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Loading and Release of 6-Busulfan from Functionalized Multiwalled Carbon Nanotubes Using Incipient Wetness Impregnation Method



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ABSTRACT

Most of the existing anticancer drugs are very potent small molecules; their efficacy is constrained by their systemic toxicity, narrow therapeutic window, low drug loading, size control, scale up, cost of formulation but also as a result of drug resistance and limited cellular entry. In the last few years, carbon nanotubes have been projected as a promising carrier for many drugs including anticancer agents because of the high surface area and efficient targeting capabilities. The present work is an attempt to investigate the potentialities of multi-walled carbon nanotubes (MWCNT) as a carrier for targeting Busulfan to cancer tissues by loading these carriers by the Incipient Wetness Impregnation method. MWCNTs were carboxy functionalized and then loaded with Busulfan using the Incipient Wetness Impregnation method to produce Busulfan loaded CNTs. The conjugates were characterized for drug loading efficiency, *in vitro* drug release and release kinetics. The result indicated that a maximum of about 78% entrapment was achieved. The loaded nanotubes were shown to release the drug for more than 20 hours and thus controlling the release. The release pattern for all the formulations was found to follow the Hixson Crowell release pattern. Our work established several novel and easy to prepare formulations of MWCNTs with acceptable drug loading efficiency and increased dispersibility of CNTs and thus bioavailability at cancer site with reduced systemic toxicity.



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1. INTRODUCTION

Cancer is amongst the top three killers in modern society, next to heart and cerebrovascular diseases. Treating cancer has always been a challenge because cancer chemotherapeutic agents are cytotoxic and cannot differentiate cancer cells from normal cells. This leads to the destruction or impairment of vital organs particularly those that have high rate of cell division like the liver, GI lining, hair and skin; in addition to killing of the cancer cells, if their bio-distribution is not properly controlled and the therapeutic agents not targeted towards the cancer cells or tissues. Thus targeting continues to be the Holy Grail in anticancer therapy.

Discovery of Carbon Nanotubes in 1991 by Iijima provided a ray of hope in this field. Carbon nanotubes (CNTs) are described as hollow cylinders formed by rolling single layer (single-walled CNTs; SWNTs) or multiple layers (multi-walled CNTs; MWNTs) ^[1,2] of graphene sheets into seamless cylinders. In recent years, it has been demonstrated that CNTs can not only be loaded with drugs ^[3-7], nucleic acids and peptides ^[8] by forming stable covalent bonds or supramolecular assemblies based on noncovalent interactions, but also have capacity to penetrate into the cells to promote the cellular uptake of therapeutic molecules ^[9], which has offered new opportunities for their applications in nanobiotechnology and nanomedicine. Although most of the existing anticancer drugs are very potent small molecules, their efficacy is constrained not only by their systemic toxicity, narrow therapeutic window, low drug loading, size control, scale up, cost of formulation ^[10] but also as a result of drug resistance and limited cellular entry. For this reason, the development of efficient delivery systems with the ability to enhance cellular uptake of existing potent drugs is needed. The high aspect ratio of CNTs offers great advantages over existing delivery vectors, because the high surface area provides multiple attachment sites for drugs ^[11]. Functionalization of CNTs not only makes it more soluble/dispersible in water but also provides active sites for attachment of drugs, ligands and other agents like PEG to achieve long blood circulation half-life helping to impede *in-vivo* opsonization and reduced reticulo-endothelial system uptake ^[12]. In addition, many oxygen containing groups, mainly carboxyl and hydroxyl, have been found to decorate the surface of CNTs oxidized with strong acids ^[13].

Several targeted anticancer delivery systems containing Busulfan have been reported [14-18]. In the present study, Busulfan (4-(methanesulfonyloxy)butyl methanesulfonate) (Fig 1) loaded CNTs were developed by slight modification in the Incipient Wetness Impregnation method [19]. Non-covalent functionalization of Multiwalled Carbon Nanotubes (MWCNTs) was achieved using basic treatment followed by treatment with HCl. Functionalized MWCNTs were attached with Busulfan by sonication in a suitable solvent combination. The formulations were characterized for drug entrapment, *in vitro* drug release and release kinetics.

2. MATERIALS AND METHODS

2.1. Materials

Multiwalled carbon nanotubes with 10-15 nm outer diameter, 2-6 nm inner diameter and 0.1-10 μm length were purchased from the Redex Technologies, Pvt. Ltd., Noida (UP) India. Busulfan was received as a gift sample from Salius Pharma, Navi Mumbai, (Maharashtra) India. All other chemicals were of analytical grade purchased from local suppliers.

2.2. Methods

2.2.1 Functionalization of Carbon Nanotubes:

The Carbon Nanotubes were covalently functionalized by subjecting them to three types of treatments [20].

a) Treatment with conc. Hydrochloric acid:

This method simply purifies the CNTs. In this method 500 mg of MWNCTs was placed in a 500 ml round bottom flask and 200 ml of HCl was added. The mixture was stirred using magnetic stirrer for 2 h, then diluted in water, filtered, washed with ultrapure water and then dried in vacuum at 40⁰C overnight.

b) Acidic Treatment followed by treatment with hydrochloric acid:

It is used to produce covalently functionalized MWCNTs. In this method the initial acidic treatment with nitric acid and sulphuric acid produces oxidized MWCNTs and then the treatment with hydrochloric acid produces carboxylated MWCNTs. 500 mg of MWCNTs were added to a 200 ml mixture of 98% H₂SO₄ and 65% HNO₃ (V:V = 3:1) and agitated for 12 h at room temperature. The MCWNTs were thoroughly washed with ultrapure water and

dispersed in HCl and refluxed for 24 h, then collected by filtration and washed with ultrapure water to neutral pH. The product was then dried in vacuum at 40⁰C overnight.

c) Basic treatment followed by treatment with hydrochloric acid:

It is used to produce covalently functionalized MWCNTs. In this method the initial basic treatment with ammonium hydroxide and hydrogen peroxide produces oxidized MWCNTs and then the treatment with hydrochloric acid produces carboxylated MWCNTs. 500 mg of MWCNT was dispersed in 25 ml of the mixture of ammonium hydroxide (25%) and hydrogen peroxide (30%) (V:V=1:1) in a 100 ml round bottom flask equipped with a condenser and the dispersion was heated to 80⁰C and kept for 5 h. After that, the resulting dispersion was diluted in water and filtered. Then the resulting residue was washed with ultrapure water up to neutral pH and the sample was dried in vacuum at 40⁰C overnight.

2.2.2 Selection of the best method for functionalization:

This selection was made on the basis of dispersion stability. For this 10 mg of functionalized nanotubes were dispersed into 10ml of phosphate buffer solution pH 7.4 by sonication for 2 minutes and these dispersions were then kept in sealed vials, the dispersion stability was visually analyzed after a period of 15 days.

2.2.3 Functionalization of MWCNTs by the Optimized method:

After the visual evaluation of MWCNTs it was found that the basic treatment followed by HCl treatment is the best method for functionalization and this was chosen for functionalization of the MWCNTs for the preparation of drug loaded MWCNTs and 3gms of MWCNTs were then functionalized by the basic treatment followed by treatment with HCl. The optimized CNTs were then characterized by the use of FTIR spectroscopy (Perkin Elmer Spectrum II).

2.2.4 Preparation of drug loaded carboxylated MWCNTs:

A concentrated solution of Busulfan (100 mg in 10 ml) was prepared in two different solvents (Acetone and ethanol:water 1:9) in accordance to the ratio in the formulation (Table 1) and this solution was added to carboxylated-MWCNTs. Continuous agitation, using ultra-sonicator, was applied during the addition of the Busulfan solution. The solution thus obtained was stirred for 2 hrs and then the dispersion obtained was filtered using vacuum filtration assembly fitted with membrane filter (0.5 µm, Sigma Aldrich), and then the residue was washed with ultrapure water. The products were dried at 40⁰C for 24 hours.

2.2.5 Evaluation of the Drug loaded CNTs: Prepared formulations were evaluated by following tests:

- Entrapment
- *In vitro* release studies
- Drug Release Kinetics studies

Drug Entrapment

All the formulations were subjected for determination of drug entrapment. The entrapment was determined by dispersing accurately weighed quantity of formulation (containing amount of drug equivalent to 50 mg), into 100 ml of phosphate buffer pH 7.4 and heating upto 37.0⁰c, to ensure the release of the entrapped drug. Aliquot of 1 ml was withdrawn and further diluted to 10 ml with buffer, Busulfan concentration was then determined at 280 nm by using UV-Vis spectrophotometer (UV-1700 Pharma Spec, Shimadzu).

***In vitro* Release Studies**

The *in vitro* release of Busulfan from all the formulations was studied through a dialysis membrane (molecular weight cut off 12000, Sigma Aldrich). The dissolution medium used was freshly prepared Phosphate buffer pH 7.4. An accurately weighed amount of formulation equivalent to 25 mg of drug was calculated and placed in the dialysis tube (approximately 1.2 inch in length), previously soaked overnight in the dissolution medium and the ends were tied to form a pouch. The dialysis tube pouches were then placed in conical flasks containing 100 ml of phosphate buffer pH 7.4, placed in the shaking water bath (HICON, New Delhi) and maintained at 37⁰C with a frequency of 50 shakings per minute. Aliquots, each of 5 ml volume, were withdrawn at regular intervals and replaced by an equal volume of the dissolution medium. The aliquots were then suitably diluted (10 times) and analyzed by UV-Vis spectrophotometer at 280 nm.

Drug Release Kinetics studies

The Drug release data obtained from all the formulations were fitted into various mathematical models given below in order to determine the Drug release kinetics of prepared formulations:

- Cumulative percent drug released V/s. Time [Zero order rate kinetics].
- Log percent drug remaining to be released V/s. Time [First order rate kinetics].

- Cumulative percent drug released V/s. Root Time [Higuchi matrix].
- (Amount remaining to be released)^{1/3} V/s. Time [Hixson-Crowell erosion equation].

To find out the mechanism of drug release, 60% drug of release data was first fitted in the Korsmeyer-Peppas model. Where Log of cumulative percent drug released was plotted against Log Time. The model was used to study the drug release mechanism by analyzing 'n' as the diffusion exponent. According to this model if 'n' is below 0.45 then Fickian mechanism governs drug release, if between 0.45 to 0.89 then Non-Fickian mechanism governs drug release and if n is 0.89 or greater than 0.89, then release mechanism is governed by case-II transport or super case II transport mechanism respectively^[21].

3. RESULTS

3.1 Functionalization of CNTs and selection of the best method for functionalization

The CNTs were functionalized as per the three methods namely; treatment with conc. Hydrochloric acid, acidic treatment followed by treatment with hydrochloric acid, basic treatment followed by treatment with hydrochloric acid. This selection was made on the basis of dispersion stability. For this 10 mg of functionalized nanotubes were then dispersed into 10 ml of phosphate buffer solution pH 7.4 by sonication for 2 minutes and these dispersions were then kept in sealed vials, the dispersion stability was visually analyzed after a period of 15 days. The best method was found to be the basic treatment followed by treatment with hydrochloric acid, as shown in Fig.2 and then the CNTs were functionalized according to this method itself and were used to prepare the formulations.

3.2 Characterization of the functionalized MWCNTS

This was done with the help of FTIR spectroscopy and the FTIR spectra of the functionalized MWCNTs are shown in Fig. 3 (b). This shows the peaks for carboxy group at 1661cm^{-1} (range $1740\text{-}1700\text{ cm}^{-1}$) and hydroxyl group at 3432 cm^{-1} (range $3300\text{-}2500\text{ cm}^{-1}$) which are absent in pristine MWCNT Fig. 3 (a), thus proving that the MWCNTs are now carboxy functionalized.

3.3 Incorporation of the drug

100 mg of the drug was incorporated into the MWCNTs to prepare the formulations according to the formulation design table (Table 1) and then the prepared formulations were kept at 40°C for 24 hours.

3.4 Evaluation:

Entrapment

Table 2 and Fig. 4 show the percent entrapment of drug for the formulations. The entrapment was found to be in quite low, with the maximum at around 78%. Entrapment for the formulations F1, F2, F3, F4, F5 and F6 was found to be 65.66%, 72.45%, 73.66%, 62.23%, 71.45% and 78.66% respectively.

In Vitro Release Studies

The release profile for the formulation predicts how a delivery system might function and gives valuable insight into its *in vivo* behavior. The various formulations of Busulfan monohydrate were subjected to *in vitro* release studies. These *in vitro* release studies were carried out using phosphate buffer pH 7.4 as the dissolution medium.

The average cumulative drug release data obtained in triplicate (n=3) with respect to time for the various formulations were given in Table 3 and the release patterns for the prepared formulations are shown in Fig. 5.

It was found that cumulative percent drug release for F1, F2, F3, F4, F5 and F6 was 90.30%, 92.30%, 94.30%, 76.34%, 94.10% and 82.13% respectively after 20 hours.

All the formulations showed similar release patterns with very slight differences. All formulations showed an initial burst release, which may be attributed to the drug which may be loosely attached to the surface of CNTs or held within the CNTs. Overall these 6 formulations released almost all the drug content within 20 hours and thus were found to be suitable for controlled release.

The prolonged release in the later stage can be attributed to the slow release of the drug from the CNTs. The *in vitro* drug release conditions may vary from those likely to be encountered within the body particularly (the extent of agitation and other factors such as sink conditions). The bioavailability may also be lower than the values suggested *in vitro* release

because of the fast metabolism of the Busulfan in blood. However, the results clearly show that the prepared formulations have the ability to release the drug for prolonged periods of time.

Drug Release Kinetics Studies

Plots of zero order, first order, Higuchi matrix, Korsmayer Peppas and Hixson Crowell models for the formulations were plotted. The regression coefficient (r^2) values of zero order, first order, Higuchi matrix, Hixson-Crowell, Korsmayer Peppas and the 'n' values for Korsmayer Peppas are tabulated in Table 4.

Table 4 shows that for all the formulations, the best fit model was the Hixson Crowell. The 'n' exponent value, for Peppas model, for formulations F2, F3, F5 and F6 was greater than 0.45 indicating that these formulations released the drug by Non Fickian diffusion mechanism. While for formulations F1 and F4 the 'n' exponent value for Peppas model was less than 0.45 indicating that these formulations released the drug by Fickian diffusion mechanism.

4. CONCLUSION

Anticancer drug delivery by using Carbon nanotubes is a new strategy with the potential to maximize the anticancer effect of a drug and reduce systemic toxicity. In this study, we have demonstrated the effectiveness of targeting of the anticancer agent Busulfan by loading it into MWCNTs, thus increasing bioavailability at cancer site and reduction of systemic toxicity due to tumour targeting CNTs has been demonstrated. However some further studies are needed to confirm the *in-vivo* bioavailability of these products and this provides an avenue for further research.

Our work established a novel, easy to prepare formulation of MWCNTs with good drug loading efficiency and improved dispersibility of CNTs in water and provides new directions for preparation of efficient drug carriers.

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ILLUSTRATION:

TABLES

Table 1: Formulation design for preparation of drug loaded MWCNTs by the Fusion Method

| Solvent Used | Formulation Code | Quantity (w/w) | |
|---------------------|------------------|-----------------|---------|
| | | Drug (Busulfan) | c-MWCNT |
| Acetone | F1 | 1 | 1 |
| | F2 | 3 | 2 |
| | F3 | 7 | 3 |
| Ethanol:Water (1:9) | F4 | 1 | 1 |
| | F5 | 3 | 2 |
| | F6 | 7 | 3 |

Table 2: Percent Entrapment for various prepared formulations

| S. No. | Formulation | % Entrapment |
|--------|-------------|--------------|
| 1 | F1 | 65.66% |
| 2 | F2 | 72.45% |
| 3 | F3 | 73.66% |
| 4 | F4 | 62.23% |
| 5 | F5 | 71.45% |
| 6 | F6 | 78.66% |

Table 3: Cumulative Drug Release with time of the prepared formulations

| Time (Hrs) | Formulation | | | | | |
|---------------|--------------------------------|--------|--------|--------|--------|--------|
| | Mean % Cumulative Drug Release | | | | | |
| | F1 | F2 | F3 | F4 | F5 | F6 |
| 0.5 | 1.40% | 0.50% | 3.10% | 3.30% | 0.50% | 0% |
| 1 | 4.90% | 8.70% | 4.20% | 7.30% | 3.10% | 1.50% |
| 2 | 10.40% | 10.90% | 17.20% | 9.90% | 8.90% | 3% |
| 3 | 16.20% | 16.30% | 23.70% | 12.90% | 15.10% | 6.10% |
| 4 | 19.10% | 22.10% | 28.10% | 15.10% | 21.30% | 11.60% |
| 6 | 25.70% | 27.80% | 31.30% | 24.20% | 29.80% | 15.40% |
| 8 | 36.80% | 33.20% | 45.60% | 31.20% | 36.70% | 21.60% |
| 10 | 41.50% | 44.80% | 56.20% | 36.70% | 47.20% | 35.40% |
| 12 | 44.80% | 51.20% | 69.80% | 42.90% | 55.80% | 42.10% |
| 14 | 59.80% | 60.50% | 78.10% | 48.70% | 61.70% | 48.10% |
| 16 | 75.20% | 73.20% | 90.20% | 55.50% | 75.70% | 55.20% |
| 18 | 80.20% | 83.20% | 92.30% | 68.12% | 83.40% | 69.60% |
| 20 | 90.30% | 92.30% | 94.30% | 76.34% | 94.10% | 82.13% |

Table 4: Regression Co-efficients for various models for the prepared formulations

| Formulations | r ² | | | | Korsmayer | | Best Fit Model | Release Mechanism |
|--------------|----------------|-------|---------|-------|----------------|-------|----------------|-------------------|
| | Hixson Crowell | Zero | Higuchi | First | r ² | N | | |
| F1 | 0.982 | 0.963 | 0.962 | 0.942 | 0.925 | 0.382 | Hixson Crowell | Fickian |
| F2 | 0.994 | 0.980 | 0.946 | 0.975 | 0.886 | 0.468 | Hixson Crowell | Non Fickian |
| F3 | 0.986 | 0.972 | 0.886 | 0.962 | 0.942 | 0.587 | Hixson Crowell | Non Fickian |
| F4 | 0.990 | 0.966 | 0.976 | 0.886 | 0.989 | 0.412 | Hixson Crowell | Fickian |
| F5 | 0.983 | 0.942 | 0.973 | 0.925 | 0.975 | 0.581 | Hixson Crowell | Non Fickian |
| F6 | 0.996 | 0.946 | 0.972 | 0.864 | 0.993 | 0.454 | Hixson Crowell | Non Fickian |

FIGURES

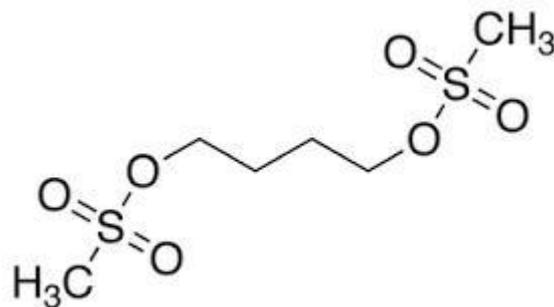


Fig. 1: Busulfan

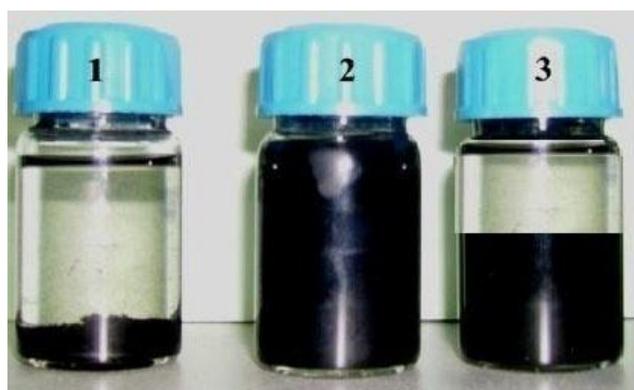


Fig. 2: Dispersions of CNTs functionalized by different methods in phosphate buffer pH 7.4 (1=HCl treatment, 2=basic treatment and 3=acidic treatment) (picture taken after 15 days)

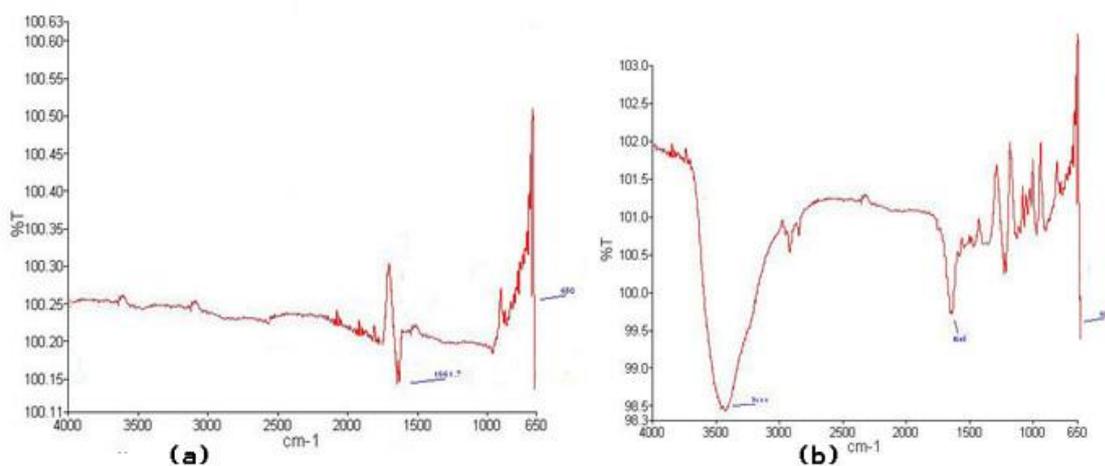


Fig. 3: FTIR spectra of (a) pristine (untreated) MWCNT and (b) carboxylated MWCNT

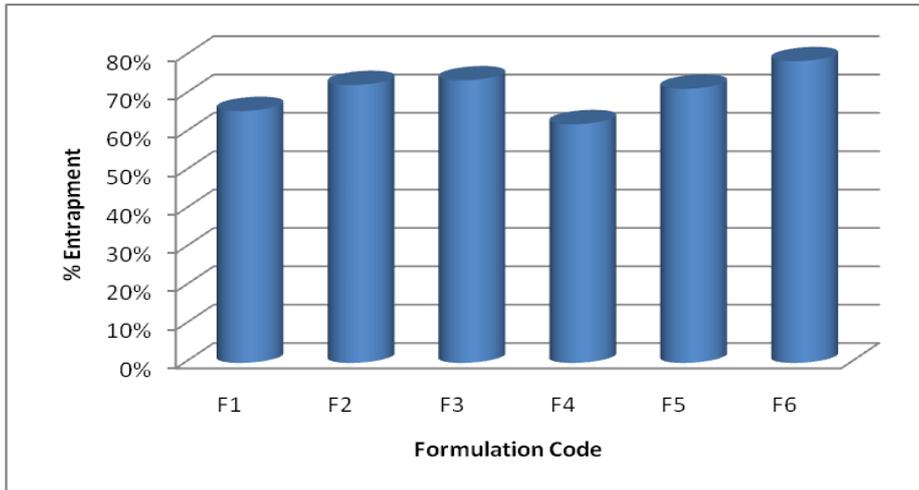


Fig. 4: Bar Chart showing Percent Entrapment for various prepared formulations

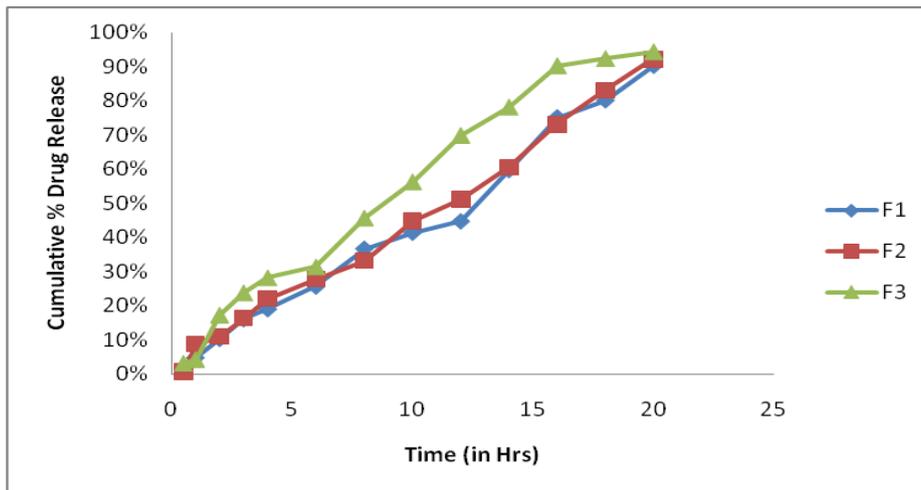


Fig. 5: Release pattern for the formulations prepared by using acetone as solvent

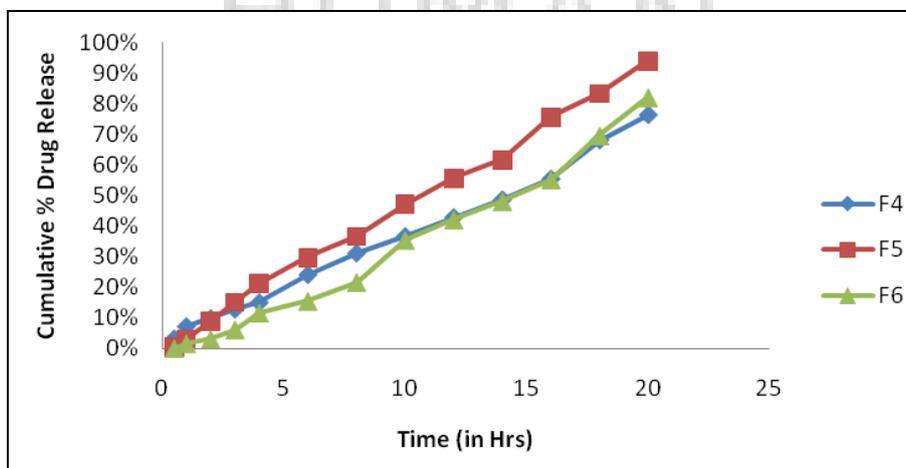


Fig. 6: Release pattern for the formulations prepared by using ethanol:water as solvent