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SOLUBILITY ENHANCEMENT OF ANTICANCER (METHOTREXATE) BY USING PAMAM DENDRIMER AS A SOLUBILIZING AGENT

Navneet Kumar Verma[1], Pushpendra Kumar Tripathi[2]*, Uma Srivastava[3], Ruchi Mishra[4]

[1]Department of Pharmaceutics, Buddha Institute of Pharmacy, GIDA, Gorakhpur, (U.P), India
[2]*Department of Pharmaceutics, Rameshwaram Institute of Technology and Management Lucknow (U.P), India
[3]Department of Mathematics & Statistics, DDU Gorakhpur University, Gorakhpur, UP, India-273009
[4]Assistant Professor, B.P.S. Educational Institution of Pharmacy, Vidya Vihar, Etah, Uttar Pradesh, India
Emai: tripathi.pushpendra@rediffmail.com

***Corresponding Author: -** Pushpendra Kumar Tripathi
*Department of Pharmaceutics, Rameshwaram Institute of Technology and Management Lucknow (U.P), India

ABSTRACT
Methotrexate (MTX) is an immunosuppressive medication used as an anticancer agent. Preformulation studies were carried out by identification test including Appearance (A bright yellow orange crystalline powder), Chemical test (Positive), Melting point (188-193C), Solubility profile (Insoluble in water, ethanol and chloroform, Soluble in methanol but freely soluble in dilute solution of Hydrochloric acid and Sodium hydroxide), Scanning of the drug (λmax was 304nm) I.R. Spectroscopy and prepared calibration curve by U.V Spectroscopy and HPLC method. Calibration curve of MTX in water was prepared by U.V.Spectroscopy and HPLC while in Biological sample and PBS was prepared by HPLC. PAMAM dendrimers (G4-NH2) 0.2% was used as a Solubilizing agent to solubilize MTX for the delivery of the drug. The effect of solvent, pH as well as temperature was calculated by HPLC method. In the Spectroscopical analysis of drug the λmax was shifted from 304 to 372.9.The transmittance of MTX shifted from 59.4 to 75.3 nm after the addition of G4-NH2 Dendrimer. The average particle size of MTX G4-NH2 dendrimer solubilized was 216.5 nm. 24.57% drug was *in-vitro* released in 24 hours. In the Pharmacokinetic study C_{max} was significantly higher than the pure drug.

Keywords: Dendrimers, MTX, PAMAM Dendrimers, Solubilizing agent.

1.1 INTRODUCTION

A novel class of systems which to a large extent fulfills such requirements, combining at the same time advantageous features such as modifiable surface groups, multifunctionality, and nanoscale monodisperse size, are polymers bearing a regular dendritic architecture, referred to as dendrimers.[1,2] These molecules can form covalent or noncovalent complexes with pharmaceutical compounds and act as vehicles for targeted drug delivery and controlled-release purposes.[3] Complex formation with other compounds can be promoted, for example, by solvophobic/solvophilic interactions, hydrogen bonding, or ionic pairing or through chemical binding (conjugation) to their surface groups [4]. A particular class of such molecules namely, poly(amidoamine) dendrimers (PAMAM)s has been widely considered for biomedical applications. Properties such as biocompatibility, [5] water solubility, versatility in modifying their functional groups,[6] and responsiveness of their conformational properties to an aqueous environment [7] provide these molecules appropriate for such uses. These attributes facilitate PAMAM dendrimers to enhance drug solubilization and to assist the transport and the controlled release of complexed pharmaceutical and

biological molecules at targeted sites.[8,9] Among other biomedical uses, PAMAM dendrimers have recently emerged as capable candidates for the encapsulation and the delivery of poorly water-soluble drugs belonging to class II compounds in the Biopharmaceutical Classification System (BCS) [10] (i.e., characterized by poor solubility but high permeability), which still create a challenge in drug formulation.[11,12,13] Poorly soluble compounds also present many in vitro formulation obstacles, such as severely limited choices of delivery technologies and increasingly complex dissolution testing with limited or poor correlation to the in vivo absorption. These in vivo/in vitro correlations are often sufficiently formidable to halt development of many newly synthesized compounds due to solubility issues. Poorly soluble drugs such as Nifedipine and Felodipine have motivated the development of drug delivery technologies to overcome the obstacles to their solubilization through either chemical or mechanical modification of the environment surrounding the drug molecule or physically altering the macromolecular characteristics of aggregated drug particles. These technologies include both traditional methods of solubility enhancement such as particle size reduction via comminution and spray drying, miceller solubilization, and cyclodextrin mediated inclusion complexes. [14, 15,16] Cyclodextins and micelles share something in common: their hydrophobic interior is capable of encapsulating hydrophobic drugs and their hydrophilic exterior is responsible for solubilization. Bountiful literature reporting cyclodextrin-mediated solubilization of drugs is available. [17] High costs and nephrotoxicity on parenteral administration limit the use of cyclodextrins. Moreover, the aqueous solubility of the most commonly usedcyclodextrin, *â*-CD (1.8 g/100 mL at 25 °C), is often insufficient to stabilize drugs at therapeutic doses.[18] The reports on micelle- and polymeric-micelle-mediated solubilization are also in abundance.[19,20] The disruption of micellar structure on dilution with body fluids below critical micellar concentration (CMC) leads to the burst release of the entrapped drugs.

2.1 MATERIAL AND METHOD

2.2 MATERIAL

Methotrexate (Sun Pharma Badodara), PAMAM Dendrimer (Nanosynthon, USA), HPLC water (Rankem), Potassium dihydrogen phosphate (Merk India limited), HPLC grade methanol (Qualigens), Chloroform (qualigens), Whatman filter paper , Magnetic stirrer with hot plate (Simco microscope),Ultrasonic bath sonicater (Relex), Double beam spectrophotometer (Systronic), HPLC (Shimadzu), Electronic balance (Vibra & Essae).

METHOD

2.2 PREFORMULATION STUDIES

2.2.1 Identification of drug

Drug was identified as per USP.

2.2.1.1 Appearance

Physical appearance of the drug was noted by visual observation.

2.2.1.2 Chemical test

Chemical test of the drug sample were performed according to USP.

2.2.1.3 Melting point

Melting point of drug sample was determined by using melting point apparatus.

2.2.1.4 Solubility profile

The solubility of drug at room temperature was determined in different solvents (Lyon 1981).

2.2.1.5 Scanning of drug

Drug solution (0.01%) in water was scanned in range 200-400nm by Systronics double beam spectrophotometer and absorption maxima was noted as per USP.

2.2.1.6 Infrared spectroscopy
The IR spectrum of drug sample was obtained by KBr disc method using shimadzu IR spectrophotometer as per USP.

2.2.2 Analytical method development
2.2.2.1 Preparation of Calibration curve of Methotrexate in water by using UV method (λmax-304nm):
The wavelength of maximum absorption (λmax) was determined by scanning the drug .The media used for Methotrexate were methanol and water.

Preparation of stock solution and standard curve for water(as per USP).
10.0 mg of methotrexate was dissolved in 3 drops of methanol and volume was made upto 10.0 ml with water (HPLC grade) in a 10.0 ml capacity volumetric flask (1mg/ml).
From the standard stock solution (1mg/ml) appropriate aliquots 0.1 to 0.5ml were transferred to a series of 10.0ml volumetric flask, and made upto 10.0ml with water. So as to get concentration 10,20,30,40 and 50µg/ml, the absorption of the solution was measured using Systronics double beam spectrophotometer at λmaxat 304nm.calibration curve was plotted.

2.2.2.2 Preparation of calibration curve of Methotrexate in water using HPLC Method(as per USP):
Equipment
The HPLC was performed with a modular system consisting of a variable wavelength UV visible detector and auto sampler.

Mobile phase
Mobile phase consisted of a mixture of methanol: water (75:25).
Preparation of stock solution and standard curve:-
Accurately weight quantity of drug (10mg) was taken in 10ml volumetric flask, dissolved in 3 drops of methanol, made upto 10 ml with sufficient quantity of HPLC grade water, this gave a stock solution of 1mg/ml. Aliquots were prepared by transferring 0.1 to 0.5ml to a series of 10 ml volumetric flasks and volume was made up to 10ml using mobile phase (methanol and water in the ratio of 75:25), then all aliquots were filtered by whattman filter paper.
The solution was then injected in the 200µl loop attached to the pump, the mobile phase was run at the rate of 1 ml/min. detection were done at 304 nm sample concentration were calculated by measuring covered area and plotting against standard concentration.

2.2.2.3 Preparation of calibration curve of Methotrexate in plasma using HPLC method
The HPLC method reported by AbolghasemJouyban et al; (2011) was followed for estimation of Methotrexate in biological sample.

Equipment

The HPLC was performed with a modular system consisting of a variable wavelength UV visible detector and auto sampler.

Mobile phase
Mobile phase consisted of a mixture of methanol: water (75:25).

Preparation of stock solution and standard curve

Accurately weight quantity of drug (10mg) was taken in 10ml volumetric flask, dissolved in 3 drops of methanol, made upto 10 ml with sufficient quantity of HPLC grade water, this gave a stock solution of 1mg/ml. Aliquots were prepared by transferring 0.1, 0.2,--------up to 0.5ml to a series of 10 ml volumetric flasks and mixed with 0.2ml of rat plasma homogenate and the volume was made up to 10ml then all aliquots were filtered by whattman filter paper. The solution was then injected in the 200µl loop attached to the pump, the

mobile phase was run at the rate of 1 ml/min. detection were done at 304 nm sample concentration were calculated by measuring covered area and plotting against standard concentration.

2.2.2.4 Preparation of calibration curve of Methotrexate in phosphate buffer solution (PBS) using HPLC method (As per USP)

Equipment

The HPLC was performed with a modular system consisting of a variable wavelength UV visible detector and auto sampler.

Mobile phase

Mobile phase consisted of a mixture of methanol: water (75:25).

Preparation of PBS:

8.0g of Sodium hydroxide (NaOH) was shaked in HPLC water until it was dissolved and then added 27.21g of Potassium hydrogen pthallate in 1000ml water (HPLC grade), pH was adjusted 7.4.

Preparation of stock solution and standard curve:-

Accurately weight quantity of drug (10mg) was taken in 10ml volumetric flask, dissolved in 3 drops of methanol, made upto 10 ml with sufficient quantity of HPLC grade water, this gave a stock solution of 1mg/ml. Aliquots were prepared by transferring 0.1 to 0.5ml to a series of 10 ml volumetric flasks and the volume was made up to 10ml with phosphate buffer solution (pH 7.4) then all aliquots were filtered by whattman filter paper. The solution was then injected in the 200µl loop attached to the pump, the mobile phase was run at the rate of 1 ml/min. detection were done at 304 nm sample concentration were calculated by measuring covered area and plotting against standard concentration.

2.3.1 METHOD OF SOLUBILITY MEASUREMENT

An excess amount of drug was added to deionized water containing increasing concentrations of PAMAM dendrimers (G4-NH2) maintained at pH 7.0. The liquid product was briefly sonicated and further agitated at 32 C at 300 rpm for 3 days after equilibrium, it was filtered through 0.45µm pore size nylon membrane and the methotrexate concentration in the sample was determined by HPLC. (Higuchi and Connors; 1965)

2.3.2 PHASE SOLUBILITY STUDY

2.3.2.1 Effect of solvent:

Different concentration of drug was prepared in water using PAMAM dendrimers (G4-NH2). Excess of drug was added to 10.0 ml of above solution and kept for 24 hr with intermittent shaking. The amount of drug solubilized was determined by HPLC.

2.3.2.2 Effect of pH on Solubility

To the dendrimer solution an excess amount of drug was added and the pH value was adjusted to 5, 7 and 9. Electrostatic interaction between hydrophobes and peripheral, as well as internal, tertiary amines of dendrimers is a major mechanism responsible for solubility enhancement. The protonation of nitrogen whether at periphery or at dendrimer interiors is influenced by pH. [21]

2.3.2.3 Effect of Temperature on Solubility

The effect of temperature on solubility enhancement of Methotrexate using G4 PAMAM dendrimers were studied the solubility enhancement of Methotrexate at different temperatures, viz., 30, 35, 40, 45, and 50^{o}C. [22]

2.3.3U.V.Spectroscopy: U.V. Spectroscopy of solubilizate was performed by using U.V. Spectrophotometer (Shimadzu).

2.3.4 Transmittance: Transmittance of solubilizate was carried out by using U.V. Spectrophotometer (shimadzu).

2.3.5Size distribution: Size distribution was measured using a particle size analyzer Malvern Master sizer 4 in reverse Fourier mode.

2.3.6 Stability study: Vials containing powder alone (4mg) solubilizate (4mg reconstituted with buffer) was kept for stability study. Stability was conducted over a month at room temperature and at 40 C and the vials were analyzed for drug content at defined intervals.[23]

2.3.7*In-vitro* release study

A treated dialysis tube was used for in vitro release studies. Formulation equivalent to 2.0 mg drug was introduced into prewashed dialysis tube and placed in a beaker containing 200 ml freshly prepared phosphate buffer solution (pH7.4). The sink condition was maintained by constantly stirring the buffer solution with a magnetic stirrer. Sample aliquots (5 ml) were withdrawn periodically and replaced with equal volume of phosphate buffer solution. Each sample was analyzed by UV Spectrophotometer at 304 nm against PBS as blank.

2.3.8*In-vivo* study

2.3.8.1 Selection of animals, caring and handling

In the present study male albino rats of average weight (150-200 gm) were used. All the animals were procured from the disease-free animal house of Central Drug Research Institute, Lucknow, India. The animals had free access to food and drinking water as per CPCSEA dietary norms. They were subjected to natural light-dark cycle (12 hours each). The animals were acclimatized for at least 5 days to the laboratory conditions prior to experimentation. The experimental protocol was approved by the Institutional Animal Ethics Committee wide its letter No-LSCP/2010/463 dated 13-04-2012. The care of the animals was taken as per the guidelines of CPCSEA, Ministry of Forests & Environment, and Government of India.

2.3.8.2 Pharmacokinetic profile

The albino rats (average wt.150-200 gm) were divided into three groups each containing six rats. First group received 500 µg of Methotrexate solution through i.p. route. Group second and third received 2500 µg free Methotrexate solution and equivalent dendrimer formulation through i.p. route. The blood sample was collected at 2, 4, 8, 12, 18, 24 hr time intervals from the tail vein in a heparinized syringe. The samples were centrifuged and plasma was collected. From each sample 200 µl of plasma were taken in different volumetric flask and volume was made up to 10 ml with mobile phase that is mixture of methanol: water (75:25) then all sample were filtered by whattman filter paper. The solution was then injected in the 200µl loop attached to the pump .The mobile phase was run at the rate of 1.0 ml/min. detection was done at 304 nm. Sample concentrations were calculated by measuring area and plotting against standard concentration. [23]

3. RESULT AND DISCUSSION

3.1 PREFORMULATION STUDIES:

3.1.1 Physical Properties of drug:

Physical properties of drug sample were investigated and all parameter in agreement with those officially reported. The identification tests were performed according to USP. All identification test was found to be positive. The drug was appeared as Bright yellow-orange crystalline powder, chemical test was found to be positive and the melting point of the drug was found to be 188-193ºC, that is shown in Table no.1.1.

Table: 1.1; Physical properties of Methotrexate

Parameter	Observation
Appearance	A Bright yellow-orange crystalline Powder
Chemical test	Positive
Melting point	188-193ºC

3.1.2 Solubility of drug

The powder drug was studied for solubility in various solvents. It was found to be soluble in methanol, dilute hydrochloric acid, freely soluble in dilute solutions of sodium hydroxide and sodium carbonate and insoluble in water, ethanol, chloroform and diethyl ether that is shown in Table no.3.2.

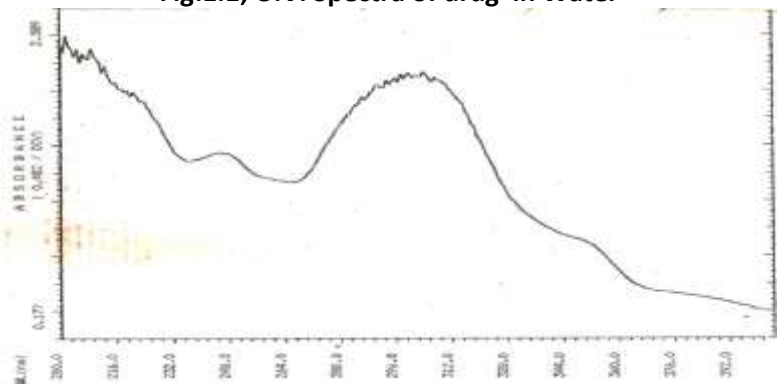
Table:1.2; Solubility of drug in different solvent

SOLVENT	SOLUBILITY
Water	Insoluble
Methanol	Soluble
Ethanol	Insoluble
Chloroform	Insoluble
Diethyl ether	Insoluble
Sodium hydroxide (Dilute Solution)	Freely soluble
Sodium carbonate (Dilute Solution)	Freely soluble
Dilute hydrochloric acid	Soluble

3.1.3 Scanning of drug

The drug sample was scan in spectrophotometer in the range λmax 200-400nm .The absorption maxima for Methotrexate was found at 245.6 and 304nm, which is fully complied with pharmacopoeia specification. The drug sample were almost 99% pure as analyze by official method that is shown in figure no.1.1.

Fig:1.1; U.V. Spectra of drug in Water



3.1.4 I.R. Spectroscopy

The infrared spectrum of the drug was performed that is shown in figure no.1.2a. These infrared spectra of Methotrexate will compared with standard spectra of drug. Which confirm the identity of drug that is shown in figure no.1.2 b.

Figure: 1.2a I.R. Spectra of Methotrexate (Sample)

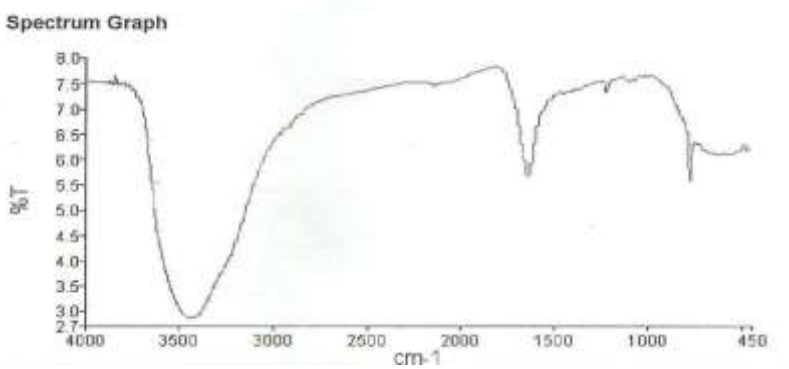
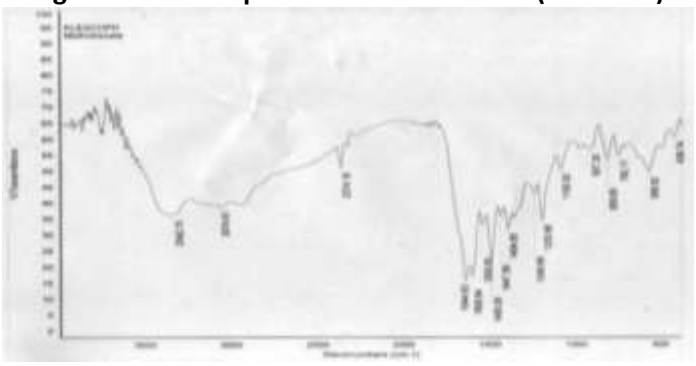


Figure: 1.2b I.R. Spectra of Methotrexate (Standard)



3.1.5 Analytical method Development

After identification on assessment of purity of sample, calibration curve were prepared in UV spectrophotometric method and HPLC method for water, phosphate buffer solution and biological sample for Methotrexate. Calibration curves using HPLC- method, for in vitro performance evaluation during formulation development that is shown in Table no.3.3, 3.4, 3.5 and 3.6 respectively and in figure no.3.3, 3.4, 3.5 and 3.6 respectively. The spectrophotometric method was selected to its sensitivity and simplicity. The absorption maxima (λmax) selected were 304 nm for Methotrexate shows excellent linearity and obeys bears-lamberts law in the concentration used (10 to 50µg/ml) for Methotrexate. The correlation coefficient values were greater than 0.99 for the drug.

Figure 1.3; Standard Curve of Methotrexate by U.V. Spectroscopy

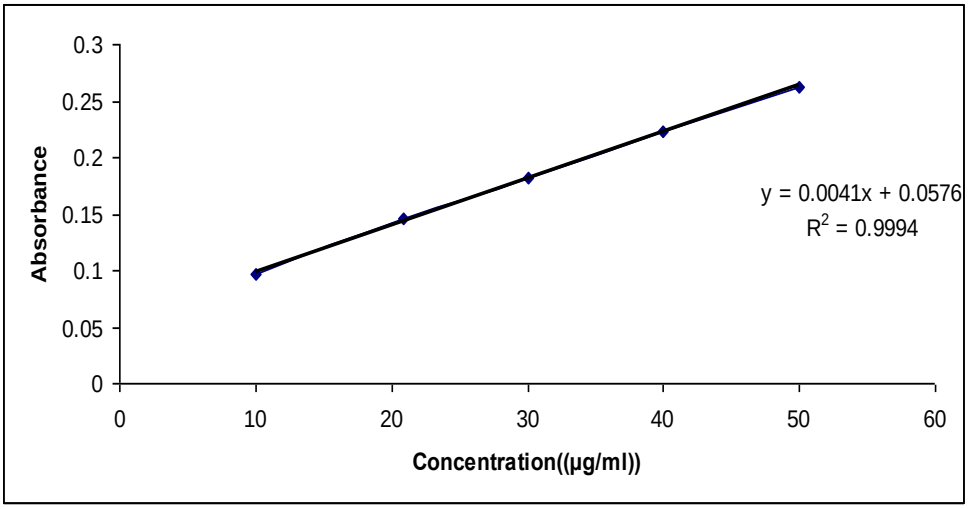


Figure.1.4; Standard curve of Methotrexate in water by HPLC method at 304nm

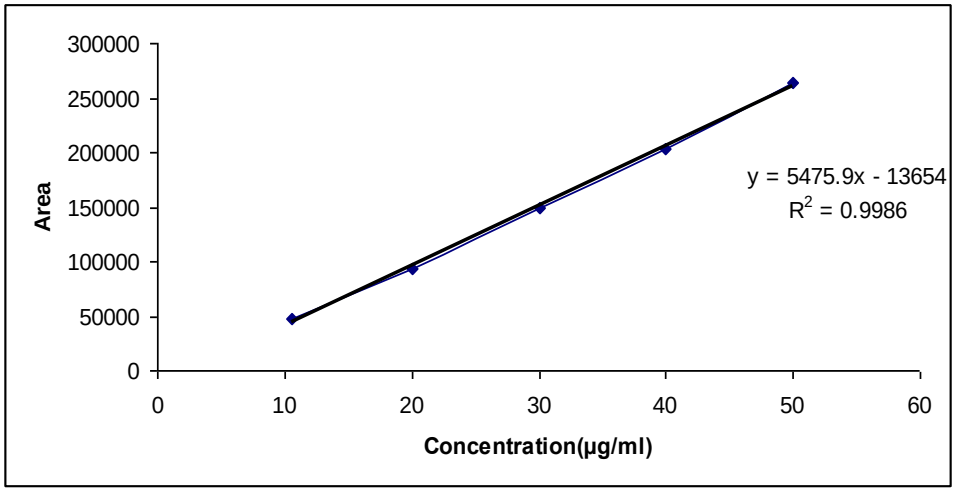


Figure: 1.5 Standard curve of Methotrexate in biological sample at 304nm

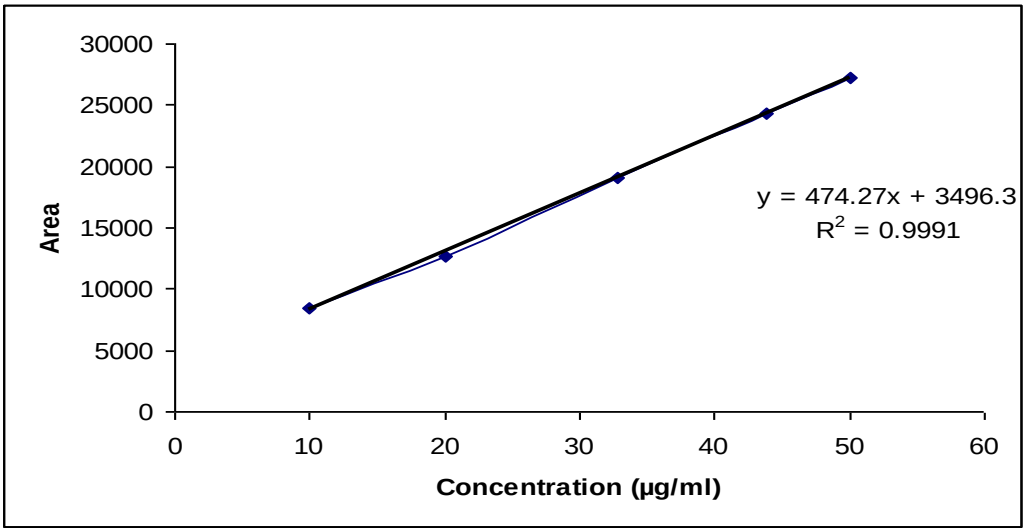
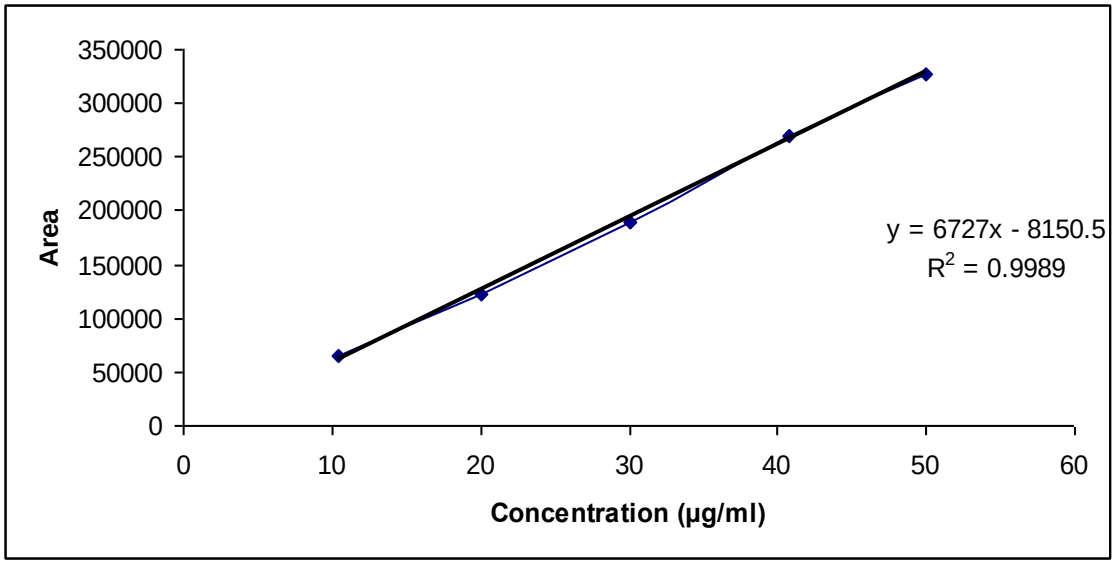


Figure: 1.6 Standard curve of Methotrexate in phosphate buffer solution (pH7.4) By HPLC method at 304nm



3.2 FORMULATION AND CHARACTERIZATION

A Solubilizate was formed, which was characterized for the different parameters of solubility enhancement.

3.2.1 Phase solubility study

3.2.1.1 Effect of Solvent;

The solubility profile of Methotrexate is a function of increasing concentration of the G4-NH2 dendrimer in aqueous solution at pH – 7.0. The result were given in the observation Table 3.7.The number of Methotrexate molecules associated with each molecule of dendrimer was calculated from the solubility data. At 0.2% w/v NH2-dendrimer concentration, 1 x 10^{-6} moles of Methotrexate was associated that was given in Table 1.3.

Table: 1.3 Effect of Solvent;

Solvent	Dendrimer molar conc^{n} 10^{-6}	MTX dissolved (mg/ml)
Water	1	0.5089
Water	2	0.7119
Water	3	1.122

3.2.1.2 Effect of pH;

Methotrexate solubility increased linearly with increasing concentration of amine-terminated PAMAM dendrimers (G4) at pH 5, pH 7 and pH 9 given in Table 1.4.

Table: 1.4 Effect of pH;

S.N.	SYSTEM	SOLUBILITY AT DIFFERENT pH		
1.	**A-NH2**	pH-5	pH-7	pH-9
		0.02	0.09	0.16
		0.05	0.19	0.29
		0.07	0.38	0.46

3.2.1.3 Effect of Temp;

The solubility of Methotrexate decreased with increase in tempreture, shown in Table 1.5.

Table: 1.5 Effect of Temp;

S.N.	TEMPRETURE	SOLUBILITY (mg/ml)
1	30	0.12
2	35	0.11
3	40	0.09
4	45	0.07
5	50	0.06

3.2.2 Spectroscopical analysis of Solubilizate

The λ_{max} of solubilized Methotrexate shifted from 304.0 to 372.9 nm. This suggests little interaction between the dendrimer and the drug (Table 1.6).

Table: 1,6: Spectroscopical analysis of Solubilizate

S.N.	Solution	λ max(nm)
1	Methanolic drug solution	304.0
2	Dendrimer solution	269.4
3	Drug+ Dendrimer + Water	372.9

3.2.3 Transmittance of Solubilizate

The transmittance of Methotrexate shifted from 59.4 to 75.3 nm after the addition of G4-NH2 Dendrimer. The observation was shown in Table 1.7.

Table: 1.7 Transmittance of Solubilizate

S.NO.	Formulation code	Transmittance at λ max 650nm	
		Without dendrimer	With dendrimer
1	A-NH2	43.6	58.8
2	B-NH2	63.4	75.3
3	C-NH2	71.3	91.8

3.2.4 Average Particle size

The average particle size of Methotrexate G4-NH2 dendrimer solubilized was 216.5 nm shown in Table 1.8.

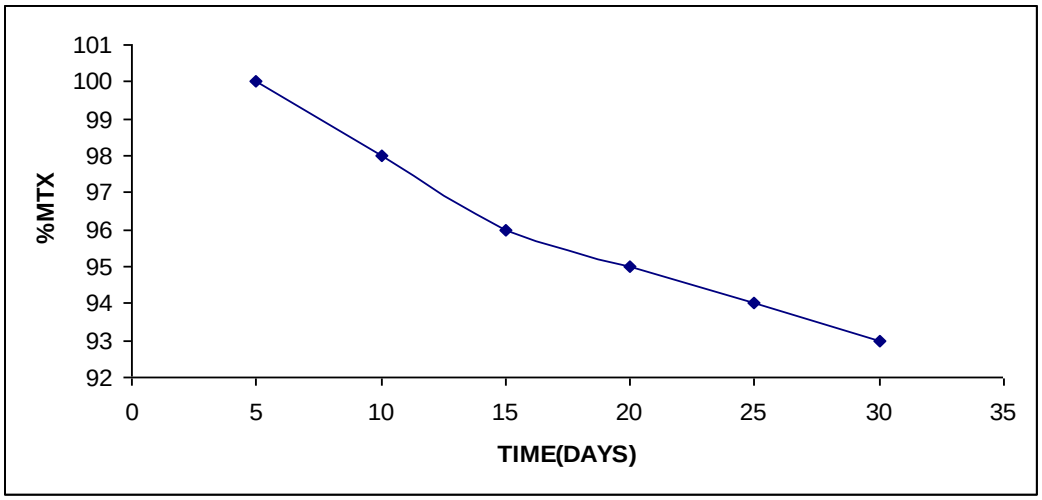
Table: 1.8 Average Particle size

S.NO.	Formulation code	Average particle size(nm)
1	A-NH2	191.6
2	B-NH2	216.8
3	C-NH2	241.1

3.2.5 Stability studies:

Stability study showed about 7% degradation in 30 days indicating relatively good stability is shown in Figure.1.7.

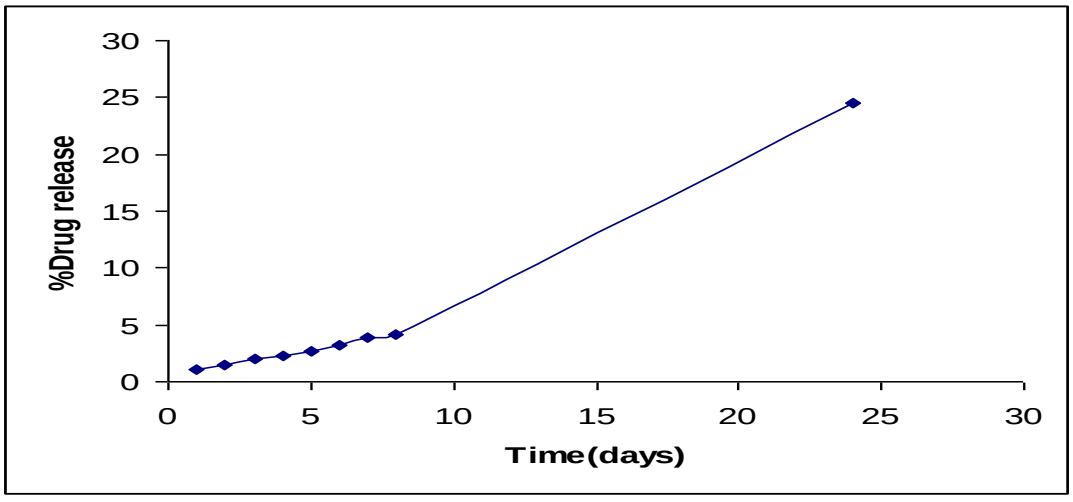
Figure.1.7 Stability graph for Methotrexate:



3.2.6 In-vitro drug release

Release study of Methotrexate were carried out in phosphate buffer solution at pH 7.4, it showed that 24.57% drug was released at 24 hours shown in Figure.1.8.

Figure.1.8*In-vitro* drug release:



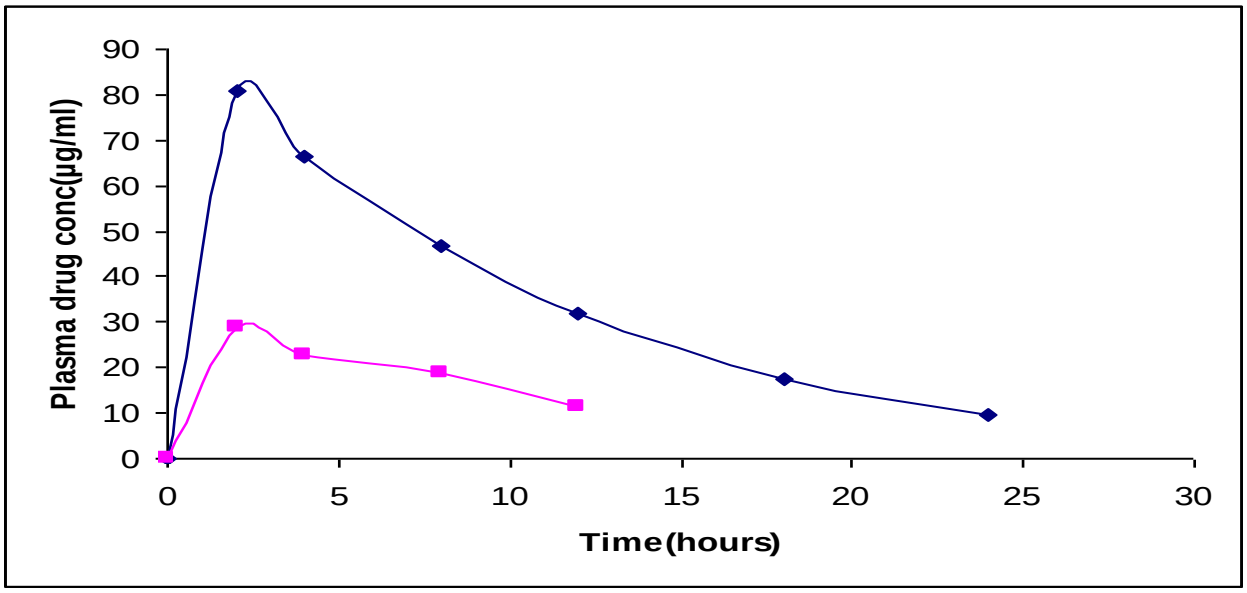
3.2.7 Pharmacokinetic study

In *vivo* performance as shown in Table 1.9, figure 1.9 as plasma profile of Methotrexate, the pharmacokinetic parameters Cmax values of G4-NH2 was significantly higher than the pure drug.

Table 1.9 Pharmacokinetic study

S.N.	Time (hours)	G4-NH2 Concentration (µg/ml)	Plane Methotrexate Concentration (µg/ml)
1	0	0 ± 0.00	0 ± 0.00
2	2	80.80 ± 2.78	28.96 ± 2.37
3	4	66.26 ± 2.56	22.91 ± 2.10
4	8	46.56 ± 2.25	18.82 ± 1.20
5	12	32.02 ± 2.03	11.39 ± 0.98
6	18	17.44 ± 2.01	--
7	24	9.67 ± 0.43	--

Figure.1.9 pharmacokinetic graph



- Plane Methotrexate
- Methotrexate with NH2-dendrimer

SUMMARY

The solubility profile of Methotrexate is a function of increasing concentration of the G4-NH2 dendrimer in aqueous solution at pH – 7.0. At 0.2% w/v NH2-dendrimer concentration, 1 x 10^{-6} moles of MTX was associated. MTX solubility increased linearly with increasing concentration of amine-terminated PAMAM dendrimers (G4) at pH 5, pH 7 and pH 9. The solubility of Methotrexate decreased with increase in temperature. The λ_{max} of solubilized Methotrexate shifted from 304.0 to 372.9 nm. This suggests little interaction between the dendrimer and the drug. The transmittance of Methotrexate shifted from 59.4 to 75.3 nm after the addition of G4-NH2 dendrimer. The average particle size of Methotrexate G4-NH2 dendrimer solubilized was 216.5 nm. Stability study showed about 7% degradation in 30 days indicating relatively good stability. Release study of Methotrexate were carried out in phosphate buffer solution at pH 7.4, it showed that 24.57% drug was released at 24 hours. In *vivo* performance as plasma profile of Methotrexate, the pharmacokinetic parameters C_{max} values of G4-NH2 was significantly higher than the pure drug.

CONCLUSION

The hydrophobicity of the anticancer drug (MTX) creates major problem during the product development and presents a major hindrance in achievement of satisfactory bioavailability. Hence, solubility enhancement of these hydrophobic drugs (MTX) has always been a challenge to the Scientists. The use of PAMAM dendrimer as solubilizing agent has attracted the attention of many scientists due to its characteristic properties. Range of PAMAM dendrimer in their original or modified form has been tried successfully for enhancing solubility of hydrophobes. Studies comparing potential of PAMAM dendrimer in solubility enhancement to improve the delivery of hydrophobic drugs. The toxicity of the amine-terminated dendrimers limits the clinical applications, yet due to its multifunctional nature. Studies are reported which show that masking the terminal amine groups by some means not only considerably improved the efficiency of PAMAM dendrimers in solubility enhancement but also made them more biocompatible. These studies suggest that pH of the medium, temperature, and solvents are the factors that influence the efficiency of dendrimers as solubilizing agent. The hydrophobic interactions are the possible mechanisms by which PAMAM dendrimers apply their solubilizing effect. The insufficiency of studies investigating the effect of temperature on dendrimer-mediated solubility enhancement is yet another aspect which needs increased concentration. As pharmaceutical products are manufactured and stored subject to various temperature conditions and they are exposed to 37 °C in the body, the necessity of relevant study design exploring these effects intensifies. The role of PAMAM

dendrimer in solubility enhancement can become meaningful only if it results in subsequent enhancement in drug bioavailability. A detailed experimentation correlating *in vitro* and *in vivo* performance of dendrimers can yield substantial information, which could be useful in their development as drug delivery. Finally, it can be concluded that PAMAM dendrimer is highly effective for solubility enhancement to improve the drug delivery.

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