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Development and *in vitro–in vivo* relationship of controlled-release microparticles loaded with tramadol hydrochloride

Muhammad Naeem Aamir[a,*], Mahmood Ahmad[a], Naveed Akhtar[a], Ghulam Murtaza[a], Shujaat Ali Khan[a], Shahiq-uz-Zaman[a], Ali Nokhodchi[b]

[a] *Faculty of Pharmacy & Alternative Medicine, The Islamia University of Bahawalpur, Punjab, Pakistan*
[b] *Medway School of Pharmacy, Universities of Kent and Greenwich, UK*

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ABSTRACT

In conclusion, the controlled-release microparticles of TmH can be developed via phase separation method. The development and optimization of controlled-release microparticles of tramadol hydrochloride (TmH) for the oral delivery and their *in vitro* and *in vivo* correlation was prime objective of the present study. Four formulations of controlled-released microparticles were developed and optimized in terms of encapsulation efficiency, dissolution study and release kinetics. Among all formulated microparticles F-3 (ratio of TmH:EC 1:2) and F-4 (ratio of TmH:EC 1:3) presented the better characteristics in reference to entrapment efficiency, release kinetics and dissolution profile compared to other formulations (F-1, F-2). For *in vivo* analysis a new HPLC analytical method was developed and validated. The optimized formulations were subjected to *in vivo* studies to calculate various pharmacokinetic parameters, i.e., C_{max}, t_{max}, $AUC_{0-\infty}$ and MRT. The *in vitro* dissolution and *in vivo* absorption data was correlated with the help of Wagner–Nelson method. F-3 showed a good *in vitro–in vivo* correlation with a correlation determination of 0.9957. Moreover, lower T_{max}, $t_{1/2}$ and MRT, and higher values of C_{max} and K_e were observed for F-3. The control formulation (immediate-release) presented lowest values of $t_{1/2}$, MRT and T_{max} but the highest values of C_{max} and K_e. The controlled-release microparticles (F-3 and F-4) could sustain the drug release within therapeutic level up to 24 h and good IVIVC is expected from them.

1. Introduction

To minimize the requirement of bioavailability studies as part of formulation design and optimization, Food and Drug Administration (FDA) has developed guidelines for both immediate and modified release dosage forms. Nowadays modified-release dosage forms are developed extensively and this necessitates development of *in vitro–in vivo* correlation (IVIVC). IVIVC is often used to minimize the development time and to optimize the pharmaceutical products. A good correlation is a useful device for forecasting *in vivo* results on the basis of only *in vitro* findings. IVIVC can also be used as a substitute for bioequivalence studies (CDER, 1997).

On the basis of data used to establish IVIVC, three main levels are defined by the FDA, i.e. level A, level B and level C. Among them, level A of IVIVC is considered the most informative and is preferred because it represents a point-to-point relationship between *in vitro* dissolution and *in vivo* absorption rate. For this reason IVIVC of controlled-release microparticles using level A was selected in the present project (Bolton, 1991; USP, 2004).

TmH, a centrally acting opioid analgesic, is used in severe acute or chronic pains (Schug, 2003). It offers good oral bioavailability but its frequent dosing results in decreased patient compliance (Mattia and Coluzzi, 2006). Therefore, its controlled-released formulations are greatly attractive and valuable. IVIVC of controlled-released formulations is also crucial and so far there is no study has been reported on the IVIVC of controlled-release microparticles of TmH. Therefore, the present study was primarily focused on the development of controlled-release microparticles of TmH and their IVIV relationship. The controlled-release microparticles were developed by using phase separation method. Among different formulations two formulations, having better and desired drug loading and drug release profile during *in vitro* testing, were carried to *in vivo* and then *in vitro–in vivo* relationship was established.

2. Materials and methods

2.1. Materials

Tramadol HCl (gifted by Ali Gohar Pharmaceutical, Pakistan), ethyl cellulose (BDH Chemicals Ltd, Poole, UK), cyclohexane, n-hexane, potassium dihydrogen phosphate, methanol, acetonitrile,

* Corresponding author. Tel.: +92 321 4527428; fax: +92 62 9255565.
E-mail address: mna19bwp@yahoo.com (M. Naeem Aamir).

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Table 1
Production yield and drug loading of different formulated microparticles.

Formulations	F-1	F-2	F-3	F-4
TmH: EC ratio	1:0.5	1:1	1:2	1:3
Production yield (%)	92 ± 1.62	92 ± 1.91	88 ± 1.47	91 ± 2.52
Drug loading (%)	74 ± 2.23	81 ± 1.53	83 ± 1.16	86 ± 1.86
Size (μm)	124 ± 8.43	141 ± 5.31	150 ± 7.72	156 ± 5.84
Zeta potential (mV)	−3.5	−5.8	−7.0	−11.6

triethylamine (TEA) and ortho-phosphoric acid 85% (v/v) were purchased from Merck (Germany).

2.2. Preparation of microparticles

Microencapsulation technique based on phase separation by temperature change was employed to prepare four different formulations of controlled-release microparticles of TmH (Table 1). First of all, ethyl cellulose (EC) was dissolved in cyclohexane at its boiling point, i.e., 80 °C and then TmH was dispersed in this polymer solution with continuous stirring of 700 rpm on magnetic stirrer (Velp Scienifca, Germany). Phase separation was achieved, under continuous stirring condition, by rapid reduction in temperature using an ice-bath. The obtained microparticles were washed with distilled water and dried in an oven at 40 °C (Mammert, Germany) for 10 min. The dried microparticles were stored in glass vials till their subsequent characterization (Naeem and Mahmood, 2010).

2.3. Percentage yield

Microparticles after drying at 40 °C were weighed to calculate the percentage yield of microparticles using the following formula:

$$\text{Percentage yield} = \frac{\text{Total amount of microparticles}}{\text{Total weight of drug and polymers}} \times 100$$

2.4. Percentage drug loading

TmH content in controlled-release microparticles was determined by dissolving 50 mg of microparticles in methanol (10 mL). Then distilled water (20 mL) was added to the methanolic solution to induce EC precipitation. The precipitated ethyl cellulose was separated by filtration and final volume was made to 100 mL and analyzed by double beam UV/VIS-spectrophotometer (Shimadzu 1601, Japan) at 271 nm (Rouini et al., 2006). The absorbance of pure TmH (working standard) with the same dilution was also determined, and then percent drug loading in microparticles was calculated by dividing the absorbance values obtained from microparticles to the absorbance value of working standard (Yan et al., 2010).

2.5. Size and zeta potential measurements

For the measurement of size distribution and zeta potential of microparticles, Zeta Sizer Nano-ZS (Melvern Instruments, UK) was used (Klose et al., 2010). Microparticles were suspended in n-hexane (0.25%, w/v) and poured into specialized disposable charge/size cuvette. The samples were vortex for 2 min and then placed in Zeta-Sizer for analysis.

2.6. In vitro release of drug from microparticles

In vitro drug release from microparticles (equivalent to 100 mg TmH) and control formulation (immediate release) was determined using USP apparatus-II (Pharma Test, Germany) at 50 rpm. To simulate gastro intestinal conditions, the release studies were conducted according to the pH change method i.e., initial 2 h in pH 1.2, next 2 h in pH 4.5, then 2 h in pH 6.8 and finally in pH 7.4 (phosphate buffer) for subsequent 18 h. The dissolution media (900 mL) were maintained at 37 ± 0.5 °C and 5 mL of samples were collected at different time intervals with an automated collector after filtering through Whatmann filters (0.45 μm). The media were replaced with equivalent amounts of fresh one already maintained at 37 ± 0.5 °C. All samples were analyzed at 271 nm for TmH assay using a double beam UV/VIS-spectrophotometer (Pramod et al., 2009; Kucuk et al., 2005).

2.7. Application of kinetic models

The dissolution data of all controlled-release microparticles and control formulation was fitted to kinetics models i.e., zero order, first order, Higuchi (Higuchi, 1963), and Korsmeyer–Peppas (Korsmeyer et al., 1983; Peppas, 1985) to find out drug release pattern and mechanism.

2.8. Fourier transform infrared spectroscopy (FT-IR)

Drug-polymer interaction was studied by FT-IR spectroscopy (Midac 2000, USA). The FT-IR spectra were recorded for TmH, EC and drug-loaded microparticles. The samples were prepared in KBr disks. The resolution was 2 cm^{-1} and scanning range was 500–4000 cm^{-1}.

2.9. Preparation of mobile phase, stock/working solution and plasma extraction method for HPLC analysis

The mobile phase comprises of phosphate buffer (potassium dihydrogen phosphate 50 mM) and its pH was adjusted to 3.5 using ortho-phosphoric acid. Then methanol and acetonitrile were added to the buffer solution containing 0.1% triethly amine. The mobile phase was sonicated (Elma D78224, Germany) and filtered through vacuum filter assembly (Sartorius Goettingen, Germany) by using cellulose acetate filter (0.45 μm).

A stock solution was prepared by dissolving 100 mg of TmH in 100 mL of methanol. Working solutions were prepared in methanol by appropriate dilutions of stock solution to 0.05, 0.1, 0.25, 0.5, 1.0, 2.5, 5.0, 10, 20 and 40 μg/mL. All the solutions were stored at −20 °C and protected from light.

To 0.5 mL of plasma, 500 ng of TmH (dissolved in 0.5 mL distilled water) was added and vortexed for 2 min. Methanol (2 mL) was added to the plasma to precipitate plasma proteins and again vortexed for 1 min. The final solution was subjected to centrifugation at 45,000 rpm for 10 min. The supernatant liquid was filtered and transferred to epindroff tube for injecting in HPLC port (Agilent 1100 Series, Germany). Working plasma samples were also prepared using serial dilution method and standard curve was constructed. Samples were introduced into a rheodyne 20 μL fixed-loop injector with a 50 μL glass syringe (Ptacek et al., 2001). Chromatographic separation was performed at ambient temperature on ODS hypersil C_{18} stainless steel analytical column, 5 μm pore size, 4.6 mm × 250 mm (Thermo Electron Corporation, UK) and Guard Column (Uniguard TEC).

2.10. Validation of HPLC method

FDA guidelines were followed to validate this developed analytical method (CDER, 2001). Following parameters were determined for the validation of newly developed reverse phase HPLC method: linearity, intra-day & inter-day, precision and freeze–thaw stability. For freeze–thaw stability testing, lowest and highest concentration levels (10 and 400 ng/mL) of TmH were spiked in human plasma and kept frozen at −20 °C. During stability testing these samples

Table 2
Application of kinetic models to access drug release behavior.

Kinetic Models	F-1	F-2	F-3	F-4	Control
Zero order model	$y = 4.8763x + 45.19$ $R^2 = 0.5171$	$y = 5.4984x + 27.217$ $R^2 = 0.7206$	$y = 4.3845x + 21.609$ $R^2 = 0.7294$	$y = 2.8858x + 11.454$ $R^2 = 0.8144$	$y = 4.6463x + 48.344$ $R^2 = 0.4808$
First order model	$y = 0.1306x + 3.1998$ $R^2 = 0.2847$	$y = 0.1537x + 2.809$ $R^2 = 0.4049$	$y = 0.1512x + 2.6072$ $R^2 = 0.4213$	$y = 0.1503x + 2.122$ $R^2 = 0.4936$	$y = 0.1253x + 3.2658$ $R^2 = 0.264$
Higuchi model	$y = 26.474x + 17.935$ $R^2 = 0.7734$	$y = 27.369x + 1.5381$ $R^2 = 0.906$	$y = 21.79x + 1.202$ $R^2 = 0.9142$	$y = 13.904x - 1.087$ $R^2 = 0.9594$	$y = 25.652x + 21.506$ $R^2 = 0.7437$
Korsmeyer-Peppas	$y = 0.7906x + 2.9371$ $R^2 = 0.4446$	$y = 0.8830x + 2.5495$ $R^2 = 0.579$	$y = 0.8815x + 2.3447$ $R^2 = 0.6105$	$y = 0.8686x + 1.8708$ $R^2 = 0.7023$	$y = 0.7593x + 3.0128$ $R^2 = 0.4133$

were left to thaw completely and after melting they were stored back into freezer. This cycle was repeated three times at different intervals in a month.

2.11. Experimental design and procedure for in vivo studies

In vivo studies were conducted on 24 healthy male human volunteers of 52–68 kg of weight and 21–28 years of age. All the volunteers agreed in writing to take part in the study after being informed about the experimental protocols. All the volunteers were in good health according to their medical history and complete medical examination. A randomized, two-period crossover design with a wash-out period of 7 days was selected. A single dose of F-3, F-4 and control formulation (equivalent to 100 mg TmH) was administered orally on an empty stomach. All the subjects were housed at the study centre from an hour before to 24 h after the dosing. Each subject was instructed to fast overnight and the standardized breakfast and lunch was given to the subjects. A 20-gauge venous catheter was inserted into a fore arm of each subject for the collection of blood samples. Three mL blood samples were taken at the following times: 0, 0.5, 1, 2, 4, 6, 8, 10, 12 and 24 h after administration and plasma was separated by centrifugation at 45,000 rpm for 10 min and the stored at −20 °C till further analysis (Elena et al., 2010). Two mL methanol was added to 0.5 mL plasma and vortexed for 1 min and then subjected to centrifugation at 45,000 rpm for 10 min. The supernatant liquid was filtered and transferred to epindroff tube for injecting in HPLC port. One week of wash out period was given for the next sampling schedule. The ethics of this study were approved by the Board of Advance Studies and Research (BASR), the Islamia University of Bahawalpur, Pakistan.

2.12. In vitro–in vivo correlation

IVIVC of the formulations was investigated by plotting the dissolved drug percentage *versus* absorbed drug percentage (Mendell et al., 1997). The values of percentage dissolved drug were taken from *in vitro* release data, and absorbed drug percentage was determined by using Wagner–Nelson equation: (Wagner and Nelson, 1963):

$$F_a = [(C_t + k_e \quad AUC_{0-t})k_e \quad AUC_{0-\infty}] \times 100$$

where F_a is the fraction of drug absorbed, C_t is its plasma concentration at time t, k_e is the overall elimination rate constant, AUC_{0-t}, and $AUC_{0-\infty}$ are areas under the curve from time zero to time t and from time zero to infinity, respectively.

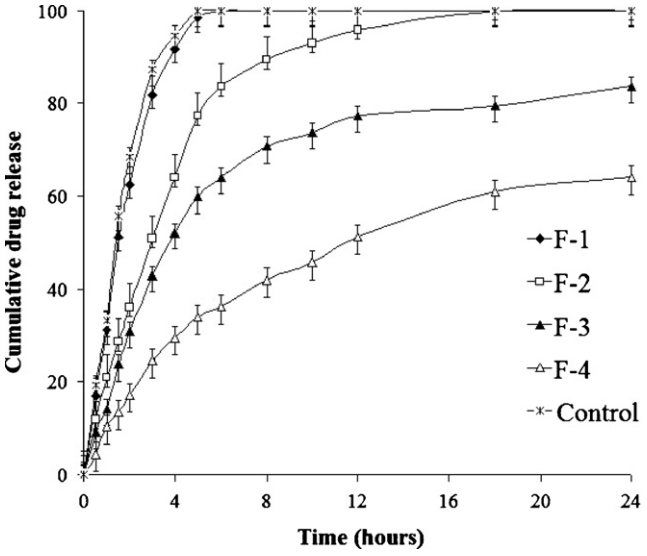

Fig. 1. Effect of polymer concentration on the release of TmH.

3. Results

3.1. Zeta potential and size of microparticles

Mean size of microparticles is given in Table 1 and mean values of zeta potential of microparticles are given in Table 1.

3.2. In vitro drug release profile of microparticles and release kinetics

The release profile study of the microparticles was studied by making them sink in dissolution medium using stainless steel sieves. After 12 h 95.78, 77.31 and 51.13% of drug was release from F-2, F-3 and F-4 respectively (Fig. 1). Whereas a rapid drug release (100% within 6 h) was observed from F-1 and control formulation.

The drug release constant (k) and correlation determination (R^2) obtained from zero order, first order, Higuchi and Korsmeyer–Peppas models are given in Table 2.

3.3. Fourier transform infrared spectroscopy

In FT-IR spectrum of TmH, the characteristic peaks of aromatic ring stretching at 1600 cm^{-1}, aliphatic CH stretching at 2900 cm^{-1}, aromatic CH stretching at 3050 cm^{-1} and OH shoulders

Table 3
The intra-day and inter-day precision ($n = 3$) for HPLC method.

Spiked TmH Conc. (ng/mL)	Intra-day		Inter-day	
	Mean Conc. Found (ng/mL) ± SD	RDS (%)	Conc. Found (ng/mL) ± SD	RDS (%)
10	9.8 ± 0.173	1.767	9.734 ± 0.252	2.586
200	199.17 ± 0.764	0.383	198.37 ± 1.528	0.770
400	398.2 ± 1.587	0.399	398.66 ± 1.155	0.289

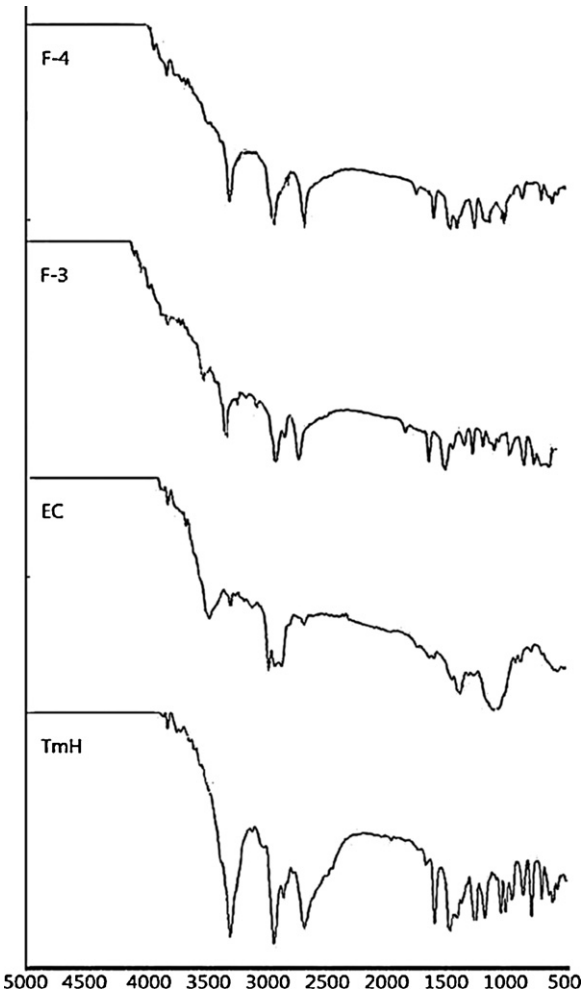

Fig. 2. Fourier transform infrared spectroscopy spectra of ethyl cellulose, tramadol and formulated microparticles.

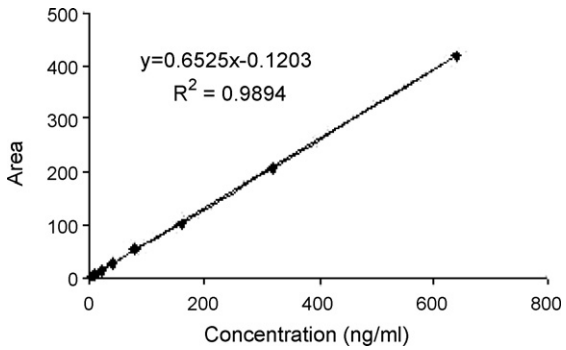

Fig. 3. Calibration curve indicating good linearity.

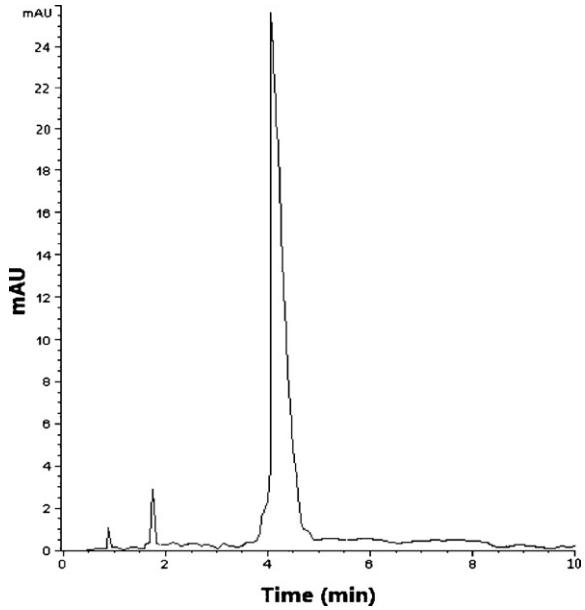

Fig. 4. A representative chromatogram of volunteer number 8 after 1.5 h of administration.

at 3300 cm^{-1} were seen (Fig. 2). EC showed two main characteristic peaks, first at 3365 cm^{-1} due to stretching vibration of OH groups at carbon number 2, 3 and 6, and second peak at 3210 cm^{-1} due to OH bond at carbon number 1 and 4. Whereas no new band was recognized in spectra of F-3 and F-4.

3.4. HPLC method validation

A linear calibration curve for TmH was obtained in the concentrations range of 10–640 ng/mL. A good linearity (R^2 = 0.9894) was observed for regression analysis of peak-area in plasma *versus* its concentration (Fig. 3).

Three TmH concentrations, low (10 ng/mL), intermediate (200 ng/mL) and high (400 ng/mL), were injected to HPLC and analyzed for intra-day precision and inter-day reproducibility. Each concentration was assayed in triplicate for both intra-day and inter-day run and relative standard deviation (RSD = 100 × SD/mean) was calculated. Inter-day and intra-day RSD in plasma were less then 5% (Table 3). These values showed that the currently developed method has high repeatability and reproducibility.

No significant variation ($p \geq 0.5$) was observed in the peak area and concentration of TmH in plasma. This showed that TmH was stable in plasma under storage conditions (Aysel and Yucel, 2005).

3.5. Pharmacokinetic parameters of selected formulations

Numbers of mobile phases with different proportion of their components were studied for sharp separation of TmH. The optimized mobile phase was Phosphate buffer (50 mM): methanol:acetonitrile at 242 nm with retention time of 4 ± 0.5 min. The optimized flow rate was 0.75 mL/min at 40 μL injection volume. A typical chromatogram is shown in Fig. 4. The important pharmacokinetic parameters were calculated for control formulation, F-3 and F-4, and the results were tabulated in Table 4.

Table 4
Pharmacokinetic parameters of F-3, F-4 and control formulation.

Parameter	F-3	F-4	Control
$t_{1/2}$ (h)	6.58	6.70	4.20
K_e (h^{-1})	0.11	0.10	0.16
MRT (h)	9.50	9.67	6.07
AUC $_{0-24\,h}$ (ng h/mL)	2893.32	2780.37	1401.6435
$AUC_{0-\infty}$ (ng h/mL)	2938.87	2947.86	1412.2446
AUMC (ng h^2/mL)	27915.41	27233.37	8574.5376
C_{max} (ng/mL)	269.15	232.99	328.35
T_{max} (h)	6.58	6.70	2.0

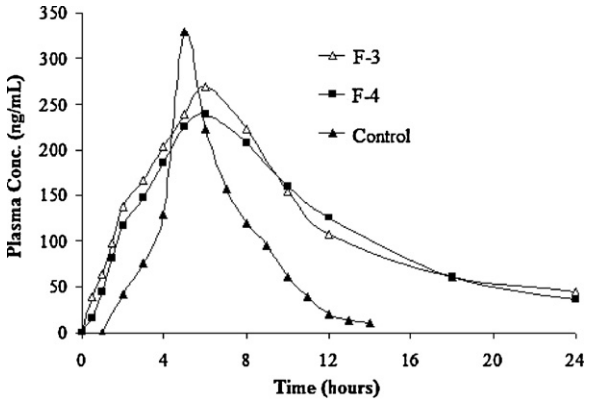

Fig. 5. Plasma profiles of TmH following oral administration of developed formulations (F-3, F-4 and control).

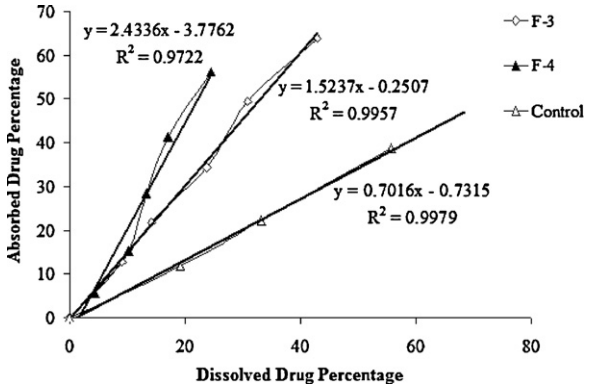

Fig. 6. Linear regression plots of percent absorption *versus* percent dissolution of F-3, F-4 microparticles and control formulation.

3.6. In vivo study and in vitro–in vivo correlation

The profile of plasma TmH concentration *versus* time obtained from the *in vivo* study clearly showed that the developed formulations of microparticles (F-3 and F-4) can maintain a constant therapeutic drug level within therapeutic range (100–300 ng/mL) over the duration of 12 h. The control formulation (immediate-release) could maintain its therapeutic level for about 8 h only.

The F-4 (the highest concentration of EC) microparticles showed lower C_{max} (but within therapeutic range) and higher T_{max} values than F-3 (Fig. 5). The values of mean residence time (MRT) and plasma half life of F-4 were higher than F-3. Whereas, compared to F-3 and F-4, the control formulation (immediate-release) presented the lowest values of $t_{1/2}$, MRT and T_{max} but the highest values of C_{max} and K_e (Table 4).

4. Discussion

A slight increase in size of microparticles from F-1 (the lowest polymer concentration) to F-4 (the highest polymer concentration) was observed due to increase in thickness of polymer coat around the core drug. Zeta potential of microparticles is the degree of electrical potential at their shear plane. The extent of zeta potential is an indication of the repulsive force and is used to assess the stability of microparticles in suspension form. Microparticles with greater zeta potential (negative or positive) in suspension will remain away from each other. A negative charge value is due to the presence of negative hydroxyl groups in polymeric film on the surface of microparticles. Low values presented that these microparticles will be less stable in suspension form. This indicated that for extended stability microparticles need to be kept in solid state.

TmH release was decreased as the concentration of polymer increased, because of an increase in hydrophobic encapsulating wall thickness around the drug. The drug release became possible when EC swelled up and channels were created as the passageway for drug and then ultimately TmH leached out into dissolution medium. A smooth release in all media was observed because TmH is a basic drug (pKa = 9.3) with acid salt and its release was independent of pH of the dissolution media (Musshoff and Madea, 2001).

The drug release pattern, from all the formulated controlled-release microparticles and control formulation, was followed and supported by Higuchi's equation as it presented highest values of correlation coefficients, R^2 (Table 2). The drug release kinetics described the release of TmH from microparticles as a square root of time-dependent process based on Fickian diffusion law. The values of n in Korsmeyer's plots for all the microparticles were higher than 0.45 and lower than 0.89 (Shinichiro et al., 2010) which was the indication of an anomalous diffusion mechanism or diffusion coupled with erosion of polymer, i.e., TmH release from controlled-release microparticles was controlled by more than one process (case-II transport).

There were no new bands identified in FT-IR spectra of F-3 and F-4 indicating no chemical interaction between TmH with EC in microparticles. Therefore, the drug remained chemically intact in new controlled-release formulations (Zhang et al., 2009).

An *in vitro–in vivo* correlation was assessed by using the Wagner–Nelson method (Sinchaipanid et al., 2003). The percentage drug dissolved *versus* percentage drug absorbed was plotted for control formulation, F-3 and F-4 (Fig. 6). IVIVC for F-3 showed a good correlation coefficient (R^2 = 0.9957). In case of F-4 the value of correlation coefficient (R^2 = 0.9722) indicated a weaker IVIVC compare to F-3 (Fig. 6). The highest correlation coefficient value (R^2 = 0.9979) was observed for the control formulation which clearly showed its superior *in vitro–in vivo* correlation compared to F-3 and F-4. Lower C_{max} for F-4 compared to F-3 could be due to slow drug release pattern as a result of increase in coating wall of polymer. The values of C_{max} of F-3 and F-4 were lower than the control formulation because of polymer layer that sustained the drug release. Similarly, because of high elimination rate constant, control formulation presented the lowest MRT and $t_{1/2}$ as compared to F-3 and F-4.

5. Conclusions

The controlled-release microparticles of TmH can be developed via phase separation method. The drug and polymer are compatible as indicated by fourier transform infrared spectrum. This study suggests that the developed optimized controlled-release microparticles can maintain a therapeutic level of TmH for about 24 h. Additionally, these microparticles are capable to provide smooth delivery of drug in plasma along with a good *in vitro–in vivo* correlation.

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