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Research Article

FORMULATION AND EVALUATION OF SUSTAINED RELEASE IMPLANTABLE MICROSPHERES OF TEMOZOLOMIDE FOR BRAIN TARGETING PREPARED BY A NOVEL TECHNIQUE

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ABSTRACT

Temozolomide (TMZ) is an alkylating agent with broad spectrum anti tumor activity. It is usually administered as oral capsules for treating malignant gliomas. The half life of TMZ is 1.6 hrs, necessitating the administration of TMZ every day for 5 days and repeating the cycle every 4 weeks. TMZ is associated with many side effects upon oral administration. This provides the need for the preparation of sustained release dosage form of TMZ. TMZ associated systemic side effects requires the need for implantable drug delivery systems. The objective of the present work is to formulate and evaluate sustained release model for TMZ using PLGA which release the drug in a uniform pattern for one month. The influence of various preparation parameters, such as the polymer concentration and solvent ratio on encapsulation efficiency was investigated. Yield, particle size and drug release from the formulation was also studied. Scanning electron microscopy (SEM) showed that the optimised formulation had a smooth surface and a spherical geometry. The differential scanning calorimetry (DSC) results indicated that TMZ trapped in the microparticles existed in an amorphous state in the polymer matrix. The release profiles of TMZ from microparticles resulted in biphasic patterns. After an initial burst, a continuous drug release was observed for 1 month. The results showed that PLGA microspheres demonstrated Sustained release of TMZ in its intact form.

Keywords: Microspheres; PLGA; Solvent extraction; Solvent evaporation.

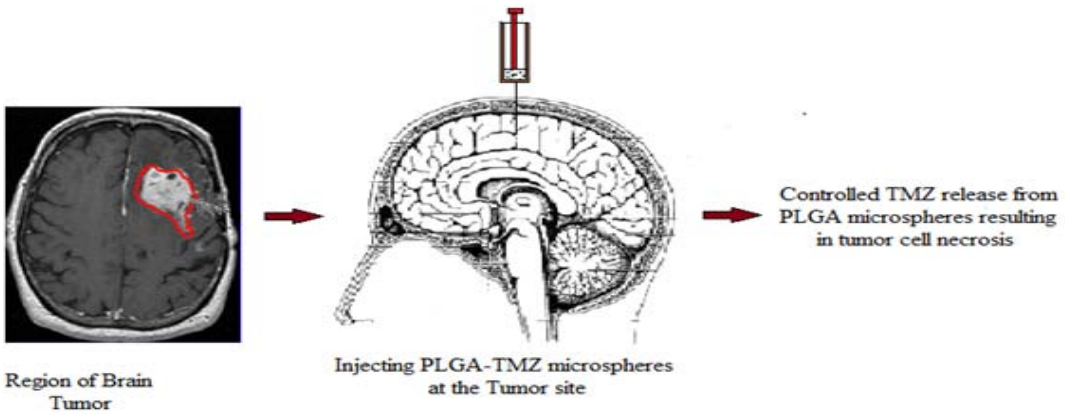
INTRODUCTION

In the past, drugs were frequently administered orally, as liquids or in powder forms. To avoid problems incurred through the utilization of the oral route of drug administration, new dosage forms were introduced. As time progressed, there was a need for delivery systems that could maintain a steady release of drug to the specific site of action. Implantable drug delivery systems have been developed to localize the drug release thereby reducing the toxicity and release drug for a prolonged period of time in a sustained or controlled fashion[1]. The major advantages of these systems include targeted local delivery of drugs at constant rate, less dose of drug required to treat the disease state, minimization of possible side effects, and enhanced efficacy of treatment[2].

Temozolomide (TMZ) is an alkylating agent with broad spectrum anti-tumor activity used for treating Brain tumors specifically Glioblastoma multiformae (GBM), Astrocytoma[3]. These tumours have indistinct margins and cannot be completely removed by surgery. It always returns, often reaching its pre-surgery size within months[4], thus necessitating the development of a drug delivery system that releases the drug in a sustained manner. With higher malignancy grades, the BBB gets leaky as the tumor degrades the tight junction by secreting soluble factor. GMBs consist of heterogeneous areas of rapid proliferation as well as areas of hypoxia and necrosis. An increasing pressure gradient is built up during tumour progression, leading to capillary and venous collapse forming a serious obstacle for drug penetration through the blood brain barrier[5].

Polyesters, such as poly (lactic acid) (PLA) and poly (lactic-co-glycolic acid) (PLGA), are examples of biomaterials which undergoes biodegradation by hydrolysis[6]. The most common areas of use of these materials include cancer chemotherapy and cancer pain management[6,7]. PLGA microspheres serve as a potential drug delivery system which releases the drug in sustained or controlled manner.

Our research was focussed on developing PLGA-based microspheres of TMZ using a novel emulsifying solvent extraction-evaporation method and evaluation of the same. The influences of several parameters, such as polymer concentration and stirring speed on yield, encapsulation efficiency, particle size were investigated. Erosion and swelling behaviour of the optimised formulation was also investigated. The physical characteristics of TMZ-loaded PLGA microspheres were studied using scanning electron microscopy (SEM), and differential scanning calorimetry (DSC).



MATERIALS AND METHODS

Materials

PLGA (76:24 mole ratio of lactide to glycolide) with viscosity 0.77 dl/g in chloroform at 20^{0}C was purchased from Pursac biomaterials (Netherland). TMZ was a kind gift from Celon life sciences Ltd (Hyderabad, India). Polyvinyl alcohol (PVA) (88% hydrolyzed) was used as the emulsifying agent, Methylene chloride (CH_2Cl_2) and Dimethyl Sulfoxide (DMSO) were obtained from SD Fine Chemicals. All other chemicals were reagent grade. Deionized water was prepared by a Milli-Q purification system from Millipore (Molsheim, France).

Microsphere Preparation

A modified oil-water (O/W) solvent extraction- evaporation method[8-10] was employed to prepare microspheres containing TMZ. Initially PLGA was dissolved in various DMSO: DCM solvent ratios. TMZ was then dissolved in the above polymeric solution. This organic phase was then added under stirring (Ika Ultraterrax) at 400 rpm to the aqueous phase (100 ml of PVA).The emulsion was continuously stirred at 15^{o}C for 1hour to completely remove traces of the organic solvents. The emulsion solidified gradually to form microspheres. The microspheres were washed twice with deionised water to remove traces of DMSO, recovered by filtration and then dried. The detailed procedure is given in [Fig-1].

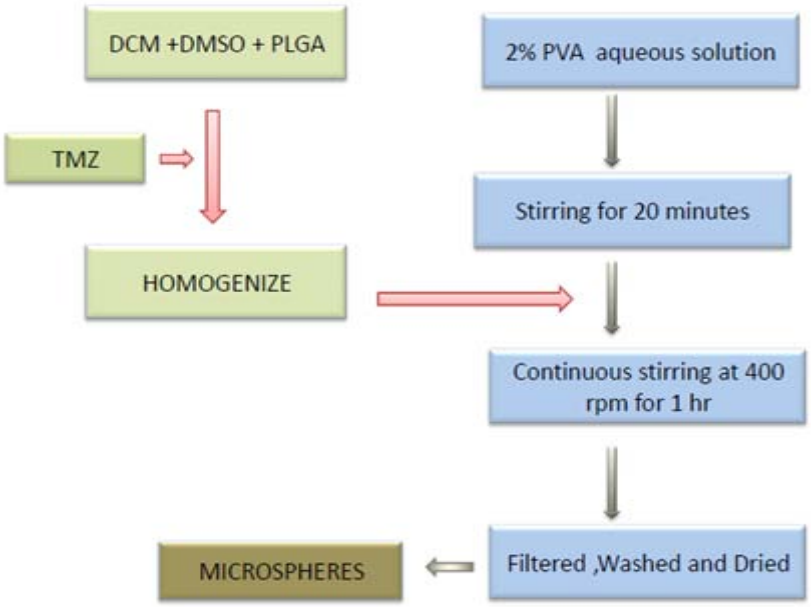


Fig. 1: Preparation of TMZ microsphere

Microspheres prepared by solvent extraction-evaporation technique

Evaluation of Microparticles

Determination of particle size[11]

The particle size was determined by optical microscopy using calibrated eyepiece and stage.

Determination of Shape and Surface morphology

The shape and surface morphology of the microspheres were examined by a scanning electron microscope [SEM].

Determination of TMZ loading and encapsulation efficiency[12,13]

Drug Encapsulation Efficiency [DEE] is defined as the percentage of the actual mass of drug encapsulated in the polymeric carrier relative to the initial amount of drug loaded. In the determination of the EE, accurately weighed quantity (50mg) of microspheres was dissolved in 1 ml of DMSO by vortexing until the microspheres dissolved completely.

10 ml of 0.1 N HCl was then added to precipitate the polymer and centrifuged (Sigma 6K15, lab centrifuge) at 2000 rpm. The supernatant was collected and filtered through 0.2µ (Millipore) filter and then analyzed using UV spectrophotometer (Syntronics) at λ_{max} 330 nm. These results were further used to determine the percentage of drug loading. Each sample was analysed in triplicates.

The encapsulation efficiency (EE) was calculated from the following equation

$$DEE = \frac{\text{Amount of drug actually present}}{\text{Theoretical drug load expected}} \times 100$$

Percentage yield for microspheres

The yield of microspheres was calculated from the amount of microspheres obtained divided by the total amount of all non-volatile components

$$\% \text{ Yield} = \frac{\text{Actual weight of the microspheres}}{\text{Total weight of all non-volatile components}} \times 100$$

Thermal Analysis

DSC analysis of microspheres was carried out using Perkin Elmer Thermal Analyzer equipped with a monitor and printer. The instrument was calibrated with indium standard. Weighed quantities of about 5-8 mg of sample were placed in a closed, flat bottom, aluminium sample pans. Thermo grams were obtained by heating the sample at a constant rate 10.00°C/min. A dry purge of nitrogen gas (20ml/min) was used for all runs. Samples were heated from 30.00^{0}C to 250.00^{0}C with a hold time for 1.0 min at 32.00^{0}C. The t_g, peak maxima, appearance of any new peak and peak shape was noted.

In vitro release study[14]

The in vitro release of TMZ loaded microsphere was carried out in phosphate buffered saline (PBS, pH 7.4) in triplicate at temperature of 37°C. TMZ loaded microspheres were suspended in 10ml of PBS

in 15ml vials, which were placed in orbital shaker incubated (Stuart Shaking incubator) at 37°C and shaken at 60 rpm. At specific time points samples were taken out and centrifuged at 2000 rpm for 5min. The supernatant was withdrawn and replaced with fresh media. Due to the instability of TMZ in the release test condition the amount of TMZ released into PBS was calculated by the amount of TMZ remained in after specific release test period.

Swelling index (water uptake) (SI)[15]

Swelling of the microspheres was determined under conditions identical to those described for the dissolution testing.

Water uptake was determined gravimetrically according to the following equations:

$$\text{Degree of swelling (water uptake)} = \frac{\text{Wet weight} - \text{original dry weight}}{\text{Original dry weight}}$$

At predetermined time points the hydrated microspheres were carefully filtered from the dissolution vials and tapped gently with tissue paper to remove excess surface water. After determining the wet weight, the microspheres were dried at room temperature until constant weight, before reweighing to determine the remaining dry weight. Experiments were performed in triplicate.

Biodegradation[15]

Erosion of the microspheres was determined under conditions identical to those described for the dissolution testing. Erosion was determined gravimetrically according to the following equation:

$$\text{Erosion (\% mass loss)} = \frac{\text{Original weight} - \text{remaining dry weight}}{\text{Original weight}} \times 100$$

At predetermined time intervals the hydrated microspheres were carefully filtered from the dissolution vials and tapped gently with tissue paper to remove excess surface water. After determining the wet weight, the microspheres were dried at room temperature until constant weight, before reweighing to determine the remaining dry weight. Experiments were performed in triplicate.

RESULTS AND DISCUSSION

Effect of preparation conditions on size, shape and entrapment efficiency

The present study was carried out to investigate the feasibility of preparing biodegradation TMZ microspheres using a novel solvent extraction evaporation technique.

Effect of PVA concentration in the external water phase

PVA concentration in the external water phase is known to be a key factor affecting the size of microspheres[10]. In the present work, 1, 1.5 and 2% PVA solutions were used as the external water phase to examine the effect of PVA concentration on the characteristics of the microspheres. The results are summarized in [Table 1] (formulation A, B and C). The size of microspheres fabricated at 1, 1.5, and 2% PVA concentration ranged from 128 to 74 µm respectively. Evidently, a significant decrease in particle size was achieved by increasing the concentration of PVA in the continuous phase. A higher PVA concentration could increase the stability of emulsion droplet formed during homogenization because the increased viscosity of the external water phase would prevent emulsion droplets from coalescence, resulting in formation of smaller emulsion droplets. These emulsion droplets gradually hardened to form microspheres as the solvent in the emulsion droplets continued to diffuse and evaporate. Therefore, the size of the microparticles was dependent on the size of the emulsion droplets formed during homogenization. Moreover, a significant increase in TMZ encapsulation efficiency from 65% to 82% occurred as the concentration of PVA increased in the external water phase. This may be due to the fact that higher PVA concentration increased the viscosity of the external water phase, which decreased the rate of solvent and TMZ diffusing from the inner organic phase to the outer aqueous phase, i.e. hindered the mass transfer of TMZ to the surrounding region. This might have led to more even distribution of the drug in the inner region of the microspheres resulting in the increased encapsulation efficiency.

Effect of solvent ratio on drug entrapment efficiency

The ratio of the solvents DMSO as well as DCM had a great impact on the amount of TMZ encapsulated. The results are summarised in [Table 2]. As the amount of DMSO reduced encapsulation efficiency increased. The amount of TMZ encapsulated decreased as some amount of TMZ escaped along with DMSO during solvent extraction due to the formation of large sized pores on the surface as observed in SEM analysis [Fig-4].

Effect of polymer concentration in the oily phase

With the increase in the PLGA concentrations in formulations G, H and I the size and encapsulation efficiency of the microspheres was increased [Table 3]. It was demonstrated that the size of emulsion droplets depends on the balance between stirring shear force and droplet cohesion. It was considered that the higher concentration of PLGA, at a fixed stirring shear force, results in a higher viscosity of the solvent phase, which makes it difficult for small o/w emulsion droplets to form and become larger particles. The increase in the drug entrapment efficiency may also be attributed to the increase in both oil phase viscosity and the larger size of the oil droplets, which prevented leaching of the drug to the outer aqueous phase.

By increasing the drug to polymer ratio percentage of drug encapsulated also increased. This may be attributed to the availability of more coat material per drug molecule. By increasing the stirring speed percentage drug entrapment efficiency of PLGA microspheres slightly decreased. It was observed that by increasing the speed from 400 to 600 the drug loading reduced this may be due to the diffusion of the drug into the external phase and at higher speed rapid diffusion might have occurred during solvent extraction [Table 4].

Effect of stirring speed

From the results in [Table 4] it is clear that the particle size slightly decreased with increase in the stirring speed. As the agitation speed increased from 400 to 600 rpm, the size of the microspheres reduced due to the breakdown of the emulsion into smaller droplets caused by increased shear.It is also observed that the particle size of the microspheres increased with the increase in the polymer concentration. The increase in the viscosity with increasing polymer concentration resulted in the formation of larger emulsion droplets which led to immediate solidification and probably the subsequent formation of bigger microspheres.

In vitro release studies

The influence of the concentration of polymer (PLGA 74 / 26) on the release characteristics is shown in [Fig-2]. The PLGA microspheres showed a typical three phase in vitro release. The release profiles showed a characteristic initial burst release followed by a lag period and further initiation of erosion-controlled release. After the initial lag, a nearly linear and continuous release was observed over 10–13 days, a continuous release then followed up to 100% at day 30. The initial burst release was highly dependent on the formulation parameters. When the concentration of polymer solution increased from the lowest and highest optimised concentrations i.e. 5.3 % to 13.3 %, the initial burst release decreased from 40% to 15% respectively. The increasing concentration of polymer led to a significant decrease of impact on the initial burst release of TMZ from microspheres. This may be attributed to the higher polymer concentration leading to the formation of a dense polymer matrix structure in the microspheres resulting in smaller pores.

A triphagic release pattern was observed. The release pattern may be divided into 3 regions. Initial burst release, a lag time followed by constant drug release. The initial burst release was as high as 42 % for F1 formulation prepared at 400 rpm. This burst release may be accounted to various reasons namely porosity, drug polymer ratio, polymer concentration, and diffusion of the drug through the polymeric matrix and the erosion of the polymer,

The initial burst release for microspheres prepared at 400 rpm was highly dependent on the formulation parameters. When the concentration of polymer solution increased from 5.33% to 13.33%, the initial burst release decreased from 42% to 11%. The concentration of polymer had a significant impact on the initial burst release of TMZ from microspheres.

Similarly for microspheres prepared at 600 rpm was highly dependent on the formulation parameters. When the concentration of polymer solution increased from 5.33% to 13.33%, the initial burst release decreased from 53% to 20%. The concentration of polymer had a significant impact on the initial burst release of TMZ from microspheres. This may be due to the higher polymer concentration leading to the formation of a dense polymer matrix structure in the microspheres, resulting in smaller pores and a more tortuous structure.

With the increase in polymer concentration the drug release could be prolonged up to 30 days for both spheres prepared at 600 and 800 rpm.

The greater burst release of microspheres prepared at 600 rpm may be due to initial surface pores in the polymer (confirmed by SEM) and pores created during water entry and polymer swelling, which can potentially increase the uptake of the release medium into the particles and accelerate the drug pore-diffusion and release. The DSC studies indicate a change in the t_g of the optimised formulation.

The burst release from the microspheres could be reduced by compressing the microspheres. Initially a lag time was obtained for 15 days, after which the drug released in a controlled fashion.

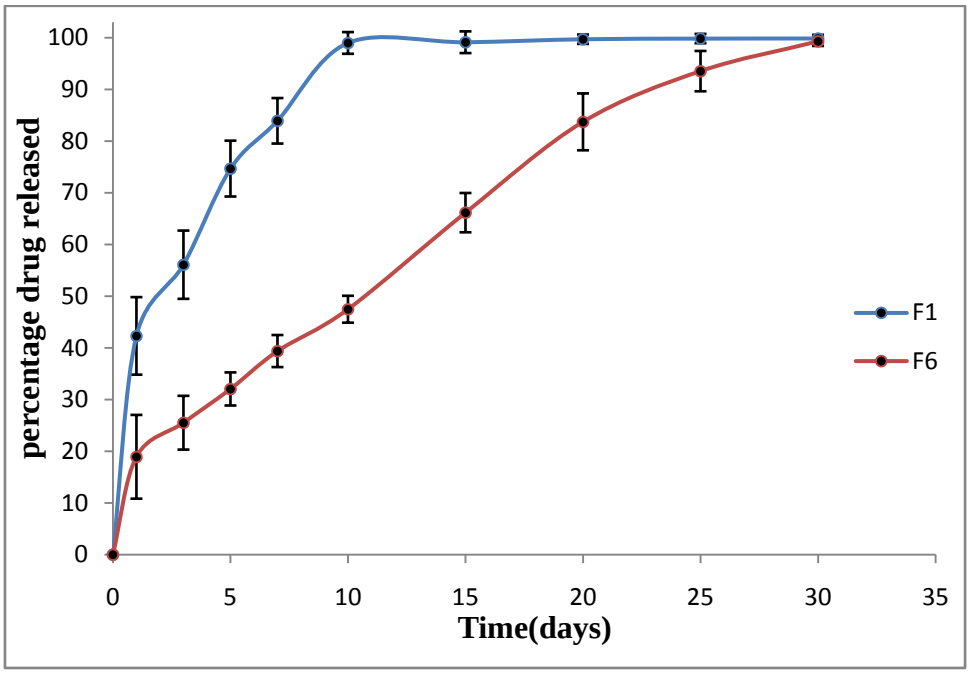


Fig. 2: In vitro drug release profile

F1- release profile of microspheres prepared with 5.3% PLGA, F6- release profile of microspheres prepared with 12% PLGA.

Drug release kinetics

Dissolution data derived from PLGA microspheres were subjected to curve fitting by non linear regression using Sigma plot 11. [Table 5] outlines the model used and illustrates the data generated by use of the kinetic models. All the models mentioned in [Table 5] were applied using dependant variable (% drug release) and independent variable (time). The selection of the most appropriate model for drug release kinetics was based on Akaike Information Criteria (AIC) and Schewartz Information Criteria (SC). The Akaike Information Criterion (AIC) is a measure of the goodness of fit of a particular model based on the maximum likelihood[16]. When comparing several models for a given set of data, the model associated with the smallest value of AIC is regarded as the best fit out of that set of models and if two models of one formulation had similar values then a model with less number of parameters is considered as the best fit. AIC and SC were calculated using the following equation.

$$AIC = n \times \ln(SSR) + 2P$$
$$SC = n \times \ln(SSR) + p \times \ln(n)$$

Where,

SSR = sum of squares of residues obtained directly from the software
n = number of dissolution points
P = number of estimated parameters of model

Using Peppas Model n value for F1 formulation 0.27 was obtained indicating that drug release from PLGA microspheres was regulated through Fickian diffusion. As the concentration of the polymer increased the n value also increased from 0.27 to 0.57 indicating that the drug release pattern changed from Fickian to non fickian diffusion, suggesting that the polymer concentration affected the drug release pattern. These results confirmed that diffusion and polymer erosion were the primary operating release mechanism for the optimised formulation. These conclusions are supported by experimental data generated from erosion studies where 33 % polymer erosion was observed within 30 days.

Swelling and Erosion study

The swelling and erosion behaviour of the optimised TMZ microspheres in phosphate buffered saline pH 7.4 is shown in [Figure-3]. From the graph it is inferred that the PLGA microspheres displayed a limited amount of swelling throughout the study. The maximum degree of swelling was observed on the first day. This may be attributed to the inherent hydrophobic nature of PLGA. Thereafter, minimal changes in the degree of swelling were observed which may be attributed to the inherent hydrophobic nature of PLGA. In addition the erosion of the microspheres which occurred throughout the test period also might have contributed to the lower swelling values. Erosion of the microspheres occurred through the test period.

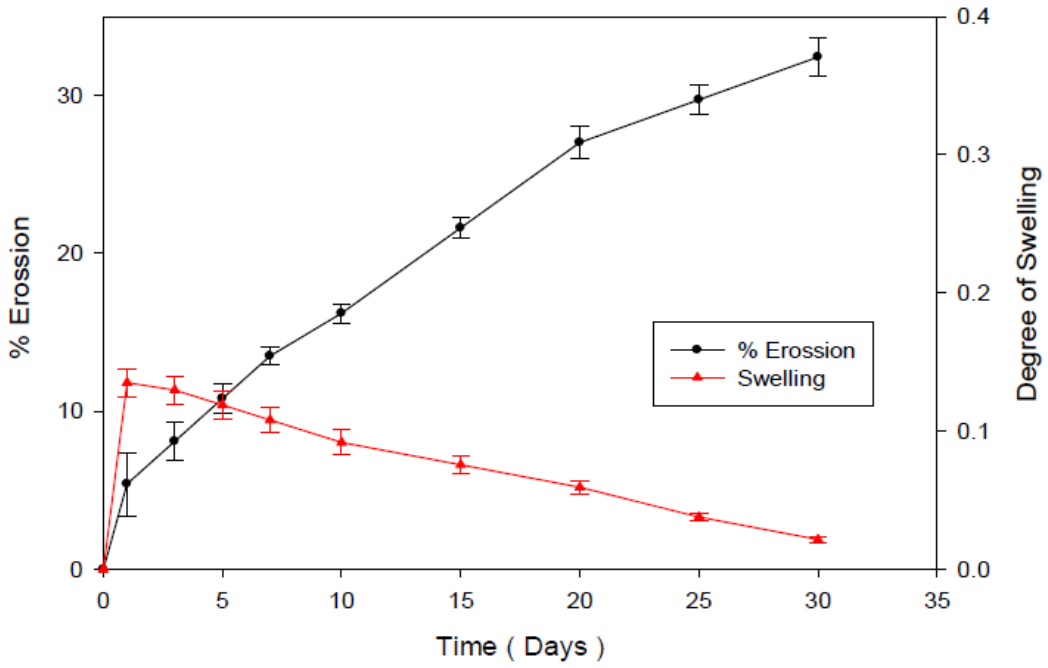


Fig. 3: Degree of swelling and erosion of formulation F6

Scanning electron microscopy [SEM]

SEM was performed on microspheres prepared with 3:1 (DMSO: DCM) solvent ratio [Figure 4], optimized PLGA microspheres prepared at 400 rpm at 350 X magnification [Fig 5-A], at 500 X [Fig 5-B], optimized PLGA microspheres at 350 X [Fig 6-A] magnification and 750 X magnification [Fig 6-B]. The SEM phssotographs showed that the shape of the microspheres was nearly spherical. The surface was found to be slightly porous with depressions in certain areas. With the increase in the RPM from 400 to 600 the reduction in the particle size is clearly observed.

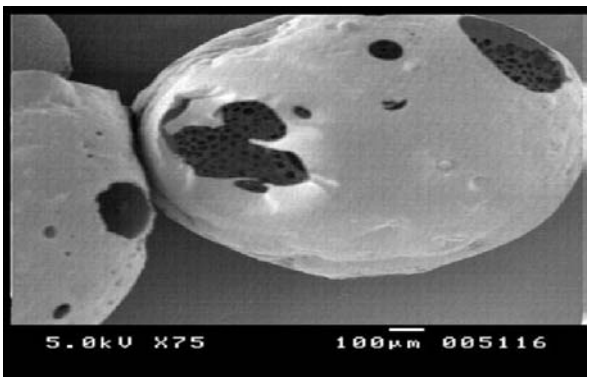


Fig. 4: Microspheres prepared with 3:1 (DMSO: DCM)

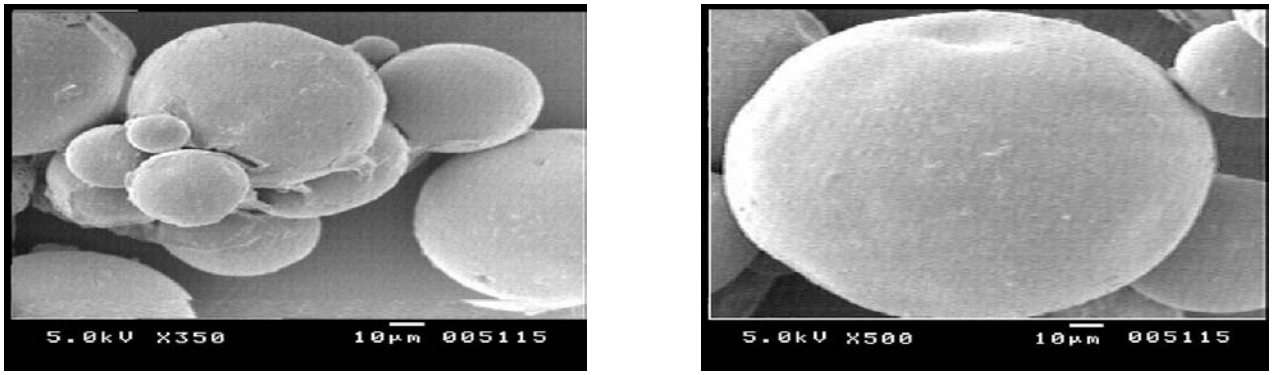


Fig. 5: Microspheres prepared at 400 rpm

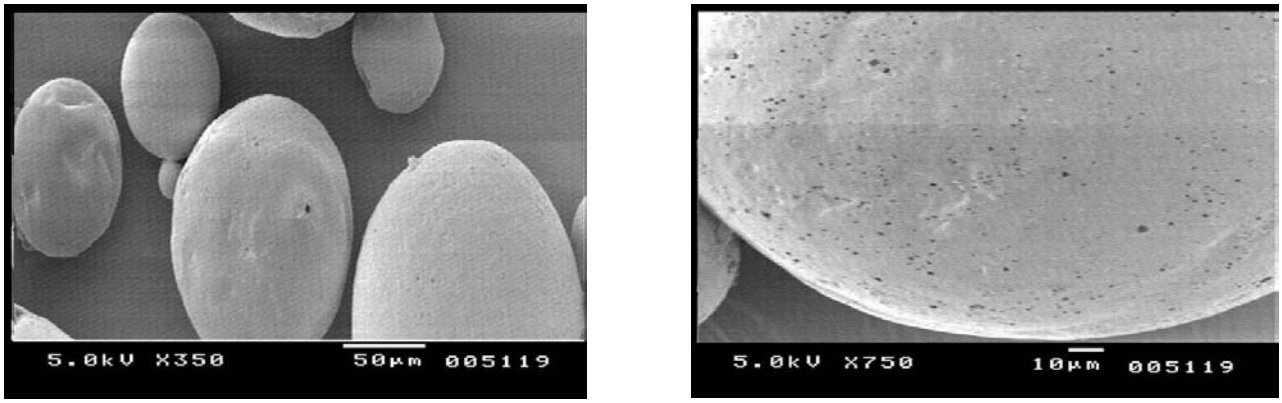


Fig. 6: Microspheres prepared at 600 rpm

Differential Scanning Calorimetry studies [DSC]

In the present investigation, DSC thermogram of the pure drug and drug loaded PLGA microspheres F1 respectively were investigated for drug polymer interactions. The thermogram of pure drug TMZ shows an endothermic melting point at 211.29°C, which corresponds to its melting point [Fig 5]. Placebo showed an endothermic peak at 54.6°C which corresponds to its t_g. Drug loaded PLGA microspheres did not show any peak indicating that the drug might be distributed in the polymeric matrix in an amorphous form.

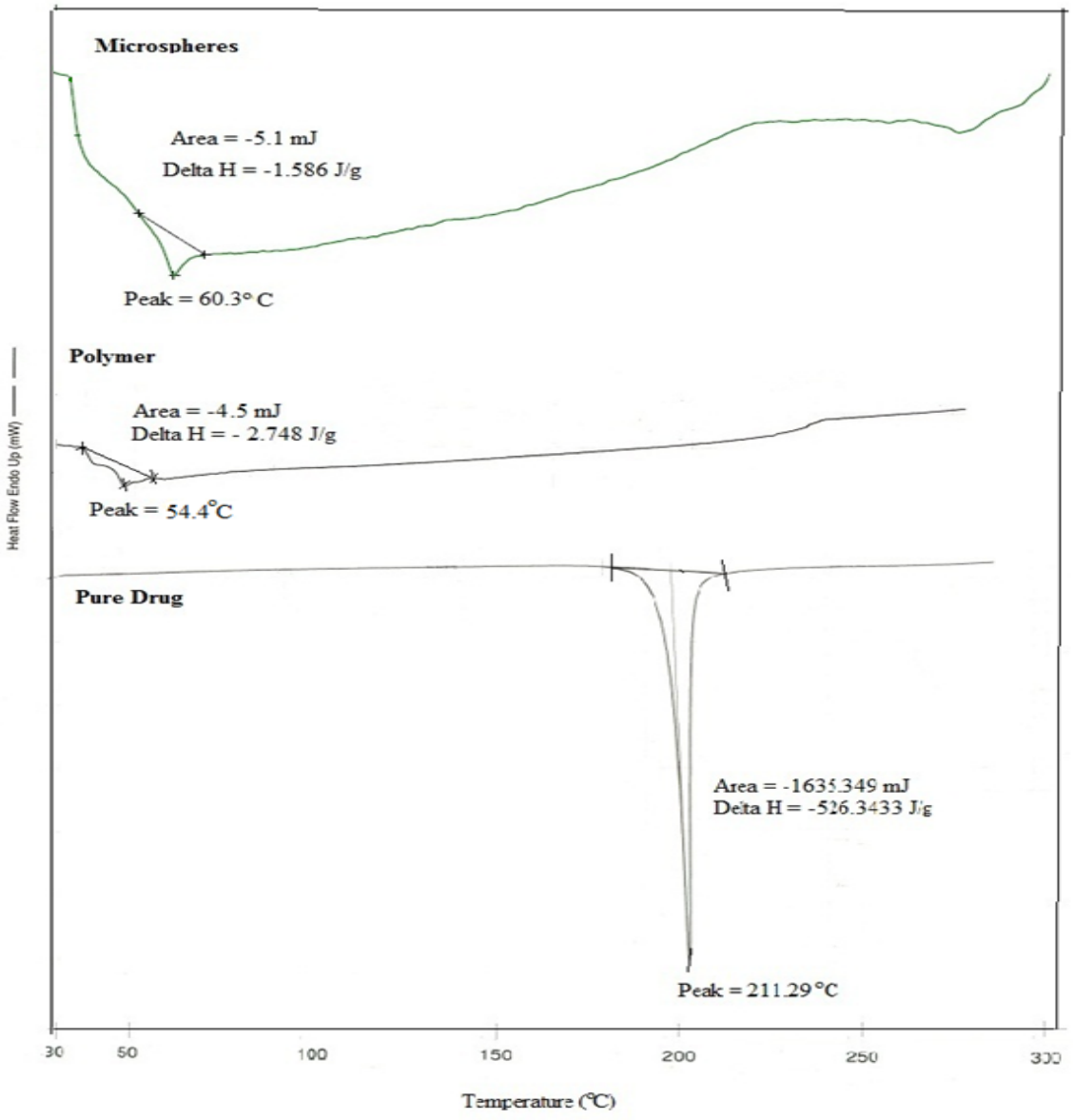


Fig. 7: DSC scan of pure drug, placebo, and microsphere formulation

Table 1: Effect of PVA concentration on particle size and drug encapsulation efficiency

CODE	% PVA	Particle Size	%Encapsulation
A	2 %	74.12	82.24
B	1.5 %	93.56	73.36
C	1 %	128.22	65.15

Table 2: Effect of solvent ratio of on drug encapsulation efficiency

Formula Code	Solvent Ratio (DMSO:DCM)	Encapsulation Efficiency
D	3:1	43.12%
E	2:1	58.35%
F	1:1	76.30%

Table 3: Effect of polymer concentration

Code	Polymer concentration	Particle size	Encapsulation
G	5.33	41.12	67.30
H	9.33	55.11	76.30
I	13.33	74.12	82.24

Table 4: Effect of speed on the particle size

Formula code	polymer	Particle size	Formula code	Particle size
F1	5.3	41.12	F8	38.12
F2	6.7	47.76	F9	44.36
F3	8	51.29	F10	48.65
F4	9.3	55.11	F11	51.88
F5	10.6	61.67	F12	56.19
F6	12	68.33	F13	63.65
F7	13.3	74.12	F14	69.48

Table 5: Mathematical modeling and drug release kinetics

Model	Equation	Plot of graph	Parameters
Zero order	$M_t = M_0 + K_0 t$	Mt/M_0 Vs t	K_0 – zero order rate constant
First order	$M_t = M_\alpha (1\text{-}e^{-k_1 t})$	Log M_t Vs t	K_1 – first order rate constant
Higuchi	$M_t = K_H t^{1/2}$	M_t Vs $t^{1/2}$	K_H – Higuchi constant
Peppas	$M_t/M_\alpha = K.t^n$	Log M_t/M_α	K – Kinetic constant n - exponent characterising Diffusional mechanism.

Table 6: Drug release kinetic data derived from various mathematical models

Code	AIC					
	Zero order	First order	Higuchi	Peppas	K	n
F1	67.44	1.94	62.37	-34.10	0.9006	0.2666
F2	63.67	-8.13	55.95	-42.19	0.9655	0.2997
F3	57.31	-2.89	50.01	-39.79	0.9692	0.3614
F4	53.71	-6.85	47.71	-35.36	0.964	0.4265
F5	51.82	-10.21	44.14	-38.39	0.9804	0.4917
F6	45.22	-4.40	47.78	-30.43	0.9604	0.5345
F7	38.92	-22.40	46.74	-30.98	0.9675	0.5738

Table 7: Drug release kinetic data derived from various mathematical models

Code	SC					
	Zero order	First order	Higuchi	Peppas	K	n
F1	72.71	-0.064	67.08	-46.57	0.9006	0.2666
F2	68.53	-11.26	59.94	-56.46	0.9655	0.2997
F3	61.46	-5.44	53.35	-53.53	0.9692	0.3614
F4	57.46	-9.84	50.79	-48.11	0.964	0.4265
F5	55.36	-13.57	46.82	-51.82	0.9804	0.4917
F6	48.02	-7.12	50.87	-42.08	0.9604	0.5345
F7	41.02	-27.12	49.72	-42.76	0.9675	0.5738

CONCLUSION

TM-loaded PLGA microparticles were prepared by emulsifying-solvent extraction evaporation technique. Parameters, such as polymer concentration and stirring rate were found to have played a predominant role in the preparation. The formed microspheres were found to be spherical in shape. SEM and DSC results indicate that TMZ trapped in the microparticles must be existing in an amorphous state in the polymer matrix. The release profiles of TMZ from microparticles showed biphasic pattern.

The burst release from TMZ from PLGA microparticles decreased with the increase in drug to polymer ratio. The release of TMZ could be sustained for 30 days. The optimized formulations exhibited in vitro sustained release for one month, compared to controlled marketed preparation[Temdor-20mg] (conventional capsules) which showed 85 % of the drug release within 30 min. From the experimental results it is evident that the microspheres of TMZ can be successfully formulated as a promising candidate for treatment in brain tumors. The set objective of formulating a sustained release dosage form of TMZ was achieved as a first step to be further supported by in vivo evaluation.

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