tablets vis-à-vis marketed preparation (5 µg/mL) represented its good antimicrobial activity.

Uniterms: Chronotherapeutics. Time-dependent release. *In vitro* dissolution. Minimum inhibitory concentration. Zone of inhibition.

O objetivo do presente estudo foi avaliar a atividade antimicrobiana de formulações de comprimidos de dupla camada contendo amoxicilina triidratada para liberação tempo dependente e avaliação da liberação *in vitro* do fármaco pelo ensaio de atividade antimicrobiana utilizando o método de difusão em placa de ágar. Os comprimidos de dupla camada consistem em uma camada para liberação retardada e outra sustentada. O método de compressão direta foi usado para a preparação dos comprimidos de dupla camada contendo Eudragit-L 100 D55 como polímero para liberação retardada e HPMCK4M ou HPMCK15 como polímeros para liberação sustentada. As formulações de comprimidos de dupla camada contendo amoxicilina triidratada foram avaliadas quanto a dureza, espessura, friabilidade, variação de peso e conteúdo de fármaco. Além disso, a liberação do fármaco *in vitro* foi avaliada por ensaio de atividade antimicrobiana usando *S. aureus* e *E. coli* como microrganismos teste. A alíquota das amostras do estudo de liberação do fármaco *in vitro* demonstrou ser efetiva contra ambos os microrganismos por um período de 16 horas devido à ação sustentada. O estudo de liberação do fármaco *in vitro* e o ensaio de atividade antimicrobiana mostraram que os comprimidos de dupla camada tiveram um perfil de liberação sustentada do fármaco com um pico de liberação após 2 horas de ensaio. O menor valor de MIC (2 ug/mL) dos comprimidos de dupla camada quando comparados à formulação comercial (5 ug/mL) representa uma boa atividade antimicrobiana.

Unitermos: Cromoterapia. Liberação tempo-dependente. Dissolução *in vitro*. Concentração inibitória mínima. Zona de inibição.

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INTRODUCTION

Time-dependent release systems are designed to deliver drugs after a particular lag-time period. Lag-time equates to the time taken for the drug to release from a dosage form at the absorption site (Aurora, Talwar, Pathak, 2006; Singh *et al.* 2010). Over the past few years, pharmaceutical formulators and scientists have shown increasing interest in the development of time-dependent release systems for controlled release delivery of drugs (Akhgari, Sadeghi, Garekani, 2006, 2009).

Bacterial infections are the chronobiological diseases whose progression depends upon the circadian rhythm of the body. Growth cycle of bacteria involves four different phases such as lag phase, exponential phase, stationary phase and decline phase. The bacterial growth is higher during reproductive phase specifically in the early daytime, which is a chronobiology-mediated phenomenon. The time-dependent release systems of antibiotics are useful as they provide drug release from the dosage form by mimicking the bacterial reproduction cycle to achieve higher C_{max}, AUC and to reduce the bacterial population (Cha, Rybak, 2004; Sun, Lee, Banevicius, Du, Maglio, Nicolau, 2005; Leuthner, Cheung, Rybak, 2006).

Amoxicillin trihydrate (AMT), chemically α-amino hydroxyl benzyl penicillin, is a broad spectrum semi-synthetic penicillin belonging to the β-lactam family (Hervey, 1991). It has been found to be highly effective against gram-positive and gram-negative bacteria especially for *Helicobacter pylori* by inhibiting their cell wall synthesis (Donowitz, Mandell, 1988; Sahasathian *et al.*, 2007). It binds to penicillin-binding proteins of the inner membrane of the bacterial cell wall. In actively growing cells, the binding of amoxicillin within the cell wall leads to interference with production of peptidoglycan, and subsequent lysis of the cell in an iso-osmotic environment (Papich, 1987; Yellanki, Singh, Ali, 2010). AMT is susceptible to degradation by β-lactamase producing bacteria. Further, low plasma protein binding (20%) and very low $t_{1/2}$ (1-1.5 hours) and lack of stability in gastric acidic pH calls for the design of novel pharmaceutical formulations (Wilson, Lee, Mukherji, 2002). Several formulation approaches have been explored to develop sustained release dosage forms of AMT to address the above-mentioned problems (Bonev, Hopper, Parisot, 2008; Hilton, Deasy, 1992; Patel, Amiji, 1996; Risbud, Hardikar, Bhat, 2000). The novel time-dependent release bilayer tablets comprised of a delayed release layer (i.e., Eudragit-L100 D55 as pH dependent enteric polymer which releases the drug after specific lag-time), and a sustained release layer (i.e., HPMCK4M and HPMCK15). Thus, the prime objectives behind designing the once-a-day bilayer tablet formulation of AMT were to provide gastric protection, lower the minimum inhibitory concentration (MIC) and subsequently improve the patient compliance.

The present studies, therefore, entails the method of preparation of time-dependent release bilayer tablets of amoxicillin trihydrate and evaluation of its antimicrobial activity using agar plate diffusion method.

MATERIAL AND METHODS

Material

AMT was generous gift sample from M/s Ranbaxy Laboratories Ltd., Gurgaon, India. Eudragit-L100 D55, HPMCK4M, and HPMCK15 were purchased from M/s Colorcon Asia Ltd., (Mumbai, India). Aerosil-200, silicified microcrystalline cellulose (Prosolv-HD60) and magnesium stearate were obtained from M/s FMC Biopolymer (Mumbai, India). All other reagents and chemicals used were of analytical grade. Deionized double-distilled water (Milipore Corporation, USA) was used throughout the study.

Method of preparation of bilayer tablets

The time-dependent release bilayer tablets containing divided dose of amoxicillin trihydrate in delayed release layer and sustained release layer were prepared by direct compression method. The total dose of AMT in the matrix tablet was kept as 900 mg, which was equivalent to 775 mg of amoxicillin, i.e., once-daily dose for AMT in treatment of bacterial infections. The delayed release layer contained 145 mg of AMT and sustained release layer contained 755 mg of AMT. The delayed release granules were prepared by wet granulation method. Drug was granulated with Eudragit-L100 D55 dissolved in acetone. The wet mass was passed through sieve no. 18 (#BSS), and dried in hot air oven at 40 °C for 20 min. Finally magnesium stearate was mixed with dried granules for lubrication. Similarly, sustained release granules were prepared by dry blending of drug with excipients. Finally both the layers were compressed into a single bilayer tablet by direct compression technique using (20 x 9 mm) capsule-shaped punch in a rotary tablet compression machine (Cadmach India Ltd., India). The compositions of prepared time-dependent release bilayer tablets of AMT are shown in Table I.

Characterization

Evaluation of delayed release granules

The prepared delayed release granules were evalu-

TABLE I - Composition of time-dependent bilayer tablet of amoxicillin trihydrate

Composition (Delayed release layer)	**F1**	**F2**	**F3**	**F4**	**F5**	**F6**
Amoxicillin	145	145	145	145	145	145
Eudragit-L100 D55	23	33	43	53	63	73
MCC-PH101	80	70	60	50	40	30
Mg. Stearate	2	2	2	2	2	2
Total	250	250	250	250	250	250
Composition (Sustained release layer)	**F1**	**F2**	**F3**	**F4**	**F5**	**F6**
Amoxicillin	755	755	755	755	755	755
HPMCK4	50	60	70	_	_	_
HPMCK15	_	_	_	50	60	70
Prosolv-HD60	91	81	71	91	81	71
Aerosil	6	6	6	6	6	6
Mg. Stearate	8	8	8	8	8	8
Total	900	900	900	900	900	900

*All the quantities expressed are in mg

ated for micromeritic properties like percentage loss on drying (%LOD), angle of repose, bulk density and tapped density, Carr's compressibility index and Hausner's ratio.

Evaluation of bilayer tablets

The different formulations of time-dependent release bilayer tablets were evaluated for hardness, thickness, friability, weight variation and drug content as per USPXXI specifications. For drug content evaluation, 20 tablets were weighed and crushed into powder. An accurately weighed quantity of powder was suitably dissolved in phosphate buffer (pH 7.4), appropriate dilutions were made and analysed by UV-Visible spectrophotometer to calculate the percentage drug content. The acceptance criteria of all these tests were based on the USPXXI specifications.

In vitro drug release

In vitro drug release from the prepared bilayer tablets was performed using USP Type-1 dissolution apparatus using SGF (pH 1.2) (900 mL) for first 2 hours followed by phosphate buffer (pH 7.4) (900 mL) for upto 16 hours at 50 rpm/37±0.5 ^{0}C. Aliquot samples (5 mL) were taken at various time intervals (0.5, 1, 1.5, 2, 2.5, 3, 4, 6, 8, 10, 12 and 16 hours), replaced with fresh media, filtered through 0.45 µm nylon filter (Millipore, Mumbai, India) and absorbance was determined spectrophotometrically at 273 nm. Further, the %drug release was determined and a plot was constructed between time (h) versus cumulative %drug release. Similarly, dissolution study was performed for marketed preparation Amoxil® (Dabur, India), containing AMT equivalent to 500 mg, using USP Type-I apparatus with phosphate buffer (pH 7.4, 900 mL, 50 rpm/37±0.5 ^{o}C) for 4 hours. At specific time intervals (0.5, 0.75, 1, 1.5, 2, 2.5, 3, 3.5 and 4 hours) aliquots (5 mL) were collected, filtered and analysed spectrophotometrically. The *in vitro* drug release samples were further subjected to antimicrobial activity evaluation using specific test organisms.

Assay of AMT and antibacterial activity assessment

The tablet formulations which showed optimum drug release were taken for further evaluation using antimicrobial activity. The antimicrobial assay of bilayer tablets of AMT was performed using agar plate diffusion method. The different dilutions of standard were prepared in distilled water with concentrations ranging from 1-250 µg/mL. The aliquots obtained after dissolution were filtered through 0.45 µm nylon filter (Millipore, Mumbai, India). Each 1 mL of the filtered samples was carefully transferred into the wells prepared with sterile borer on solidified nutrient agar (Himedia, India) plate in petridishes inoculated with test gram positive cocci, *S. aureus* (ATCC29213), and gram negative bacilli, *E. coli* (ATCC25922). After inoculation, petri-dishes were kept in an incubator (Remi Corporation, India) at 37 ^{o}C for 24 hours. After incubation the zone of inhibition (ZOI) for time-dependent release bilayer tablet, marketed preparation and standard dilution of antibiotic were measured with the help of slide calliper scale in mm. The concentration of AMT in aliquot samples was calculated using the following equation (1):

$$\ln(MIC) = \frac{\ln(c) - x^2}{4Dt}. \qquad (1)$$

Where, MIC is the minimum inhibitory concentration, x is the zone of inhibition (mm), c is the concentration of antibiotic (µg/mL), D is the diffusion coefficient, and t is the time required for antibiotic diffusion. A plot was made between x^2 vs. ln (c) graphs for standard dilutions of the antibiotic using each test organism. From this graph, the unknown concentration of AMT present in samples obtained after dissolution of bilayer tablets can be determined by extrapolating zone of inhibition (x) with respect to the concentration (c) for each microbial strain to find out the %drug release. This helps in establishing the correlation between the *in vitro* drug release and anti-microbial assay.

RESULTS

Evaluation of delayed release granules

The micromeritic properties of the granules are given in the Table II. The %LOD was found to be less than 13%, while other parameters like angle of repose (25-30%), Carr's index (12-16%) and Hausner's ratio (<1.25) for all the formulations are within the acceptable range, indicated good flow property and compressibility.

Evaluation of bilayer tablets

The prepared bilayer tablets were capsular in shape with good physical appearance. Thickness, hardness, friability, weight variation and drug content of all formulations were found to be satisfactory as shown in Table III. Results indicated that all batches of prepared tablet formulations met the USPXXI specifications with thickness <5%, hardness 18 kg/cm^2, friability <1% and weight variation ±10%. Drug content uniformity was within 98.9±0.35 to 102.4±0.16%, respectively.

In vitro *drug release and antibacterial activity assessment*

The *in vitro* drug release profile of different batches of time-dependent release bilayer tablet formulations are depicted in Figure 1. The formulation F2 showed optimum time-dependent release with more than 85% drug release in 16 h. This optimized formulation was subjected to antimicrobial activity evaluation using agar plate diffusion method as discussed above. According to equation (1), x^2 *vs.* ln (c) plots were drawn using zone of inhibition data of control against *S. aureus* and *E. coli* as obtained from agar plate diffusion assay in Figure 2 and Figure 3. The correlation coefficient values for these plots were found to be 0.9983 and 0.9876 indicated good linearity. From these plots, the concentration of AMT from *in vitro* drug release samples of bilayer tablets was determined by comparing their zones of inhibition (mm) data with respective concentrations (µg/mL) for each test organism (Table IV).

The *in vitro* drug release profile of time-dependent release bilayer tablets using *S. aureus* and *E. coli* as test organisms is shown in Figure 4. Further, the *in vitro* drug release profile using *S. aureus* and *E. coli* were compared by plotting y-axis and x-axis, respectively. The R^2 value was found to be 0.9939 (Figure 5), showing a positive correlation between the assay results of two different

TABLE II - Micromeritic properties of delayed release granules of AMT

Formulation code	% LOD (Loss on drying)	Angle of repose (θ)	Bulk density (g/mL)	Tapped density (g/mL)	Carr's Index (%)	Hausner's Ratio
F1	13.0	26.9	1.14	1.32	13.6	1.15
F2	12.9	28.2	1.14	1.30	12.3	1.14
F3	13.1	29.9	1.13	1.31	15.9	1.15
F4	13.0	28.6	1.14	1.31	12.9	1.14
F5	12.9	26.4	1.14	1.32	13.6	1.15
F6	13.2	27.1	1.13	1.33	15.0	1.17

TABLE III - Technological characterization of sustained release tablets of AMT (Mean± S.D, n=6)

Formulation code	Thickness (mm)	Hardness (kg/cm^2)	Friability (%)	Weight variation (mg)	Drug content (%)
F1	7.2±0.02	18.4±0.06	0.90	1151±0.06	100±0.04
F2	7.1±0.04	18.2±0.04	0.61	1149±0.08	98.91±0.14
F3	7.3±0.07	18.9±0.03	0.54	1152±0.03	100.5±0.07
F4	7.0±0.00	18.4±0.06	0.55	1151±0.07	101.9±0.06
F5	7.2±0.06	18.2±0.08	0.65	1152±0.12	101±0.06
F6	7.4±0.08	18.3±0.09	0.52	1150±0.01	98.91±0.35

TABLE IV - Antibacterial activity of control samples against *S. aureus* ATCC29213 and *E. coli* ATCC25922

Concentration (µg/mL)	ZOI in (mm ± S.D.) *S. aureus* ATCC29213	ZOI in (mm ± S.D.) *E. coli* ATCC25922
0	0	0
1	0	0
2	19.6±2.5	19.0±1.6
5	23.3±1.5	23.0±2.0
10	29.3±2.1	30.3±1.5
15	34.3±1.5	35.0±2.4
20	41.6±1.5	39.7±1.5
50	48.3±2.5	44.7±2.5
100	50.0±1.0	46.7±1.8
200	51.3±1.5	51.3±1.1
250	58.2±1.8	56.2±2.3

ZOI- Zone of inhibition; n=6

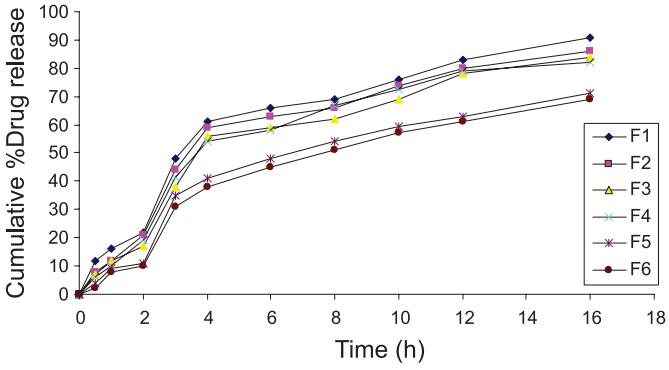

FIGURE 1 - *In vitro* drug release profile of time-dependent release bilayer tablet containing AMT by dissolution study.

organisms and also indicated that AMT estimation from the drug release samples was not affected by bacterial strains used in this investigation. Figure 6 and Figure 7 depicts the zone of inhibition of standard dilutions of AMT against *S. aureus* and *E. coli*. It was observed that zones of inhibition for these two different bacteria were almost the same. *In vitro* drug release data showed that AMT release from time-dependent release bilayer tablet was sustained up to 16 hours and delayed release occurred after 2 hours. Figure 8 and Figure 9 depicts the zone of inhibition of samples obtained from *in vitro* drug release study of time-dependent release tablet of amoxicillin trihydrate against *S. aureus* and *E. coli*.

Table IV, V represented the zone of inhibition of time-dependent release bilayer tablet and marketed preparation (Amoxil®) of AMT performed using agar

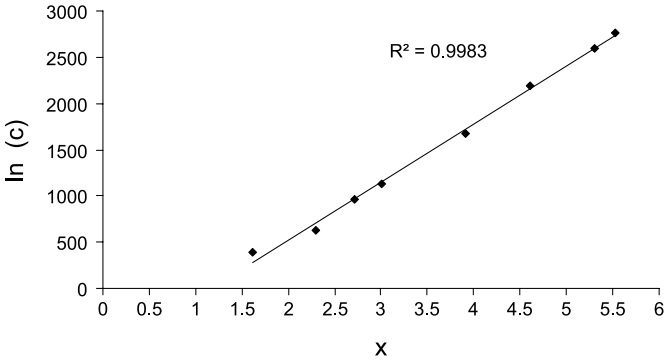

FIGURE 2 - x^2 *vs.* ln (c) plot of control (standard amoxicillin trihydrate) using *S. aureus*. x = zone of inhibition (mm); c = concentration of control samples (µg/mL)

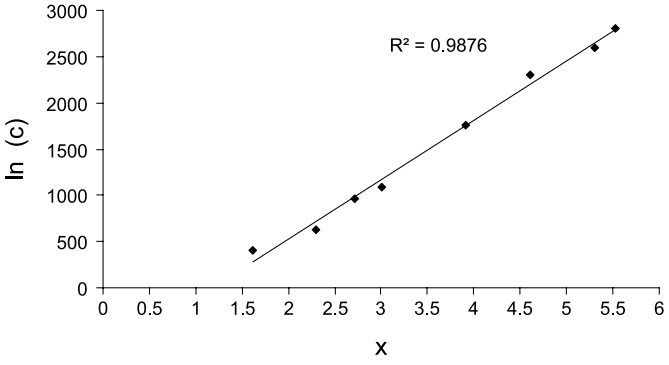

FIGURE 3 - x^2 *vs.* ln (c) plot of control (standard amoxicillin trihydrate) using *E. coli*. x = zone of inhibition (mm); c = concentration of control samples (µg/mL)

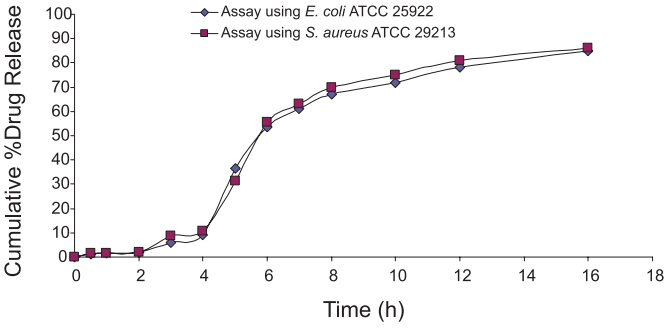

FIGURE 4 - Comparative *in vitro* drug release profiles of optimized time-dependent release bilayer tablet containing AMT using *S. aureus* and *E. coli* as test organisms.

plate diffusion employing *S. aureus* and *E. coli*. Table V represents the zone of inhibitions of standard dilutions of the AMT. The observations showed that zone of inhibition of bilayer tablets after dissolution upto 0.5 h matches with zone of inhibition of standard dilutions of pure antibiotic at 2 µg/mL, whereas zone of inhibition of marketed preparation matches with zone of inhibition of standard dilutions

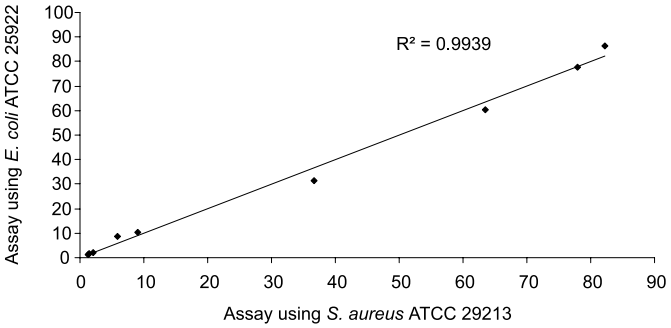

FIGURE 5 - Comparative *in vitro* drug release profiles of optimized time-dependent release bilayer tablet containing AMT using *S. aureus* and *E. coli* as test organisms.

TABLE V - Antibacterial activity indicating the ZOI of samples obtained from *in vitro* drug release study of time-dependent bilayer release matrix tablet of amoxicillin trihydrate against *S. aureus* ATCC29213 and *E. coli* ATCC25922

Dissolution time (h)	ZOI in (mm ± S.D.) *S. aureus* ATCC29213	ZOI in (mm ± S.D.) *E. coli* ATCC25922
0	0	0
0.5	27.2±3.06	25.1±2.08
1	30.3±1.53	28.4±3.51
2	32.3±1.53	32.3±3.06
3	44.0±1.00	40.1±3.93
4	46.7±1.53	44.7±2.21
5	51.7±2.08	50.0±2.92
6	57.7±1.53	57.4±1.53
7	60.2±3.06	59.7±2.01
8	61.4±2.00	60.9±1.05
10	62.5±1.13	61.5±1.17
12	66.7±1.46	64.7±1.28
16	69.1±1.33	67.8±1.77

ZOI- Zone of inhibition; n=6

of pure drug at 5 µg/mL concentration. This indicated that bilayer tablets have lower value of MIC as compared to marketed preparation in the test organisms used.

The optimized time-dependent release bilayer tablet formulation showed maximum zone of inhibition of 76.1±2.05 mm in gram-positive and 74.0±1.07 mm in gram-negative bacteria within 16 hours, while marketed immediate release tablets showed maximum zone of inhibition of 49.4±2.00 mm in gram-positive and 49.9±1.05 in gram-negative bacteria within 4 hours. Further, a prominent increase in zone of inhibition was observed with dissolution after 2 hours in time-dependent release formulation due to burst release of antibiotic to kill the viable

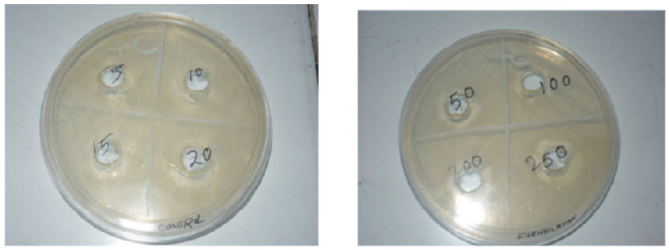

FIGURE 6 - The pictures depicting zone of inhibition of different standard dilutions of (1-250 µg/mL) against *S. aureus*.

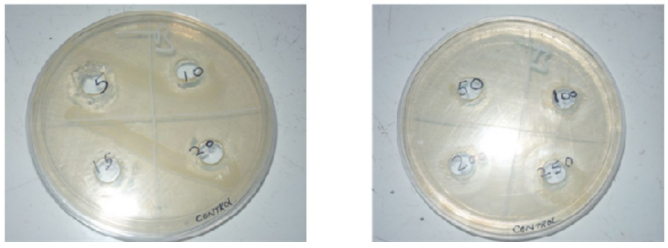

FIGURE 7 - The pictures depicting zone of inhibition of different standard dilutions of (1-250 µg/mL) against *E. coli*.

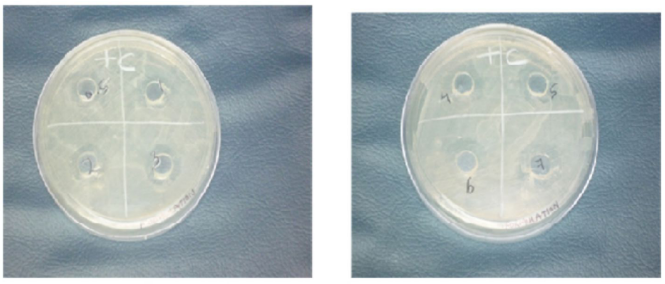

FIGURE 8 - The pictures depicting zone of inhibition of samples obtained from *in vitro* drug release study of time-dependent release bilayer tablet formulation (F2) of AMT in *S. aureus*.

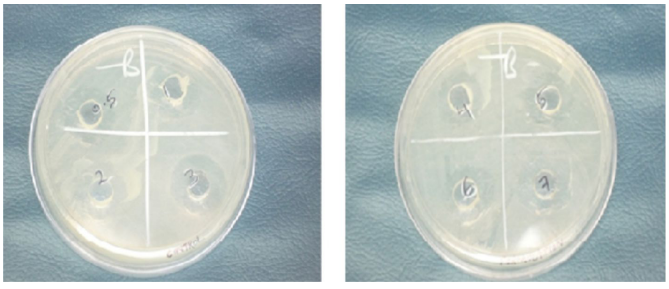

FIGURE 9 - The pictures depicting zone of inhibition of samples obtained from *in vitro* drug release study of time-dependent release bilayer tablet formulation (F2) of AMT in *E. coli*.

growth of microorganisms and showed good correlation with *in vitro* drug release data. This indicated the higher efficacy of time-dependent release bilayer tablet formulation

over conventional immediate release marketed preparation due to the low MIC and enhanced antimicrobial action.

CONCLUSION

The time-dependent release bilayer tablets of AMT were prepared and evaluated for its antibacterial activity. The *in vitro* drug release from these tablets was estimated by antimicrobial assay using agar plate diffusion method. The aliquot dissolution samples of bilayer tablet formulation were found to be sensitive against both the test organisms, *S. aureus* and *E. coli* over 24 hours study. The drug release from bilayer tablet was found to be sustained up to 16 hours with burst release achieved after 2 hours. Furthermore, the MIC of prepared time-dependent release bilayer tablets was found to be 2 µg/mL, while marketed conventional tablet preparation was found to be 5 µg/mL. The lower MIC indicated higher anti-microbial activity of the prepared bilayer tablet formulation to arrest the growth of microorganisms. Thus, chronotherapeutics by time-dependent release bilayer tablets containing amoxicillin trihydrate may be more effective alternative over conventional dosage forms for the management of bacterial infections.

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CONFLICT OF INTEREST

Authors have no conflict(s) of interest.

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