

Research Article



Synthesis and Characterization of Pamam Dendrimers Loaded With Anti-viral Drug Amantadine

P. Arshad*, Dr.P. Dineshkumar

Research Center in Pharmaceutics, Hindu College of Pharmacy, Acharya Nagarjuna University, Guntur, Andhra Pradesh, India.

*Corresponding author's E-mail: arshad24arshad@gmail.com

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ABSTRACT

The aim of the present study is to develop and explore efficiency of 5.0G EDA PAMAM dendrimers as long-duration drug release carriers and reduce the hemolytic toxicity of the drug for the treatment of virus. Amantadine (AMD) was selected as a major drug for incorporation into PAMAM dendrimers based on its anti-viral activity and hydrophobic nature. Further polyethylene glycol (PEGylated) PAMAM dendrimers were evaluated for their hemolytic toxicity and in vivo anti-viral studies. The 5.0G PAMAM dendrimers are prepared by using initiator core ethylene diamine and methyl acrylate. Furthermore, the PEGylation was done by polyethylene glycol600 using epichlorhydrin as a cross linking agent. The Amantadine loaded PEGylated 5.0G PAMAM dendrimers were characterized by FTIR and SEM analysis. The in vivo study report ensures the suitability of PEGylated dendrimer in connection to prolonged delivery of Amantadine. Moreover, PEGylated system has shown a reduced hemolytic toxicity.

Keywords: PAMAM dendrimers, Amantadine, PEGylation, in vivo PAMAM dendrimers, anti-viral studies, hemolytic toxicity.

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INTRODUCTION

Influenza is caused by infection with influenza virus. The influenza virus binds to sialic acids on the cell surface and is incorporated into the cell via endocytosis. The H⁺ influx from the endosome into the virion is mediated by the M2 protein¹. As a result, the viral ribonucleoprotein complex releases into the cytoplasm, then virus transcription and replication occurs. The newly synthesized viral proteins assemble with the newly synthesized viral RNA. Virus assembly occurs on the plasma membrane, and the virus particles are released from the cell by viral neuraminidase (NA)².

At present, two classes of anti-influenza viral medicines have been approved: M2 inhibitors (amantadine and rimantadine) and NA inhibitors (oseltamivir and zanamivir)³. However, influenza viruses have mutated and acquired resistance to these inhibitors, thereby decreasing their efficacy; for example, most of the isolates that were identified during the 2005/2006 season in the United States⁴ and Japan⁵ were found to be amantadine resistant. An oseltamivir resistant, seasonal H1N1 virus spread rapidly worldwide during the 2007/2008 influenza season. Despite the currently circulating 2009 H1N1 pandemic viruses still being NA inhibitor-sensitive, the possibility of them rapidly acquiring resistance is very high. A recent study shows

that the number of global annual influenza-associated respiratory deaths is approximately 290,000–650,000⁶. Therefore, the development of novel anti-influenza agents is urgently required.

Dendrimer represents hyperbranched, monodisperse, three dimensional macromolecules with host-guest entrapment properties having defined molecular weight. Dendrimer allows defined control of size, shape and position of functional groups⁷⁻¹⁰. For the above said reason, dendrimers have become more popular application in many fields. Amidst them, the use of dendrimers as a drug delivery carrier is more fascinating¹¹⁻¹². Dendritic architecture is one of the novel drug delivery system with the potential of delivering hydrophobic agents with better prospects. These dendrimeric macromolecules acting as static covalent micelles possess a large number of peripheral end groups and interior cavities proffering a better opportunity for drug delivery¹³. In recent years, dendritic architectures have shown promising scaffolds in many fields ranging from drug delivery to carbonyl metallo immunoassay including development of vaccines, antibacterial, antiviral and chemotherapeutics^{14,15}. Prolonged residence time of the drug in the blood circulatory system and protection of the bioactives from its environment with increased stability are the other potential advantages of dendrimeric architecture. Previous reports suggest the use of dendritic macromolecules for delivery of anti-inflammatory agents.

Cationic dendrimers have shown significant toxicity due to the presence of multiple cationic amine groups¹⁶. To circumvent the cytotoxicity and hemolytic toxicity, it is indispensable to modify the surface of amine groups



of cationic dendrimers with neutral or anionic moieties^{16,17}.

P. Dinesh Kumar et al., (2013) Developed PEGylated G4 and G5 PAMAM dendrimers for anti HIV drug lamivudine. In this study successfully prepared G4 and G5 PAMAM dendrimers with ethylene diamine core and PEGylated with MPEG for surface modifications. Further physiochemical and physiological parameter such as UV, IR, TEM, DSC, drug entrapment, drug release and hemolytic toxicity of both PEGylated and non PEGylated PAMAM dendrimers were determined and compared. Here the PEGylation of PAMAM dendrimers reduce the surface toxicity and increase the drug loading capacity of PAMAM dendrimers. Moreover PEGylated PAMAM dendrimers had released then drug in controlled and prolonged time. Hence the PEGylated PAMAM dendrimers were found as suitable drug delivery carrier for anti HIV drug lamivudine¹⁸.

Although dendrimers have vast application in biomedical field, their usage is restricted due to the RES uptake, drug leakage, immunogenicity, hemolytic toxicity, hydrophobicity etc. But PEGylation of dendrimers can generally overcome these limitations. Polyethylene glycol (PEG) conjugation or linking with the dendritic system is called PEGylation, which improves water solubility and non-statistical attachment of drug molecules. PEGylation of dendrimers is done mainly due to reduce toxicity and Amantadine (AMD) was referred as a standard drug for the treatment of influenza virus¹⁹. The current study is aiming to load Amantadine (AMD) with PEGylated 5.0G EDA-PAMAM dendrimers for the purpose of achieving significant therapeutic effect in the treatment of influenza virus.

MATERIALS AND METHODS

Materials

Amantadine hydrochloride Purchased from Yarrow Chem Products, Mumbai, Methyl Acrylate (SDFCL Fine Chem Limited, Mumbai), Ethylene Diamine (SDFCL Fine Chem Limited, Mumbai), Epichlorhydrine (Merck Specialities Pvt.Ltd, Mumbai), Poly Ethylene Glycol 600 (SDFCL Fine Chem Limited, Mumbai), Methanol 600 (SDFCL Fine Chem Limited, Mumbai), Sodium Hydroxide (Loba Chem Laboratory Reagents and Fine Chemicals, Chennai), Copper sulphate (Merck Specialities Pvt.Ltd, Mumbai), HPLC grade water (Merck Specialities Pvt.Ltd, Mumbai), HPLC grade Acetonitrile (Merck Specialities Pvt.Ltd, Mumbai).

Method

Synthesis of PAMAM Dendrimers PAMAM dendrimers were synthesized by divergent method and construction of an ethylene diamine (EDA) core PAMAM dendrimers consists of successive steps that are Michael addition of primary amine and methyl acrylate followed by amidation of multiester EDA¹⁹.

PEGylation of 5.0G EDA-PAMAM Dendrimers

The PEGylation was done by using epichlorhydrin as a cross-linking agent and in this process 1.8mM (1.8g) of epichlorhydrin and 1.8mM (10.8g) of PEG 2000 were used in 1:1 ratio¹⁹, followed by 3.5 mL solution of 5.0G EDAPAMAM dendrimer added to obtain PEGylation of 5.0G PAMAM dendrimers. To achieve this, above mentioned quantity of PEG was mixed with epichlorhydrin in separate container and kept it stirring for 2 h then it was incubated 36 h in dark place at room temperature, After that, in this 5.0G dendrimer solution was added with proper shaking and it was kept a side for 24 h, to allow the linking of PEG with 5.0G dendrimer using by cross linker epichlorhydrin. The formed PEGylated system was subjected to characterize by IR and SEM.

Drug Loading in Formulation

PEGylated and Non-PEGylated 5.0G EDA PAMAM dendrimers were mixed with methanolic solution of AMD. The solutions were incubated by continuous stirring for 24 h using Teflon beads²⁰. The free drug was removed from formulations by dialyzing in cellulose dialysis bag against double distilled water for 10 min. The estimation of solution was done through spectrophotometrically (λ_{max} 460nm) to determine indirectly the amount of drug loaded in formulations. Then lyophilization of formulations was done and it was taken into further characterization.

Scanning Electron Microscopy

The scanning electron microscopy was performed to study the particle size and surface morphology of PEGylated 5.0G EDA-PAMAM dendrimers. The samples were uniformly spread on double sided carbon tape, fixed on a stainless steel stub, and coated with gold/palladium to prevent charge buildup by the electrons absorbed by specimen.

Dissolution Studies

In dissolution studies drug release of AMD loaded PEGylated and Non PEGylated 5.0G EDAPAMAM dendrimers was carried out by dialysis method²¹. Dialysis bags were filled with a known amount of 25 μ g/mL concentration of AMD loaded PEGylated and Non PEGylated 5.0G EDA PAMAM dendrimers then it was placed in 200 mL of phosphate buffer solution (pH 7.4) at 37°C with slow magnetic stirring. Aliquots of 5 mL were withdrawn from the solution at regular intervals (5, 10, 24, 48, 72, 96, 120 h) and replaced with same volume of fresh phosphate buffer solution. The formulated drug concentrations were determined by spectrophotometrically at 460nm λ_{max} .

Hemolytic Toxicity of Dendrimer-Drug Systems

The human blood was obtained through HiAnticlot blood collection vials RBC suspension (5% hematocrit) and with this 0.5 mL of 25 μ g/mL concentration of AMD encapsulated PEGylated and Non-PEGylated formulations, drug solution, dendrimer solution and 4.5 ml of normal saline was added and it was incubated about 1h²². Other



side in separate tubes drug and dendrimers were taken, the resultant final concentrations of drug and dendrimer were equivalent in all the cases. The PEGylated system of dendrimers-drug complex should be equivalent to Non-PEGylated systems such that the final concentrations of drug and dendrimer were equivalent. The allowed comparison of hemolysis data of drug, dendrimer, AMD loaded EDA-PAMAM dendrimers and PEGylated dendritic architectures were evaluated to the effect of PEGylation on hemolysis. The centrifugation was performed, supernatants were taken and diluted with an equal volume of normal saline and absorbance was measured at 460 nm. To obtain 0 and 100% hemolysis, RBC suspension was added to 5 mL of 0.9% NaCl solution (normal saline) and 5 mL distilled water, respectively. The degree of hemolysis was determined by the following equation

$$\text{Hemolysis} = (\text{Abs} - \text{Abs}_0 / \text{Abs}_{100} - \text{Abs}_0) \times 100$$

Where Abs, Abs₁₀₀, and Abs₀ are absorbance of sample, a solution of 100% hemolysis, and a solution of 0% hemolysis, respectively.

In Vivo Study

The in vivo studies were performed to compare targeting efficiency of drug loaded nanoparticles with that of pure

drug. The animals were kept under standard conditions, with free access to food and water. For each preparation and each sampling time point, rats (Wistar albino rats) were taken (HCOP/IAEC/PR-10/2019). Rats were divided into three groups (Test, Standard, and Control). The drug loaded PEGylated dendrimers (Test), Amantadine (Standard) and dendrimers (control) were given through *i.v* route. After dosing, animals were kept in cage and blood samples through marginal ear vein 0.5 mL were collected in intervals of 0.5, 1, 2, 4, 6, 12, 24, 48, 72, 96 and 120h. The spiked plasma samples were treated with 1.0 mL of acetonitrile and shaken by the aid of vortex for one min. The samples were centrifuged for 20 min at 13000 rpm. The supernatant (1 mL) was transferred into a test tube, and 1 mL of 0.01 M NaOH was added followed by 1 mL of NQS reagent (0.2%, w/v), and the contents of the test tubes were heated in a water bath at 90±5 C for 45 minutes and then cooled in ice water for 2 min. KBH₄ solution (0.2 mL of 0.05%, w/v in methanol) was added and the reaction was allowed to proceed for 5 min at room temperature (25±5 C). The solution was diluted to 5 mL with 0.025M methanolic HCl. A volume of 20 L of the resulting solution was injected into the HPLC system.

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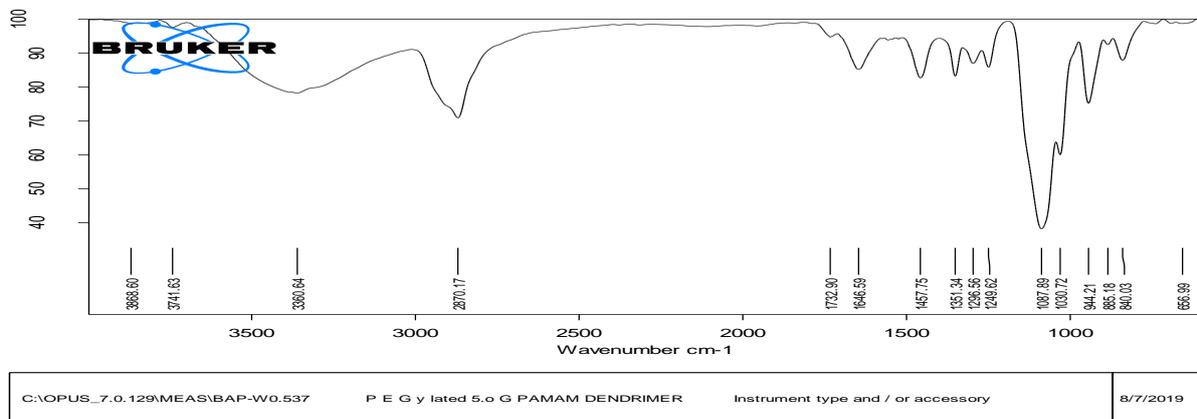


Figure 1: IR spectrum of PEGylated 5.0G EDA –PAMAM dendrimers.

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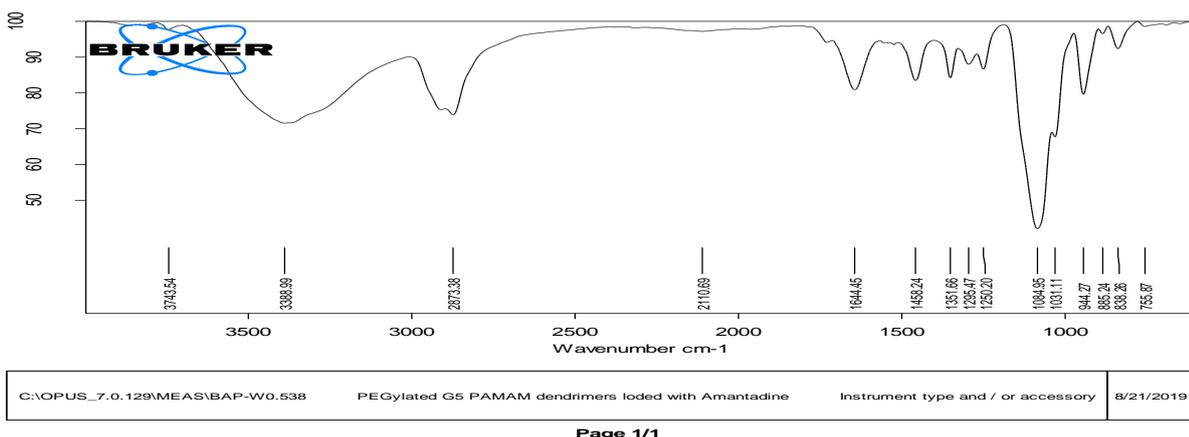


Figure 2: IR spectrum of PEGylated 5.0G EDA – PAMAM dendrimers loaded with AMD.

Stability

The stability studies were carried out for AMD loaded PEGylated 5.0G EDA-PAMAM dendrimers. In this study, sample was stored at 40°C for three months. Drug release studies were carried out to analyze the stability of formulation.

RESULTS

Table 1: Drug release, entrapment, stability and hemolytic toxicity of AMD loaded formulation.

Formulation	% Drug Entrapment	% Hemolytic Toxicity
PEGylated 5.0G EDA PAMAM dendrimers	98.83%	2.71±1.12
Formulation	% Drug Release	
PEGylated 5.0G EDA PAMAM dendrimers	81.79±0.37	
Non PEGylated 5.0G EDA PAMAM dendrimers	98.19±0.56	
Formulation	Storage Time (3 Months)	
PEGylated 5.0G EDA PAMAM dendrimers		
Appearance	Pale red	
% Drug Release	80.83±0.43	

The PEGylation of 5.0G PAMAM dendrimers was confirmed by FTIR and result was represented in (Figs. 1, 2). The IR report of synthesized dendrimers showed stretching peaks of NH stretching[2913.16], C-H alkenes[970.25], and C-N amines[1314.14].

The observed results showed that AMD entrapment efficiency in PEGylated 5.0G EDAPAMAM dendrimers was found to be 98.83% (Table 1). The SEM images (Fig.3) showed that AMD loaded PEGylated 5.0G EDA PAMAM dendrimers are in nano scale and mostly individual and surface morphology of particles found to be spherical in shape and have smooth surface. The evaluation of two formulations Non PEGylated 5.0 G PAMAM dendrimers and PEGylated 5.0G EDA-PAMAM dendrimers loaded with RIF and it was observed that drug release rate of RIF in PEGylated 5.0G EDA-PAMAM dendrimers is 81% in 120 h and in Non-PEGylated 5.0G EDA-PAMAM dendrimers it was released 98% in 72 h. Between two formulations, the indication clarified that PEGylated dendrimers showed relatively slow release as compared with NonPEGylated dendrimers (Fig.4) (Table 1).

The hemolytic toxicity studies were conducted that Non PEGylated amine terminated PAMAM dendrimers showed 14.39 for PEGylated 5.0G EDA-PAMAM dendrimers showed that 2.71. The hemolytic toxicity is less in

PEGylated PAMAM dendrimers than compared with Non PEGylated PAMAM dendrimers.

The *in vivo* study represents PEGylated 5.0G EDA-PAMAM dendrimers loaded AMD and plain AMD (standard) were administered to Wistar albino rats, plasma drug concentrations were evaluated for 6h and 120h for standard and test, respectively. The C_{max} , T_{max} , $t_{1/2}$, MRT and AUC (determined by Kinetic™ 2000 computer programme) were found to be higher for PAMAM dendrimers encapsulated drug resulting in an increase significance ($p < 0.0001$, determined by Prism 5 software) of pharmacokinetic parameters. The T_{max} was found to be 2h for standard and 48h for PEGylated 5.0G EDA-PAMAM dendrimers. After 6h there was no presence of drug in plasma in standard but in PEGylated 5.0G EDA-PAMAM dendrimers it remained for 120h. Thus, PEGylated 5.0G EDAPAMAM dendrimers loaded with drug served to ensure a slow and prolonged release of drug, a feature that could not be achieved with unencapsulated/standard drug. Finally, the drug remained in plasma for shorter time (6h) and longer days (120h) in case of standard and AMD loaded PEGylated 5.0G EDA-PAMAM dendrimers.

The end results showed that PEGylated dendrimers had maximum prolong action (Table 2, Fig.6). After storing AMD-loaded PEGylated 5.0G EDA-PAMAM dendrimers at 40±2°C for 3 months it was found that there is no change either in appearance or in its drug release (Fig.7) (Table 1).

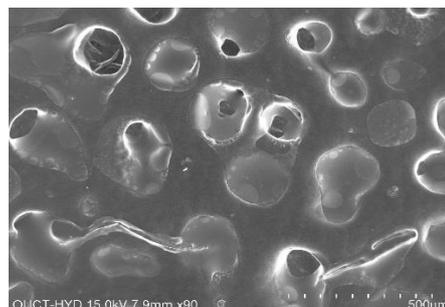


Figure 3: SEM image of PEGylated 5.0 G PAMAM dendrimers loaded with AMD.

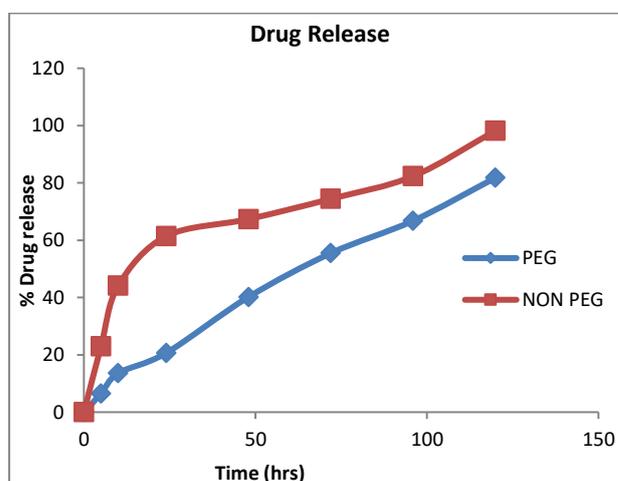


Figure 4: Dissolution of drug release in phosphate buffer pH 7.4.

P.K. Parameters	Standard	Test	P-Value Summary
C_{max} ($\mu\text{g/mL}$)	18.2 \pm 0.07	46.385 \pm 0.08	****
T_{max} (h)	10.3 \pm 0.1	48 \pm 0.1	****
AUC_{on} ($\mu\text{g/L}\cdot\text{h}$)	94.902 \pm 0.79	20038.5 \pm 0.81	****
AUC_{extra} ($\mu\text{g/L}\cdot\text{h}$)	15996.1 \pm 0.71	5941190 \pm 0.76	****
AUC_{tot} ($\mu\text{g/L}\cdot\text{h}$)	115479.5 \pm 0.8	261655 \pm 0.83	****
% AUC_{extra}	17.84835 \pm 0.03	22.88395 \pm 0.24	***
$AUMC_{on}$ ($\mu\text{g/L}\cdot\text{h}^2$)	316.523 \pm 0.56	1202970 \pm 0.71	****
$AUMC_{extra}$ ($\mu\text{g/L}\cdot\text{h}^2$)	145625 \pm 0.72	1452810 \pm 0.65	****
$AUMC_{tot}$ ($\mu\text{g/L}\cdot\text{h}^2$)	463182.5 \pm 0.81	2655685 \pm 0.81	****
$t_{1/2}$ (h)	13.14639 \pm 0.1	66.3999 \pm 0.25	****
MRT(h)	8.0106 \pm 0.2	90.73 \pm 0.12	****

*** $P < 0.001$, **** $P < 0.0001$ for significance

DISCUSSION

PEGylated 5.0G EDA-PAMAM dendrimers were synthesized and produced substantial point of add-on of PEG to dendrimers. Moreover, it is an easy, reproducible and inexpensive method and requires less time due to the presence of cross linker epichlorhydrin. The entrapment efficiency of PEGylated dendrimers was found to be higher when compared with Non PEGylated dendrimers because of hydrophobic interaction of drug and PEG at peripheral p SEM image shows that PEGylated dendrimers are mostly spherical in shape and have a smooth surface which prevents agglomeration. PEGylated dendrimers showed a reduced hemolytic toxicity, owing to the polycationic nature of EDAPAMAM dendrimers. *In vitro* studies indicate that PEGylated dendrimers showed prolonged release compared with Non PEGylated dendrimers because of greater hydrophobic interaction between drug and core of higher generation dendrimer. *In vivo* studies were performed on rats and it was divided into three groups (Test, Standard, Control). PEGylated 5.0G EDA-PAMAM dendrimers loaded with AMD (Test), AMD (Standard) and pure dendrimers (Control) were given to rats and hydrochlorothiazide was used as the internal standard. PEGylated dendrimers showed a prolonged release for longer time when compared with standard. Results demonstrated that the dendrimerization of drug generates an efficient nanotransport system, prolonged and targeted drug release.

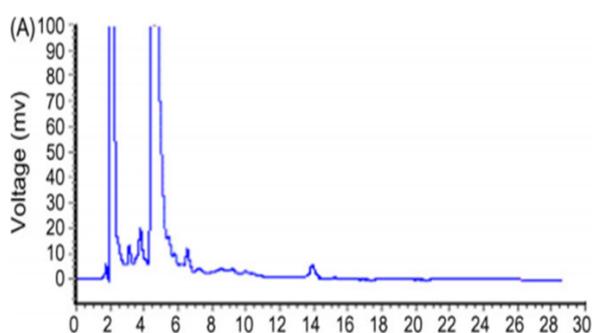


Figure 5: Chromatogram of Amantadine

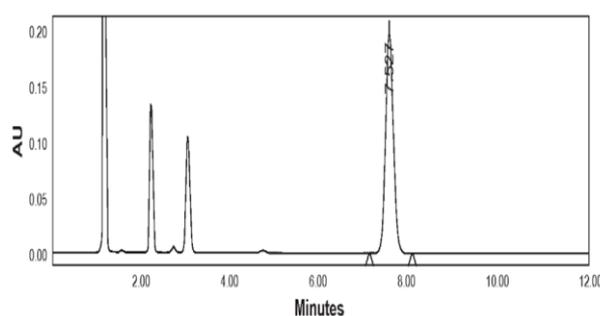


Figure 6: Chromatogram of PAMAM dendrimers loaded with Amantadine (AMD)

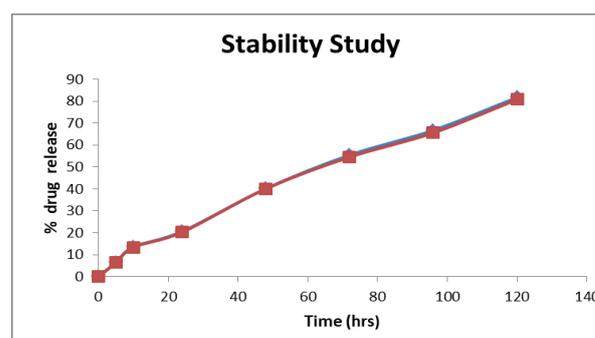


Figure 7: Drug release of PEGylated 5.0G EDA PAMAM dendrimer loaded with Amantadine (AMD) stored after 3 months

CONCLUSION

The PEGylated 5.0G EDA-PAMAM dendrimers showed prolonged longer drug release and to reduce the hemolytic toxicity compared with non PEGylated dendrimers. The *in vivo* studies proved that PEGylated dendrimers remained in the plasma for longer days, showing prolonged action in comparison with standard AMD. Based on the above findings we are conclude that the PEGylated dendrimers may be a potential input for targeted and prolonged drug release and to reduce the hemolytic toxicity. Further extensive *in vivo* studies are to be performed in RBC erythrocytes to ensure dendrimers as a promising tool for targeted drug release.

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