Synthesis and Characterization of Methotrexate Polyethylene Glycol Esters as a Drug Delivery System

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Methotrexate (MTX) is one of the most common anticancer drugs used for chemotherapy so far. However some problems such as high toxicity and short plasma half-life, have limited its use. To overcome these limitations, conjugation with polymers such as polyethylene glycol (PEG) is one the efficient approaches which has been attempted in recent years. The aim of the present study is to synthesize esters of MTX with PEGs of different molecular weights and to characterize their physicochemical properties. Polymeric esters (MTX-PEGs) of MTX with low, medium and high molecular weight PEGs (750 D, 5000 D and 35000 D, respectively) were synthesized using dicyclohexylcarbodiimide (DCC) as coupling agent and triethylamine (TEA) as catalyst. The purification was carried out using preparative TLC. Purified esters were characterized by UV, IR and ^{1}H-NMR spectroscopy methods and their thermal behavior was studied by differential scanning calorimetry (DSC). Also, an isocratic HPLC method with three mobile phase systems was set to determine the partition coefficient of the esters (log P). Gel permeation chromatography (GPC) was utilized for molecular weight determination of esters, which proved 1 : 1 conjugation of drug with each polymer. The stability and solubility of esters were determined in different pH values. The spectroscopy results indicated that esteric bond between MTX and PEGs were formed. The sharp endothermic peaks for MTX-PEGs were obtained in DSC which are similar to pure polymers, whilst a wide peak was observed for MTX. The values of log P for MTX-PEGs (+4.3, obtained by HPLC method) were remarkably different from log P of MTX (−1.4, obtained by shake-flask method). The stability results showed a pH range of 3—4 and an optimum polymer mw of 5000 for maximum stability of esters. A parabolic profile obtained from solubility studies that indicated the more solubility of MTX in alkaline condition (pH>5) and MTX-PEGs in acidic conditions (pH<5). Based on our results, it is concluded that MTX-PEGs were formed on an equimolar ratio of MTX and PEGs. The higher log P observed for the esters indicated dramatic physicochemical differences between MTX and its PEG conjugates and the higher stability and solubility in acidic medium showed a promising approach to improve the drug delivery of the conjugates, specially MTX-PEG5000 in the future.

Key words methotrexate; pegylation; stability; drug targeting

Methotrexate (amethopterin, 4-amino-N^{10}-methyl petroyl glutamic acid, MTX) is a drug that has been used clinically since 1953 for treatment of many tumors especially acute lymphoblastic anemia. It has also been used for several other diseases like choriocarcinoma, psoriasis, sarcoidosis and trophoblastic tumors.[1,2] However some limitations restrict the use of methotrexate including high toxicity due to its low specificity and short plasma half life. In recent decades some methods have been developed to overcome these limitations that polymer conjugation has been of more importance. Conjugation of methotrexate with natural and synthetic long chain polymers like human and rabbit serum albumins,[3,4] poly L-lysine,[5,6] dextran,[7] poly hydroxyethyl methacrylate (HEMA),[8] poly spartamide[9] and gelatin[10,11] proves the importance of methotrexate as an strategic drug and its necessities of polymer conjugation.[29] Polyethylene glycol (PEG) is the most common polymer ever used for conjugation. The researchers have mentioned many rationales for conjugation with PEG (pegylation) including increased water solubility, controlled permeability through biological barriers, longevity in blood stream, controlling of release, overcoming tumor resistance, increased antitumor effect and reduced protein immunogenicity.[12—15] Among the small molecules, anticancer and antiviral drugs have been in priority for pegylation such as paclitaxel,[16,17] podophyllotoxin[18] and 6-mercaptopurin[19] for increasing water solubility, amphotericin B for decreasing drug toxicity and side effects,[20] doxorubicin for increasing efficacy[21] and in case of campthotecin for increasing solubility, efficacy and plasma circulation half life.[22,23] PROTHECAN®, the ester of campthotecin and mPEG40000D is a typical conjugate that is under clinical phase II.[24]

In despite of extensive researches on physicochemical properties of produced pegylated forms of other drugs including water solubility, *in vitro* and *in vivo* bond stability (the study of different linkages and spacers between drug and PEG)[17,25—27] and *in vivo* fate of conjugates,[12,21,28] there is no enough information about different polymer conjugates of methotrexate.[29] Furthermore, as the most of polymer conjugates contain a link between α-carboxyl group of methotrexate and amino or hydroxyl group of synthetic or natural polymers, it is expected to obtain the similar physicochemical properties including partitioning and solubility in different conjugates. In the current study, as part of our aim to improve physicochemical and biopharmaceutical properties of MTX to achieve a better drug delivery system, synthesis of esters of MTX with PEGs of different molecular weight (at low, medium and high molecular weight) and evaluation of the physicochemical properties of MTX-PEGs were described. To the best of our knowledge, no reports on the synthesis and application of the esters of MTX-PEGs as novel drug delivery for MTX has been published.

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Experimental

Materials Monomethoxy PEG 750D and 5000D and Diol-PEG 35000D were purchased from Sigma-Aldrich (St. Louis, Missouri, U.S.A.). MTX was kindly gifted by HEUMANN PCS Company (Feucht, Germany). Dicyclohexylcarbodiimide (DCC), triethylamine (TEA), *N*,*N*-dimethylformamide (DMF), dichloromethane (DCM), chloroform, methanol, acetonitrile, *n*-octanol and TLC silica gel $60F_{254}$ were at least reagent grade and obtained from Merck KGaA company (Darmstadt, Germany). The polystyrene standards for gel permeation chromatography (GPC) were supplied by Knauer Company (Berlin, Germany).

Methods. Synthesis and Characterization of MTX-PEGs Synthesis of MTX-PEGs: Two different methods were used for conjugation of MTX. In method 1, 1.0 mmol of each of MTX and PEG were separately dissolved in dried DMF and then 1 mmol DCC as activator and 0.05 mmol triethylamine (TEA) as catalyst were added to the reaction medium. The mixture was stirred and a white precipitate of dicyclohexyl urea (DCU) was formed after 30 min. The reaction was continued for 12—24 h at 40 °C and monitored using TLC and HPLC until the reaction was completed. In method 2, the MTX and DCC were dissolved in dried DMF and stirred at cold temperature (2—8 °C). After 12 h the white precipitate (DCU) was separated by centrifugation at 12000 rpm for 5 min. The supernatant was taken and after addition of appropriate PEG and TEA, the reaction proceeded for 12 h at 40 °C.

Purification of MTX-PEGs: The purification was carried out on preparative TLC plates coated with silicagel 60F as stationary phase (600 μm thickness). A mixture of chloroform : methanol at a ratio of 85 : 15 (v/v), respectively, was used as mobile phase to separate MTX-PEGs from the reaction mixture. Before separation, the reaction medium was washed using 100 ml of distilled water. The MTX-PEGs were extracted by 20 ml of dichloromethane (DCM). The organic solvent was dried using sodium sulfate, filtered and evaporated under vacuum. The residue was redissolved in 2 ml of chloroform and loaded on TLC plates. The major product was separated and extracted from silica by 50 : 50 (v/v) of methanol–DCM. The solvent was dried and the precipitated product was dissolved in a small volume of DCM and reprecipitated using DCM–hexane.

HPLC Method for the Characterization and Quantification of MTX and MTX-PEGs For analytical HPLC a Merck Hitachi system was used consisting of a pump L-7100, an UV–Vis detector L-7420 and an interface D-7000. The injector system was fitted with a Rheodyne 50 μl loop. The MTX and MTX-PEGs in crude reaction mixture were analyzed by a column (250×4.6 mm) packed with Lichrospher® 100 RP-18 and 10 μm particle size (Merck kGaA, Germany). The mobile phase was phosphate–citrate buffer (pH=6) : acetonitrile (90 : 10, v/v). The wavelength of UV detector was set at 302 nm and flow rate was 1.2 ml/min. For purified esters, a Knauer MZ-analytical column (150×4.6 mm) packed with RP-8 perfectsil (5 μm particle size) and a mobile phase of phosphate–citrate buffer (pH=4) : methanol (30 : 70, v/v) was used. The λ_{max} was set in 342 nm and flow rate was 0.8 ml/min. Each method was validated and the standard curves for quantitative purposes were plotted for MTX and MTX-PEGs separately.

Spectroscopy Characterization of the Purified MTX-PEGs UV spectra of MTX and MTX-PEGs were obtained by dissolving them in the relevant HPLC mobile phases using a single beam UV spectrophotometer model CECIL CE 2021. The infrared absorption spectra of the MTX, PEGs and MTX-PEGs were obtained using a Perkin-Elmer model 843 IR Spectrophotometer over a range of 600 to 4000 cm^{-1}. For this purpose, the MTX and solid esters (MTX-PEG5000 and 35000) were pressed into potassium bromide pellets and the oily MTX-PEG750 was dissolved in chloroform. The ^{1}H-NMR spectra were recorded in $CDCl_3$ solution with TMS as a reference standard at 400 MHz by Bruker Spectrospin NMR spectrometer.

Differential Scanning Calorimetry (DSC) The thermal behavior of MTX and MTX-PEGs were studied by a DSC-60 Shimadzu instrument over a range of 10 to 200 °C.

Gel Permeation Chromatography of the Purified MTX-PEGs for Determination of Their Molecular Weight To determine average molecular weight of MTX-PEG esters, a Knauer GPC system was used consisting of a PLgel 5 μm column (300×7.5 mm) and equipped with a RI detector model 2300. The mobile phase was tetrahydrofuran (THF) and flow rate was set at 1 ml/min. The standard curve was obtained using the standard solutions in THF of polystyrene with molecular weights ranging from 10^2 to 10^6 D (Knauer Co. Germany). All samples were dissolved in THF and injected to HPLC. Then, the average molecular weights of MTX-PEGs were obtained using the obtained standard curve.

Determination of Partition Coefficient of MTX and MTX-PEGs Shake-Flask Method: Partition coefficients of MTX and MTX-PEG esters were first determined between *n*-octanol and isotonic phosphate buffer (composition: KH_2PO_4 1.9 g; Na_2HPO_4 8.1 g; NaCl 4.11 g made up to 1000 ml with water, pH=7.4) at room temperature. The method was set and applied in accordance to the guidelines for testing of chemicals.[30] The identical volumes of buffer and *n*-octanol were mixed and shaken for 24 h to equilibrate. Then, 1 mg of each of MTX and esters was solubilized in enough volume of buffer (for MTX) or in *n*-octanol (for esters) and shaken with the opposite phase for 30 min. Finally, two phases were separated by centrifugation and the amount of drug and esters were assayed in the aqueous phase by HPLC.

Table 1. The Reference Substances Used to Prepare Standard Curve for Determination of log P of Esters

Compounds	log P
2-Naphthol	2.70
3,4-Dichloroaniline	2.80
Benzophenone	3.20
Naphthalene	3.60
Biphenyl	4.00
1,2,4-Trichlorobenzene	4.20

HPLC Method: In this study, we used a RP-8 column and mobile phases with three combinations of 30 : 70, 40 : 60 and 50 : 50 v/v of buffer and methanol, respectively. The pH of each mobile phase was adjusted to 6. Six reference standards were selected, based on the data obtained from literature, covering the range of log P 2.7 to 4.2 (Table 1). A mixture of standards and esters were prepared and injected to HPLC system and monitored at 254 nm. This process was repeated with all three mobile combinations and the retention times were recorded.

Study of MTX-PEGs Stability The chemical stability of esters was studied in buffered solutions including various proportions of 0.2 M disodium hydrogen phosphate and 0.1 M citric acid according to McIlvaine buffer ranging from pH 2.2 to pH 8.[31] A constant ionic strength 0.5 was maintained by adding different amounts of potassium chloride to each pH. Concentrate stock solutions of MTX-PEGs were prepared in acetonitrile. Three separate vials containing 5 ml buffer for every pH were considered and 2% sodium azide was added for preserving against microbial growth. The degradation reactions were initiated by adding 50 μl stock to each vial. The solutions were kept in room temperature and samples were taken at certain intervals and the remaining esters were assayed by HPLC. The area under the curve at time of addition of stock to the buffers was assumed as t_0 and later quantities were compared to it. The degradation kinetic of esters was determined by plotting percentage of the remaining ester *versus* time.

Determination of MTX and MTX-PEGs Solubility Insilico Methods: The aqueous solubility of MTX-PEG esters were determined using three mathematical equations developed by Yalkowsky and his colleagues. These equations apply partition coefficient (log P) and melting point (mp) to calculate molar solubility of compounds.[32—34]

Experimental Method: The solubility of drug and esters was determined in a pH range of 2.2 to 8. The buffers were prepared similar to the pH stability test. An excess amount of each compound was added to the vials containing buffers at each pH value and incubated at 37 °C and stirred for 24 h. Then, contents of the vials were centrifuged at 12000 rpm for 5 min and the supernatants were subjected to HPLC.

Results

Synthesis and Purification of MTX-PEGs Methotrexate is an amino acid like drug with two aromatic NH_2 and two aliphatic COOH groups (α and γ) that can be used for conjugation. Although both of α and γ carboxylic groups of MTX can be esterified with hydroxyl group of mPEG, the α group is much more active and therefore, in equimolar ratio, α carboxylic group is preferred to be esterified.[9] In this study, esterification of carboxylic acid group was performed, in equimolar ratio, using DCC as coupling agent and TEA as catalyst. The TEA, a strong acid scavenger base, increased the efficiency up to 50%. In addition, preactivation of MTX by DCC in cold temperature increased the reaction efficiency (method 2). Purification of MTX-PEGs on TLC plates re-

quired at least 15% methanol in the mobile phase. More quantities of methanol led to spreading of spots on plates and decreased resolution. Under these conditions, the *Rf* values were 0.7, 0.5 and 0.3 for esters of MTX with PEGs of 750 D, 5000 D and 35000 D respectively, whilst MTX was not displaced from the original spot. At room temperature MTX-PEG5000 and MTX-PEG35000 were lemon-yellow solids and MTX-PEG750 was oil. All of the conjugates are freely soluble in the organic solvents, such as ethanol, methanol, chloroform and dichloromethane, but are less soluble in water.

Spectroscopy Studies of MTX-PEGs Comparison between UV spectra of MTX and esters revealed a 40 nm shift in λ_{max} such that the esters had a maximum absorbance around 340 nm compared to MTX that its λ_{max} lays around 300 nm. The IR spectrum of MTX-PEG esters showed two strong peaks in 1100 and 2800 cm^{-1} that were not observed in MTX spectrum (see Fig. 1 which shows the IR spectrum of MTX-PEG5000). ^{1}H-NMR spectra for MTX-PEG750 and 5000 were as follows: MTX-PEG750: ^{1}H-NMR (400 MHz, $CDCl_3$): δ ppm=8.62 (s, 1H), 7.63 (d, 2H), 6.72 (d, 2H), 4.73 (s, 2H), 4.36 (m, 1H), 3.72—3.52 (m, 64H), 3.36 (s, 3H), 1.80—2.2 (m, 4H), (d, 3H, J=7.1 Hz), MTX-PEG5000: ^{1}H-NMR (400 MHz, $CDCl_3$): δ ppm=8.60 (s, 1H), 7.63 (d, 2H), 6.72 (d, 2H), 4.79 (s, 2H), 4.35 (m, 1H), 3.78—3.56 (m, 440H), 3.39 (s, 3H), 1.80—2.15 (m, 4H), MTX-PEG35000: ^{1}H-NMR (400 MHz, $CDCl_3$): δ ppm=8.62 (s, 1H), 7.62 (d, 2H), 6.72 (d, 2H), 4.81 (s, 2H), 4.31 (m, 1H), 3.86—3.40 (m, 3100H), 3.28 (s, 3H), 2.10—2.22 (m, 4H). Figure 2 shows the ^{1}H-NMR spectrum of MTX-PEG5000.

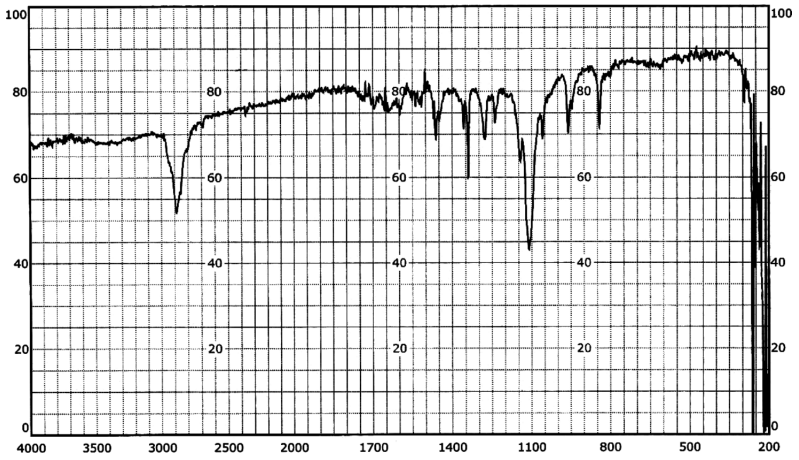

Fig. 1. The Infrared Spectrum of MTX-PEG5000

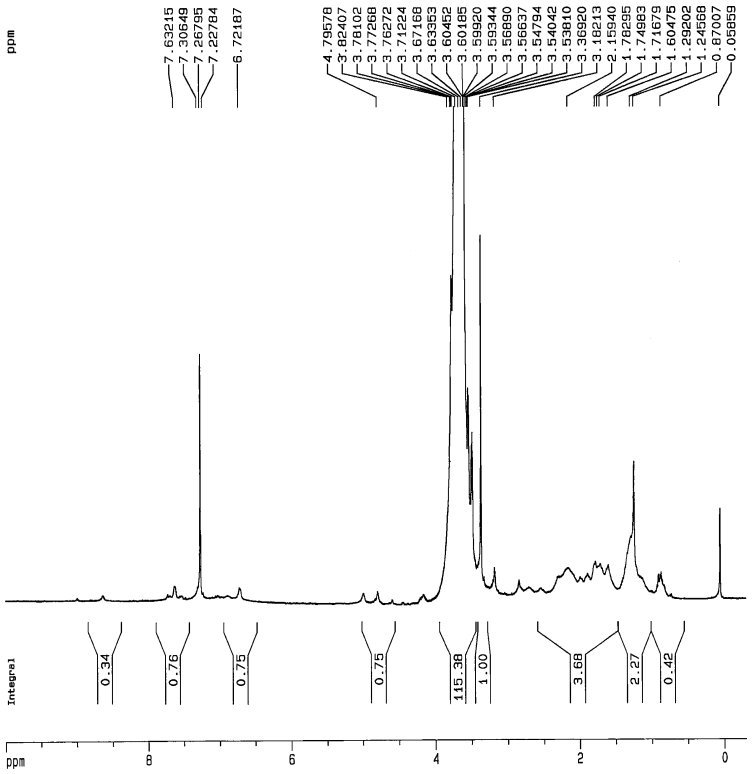

Fig. 2. The ^{1}H-NMR Spectrum of MTX-PEG5000

Differential Scanning Calorimetry (DSC) Compared to MTX, an apparent difference was observed in their thermal behaviors of the esters. MTX had a wide endothermic peak with an onset above 100 °C, whereas MTX-PEGs showed sharp peak with onsets of 34, 55 and 60 °C for MTX-PEG750, 5000 and 35000, respectively (see Fig. 3).

Molecular Weight Determination of MTX-PEGs by GPC The average molecular weights (MW) of MTX-PEG750 and MTX-PEG5000 were found 1108 and 5720, respectively. In the case of MTX-PEG35000 the obtained wieght was variable but lower than 35000 (mean 30000). Figure 4 demonstrates GPC profile of MTX-PEG5000.

Determination of Partition Coefficients of MTX and MTX-PEGs Shake-Flask Method: The partition coefficients were calculated using the following equation:

$$\mathrm{P} = \frac{c_\mathrm{i} - c_\mathrm{w}}{c_\mathrm{w}} \times \frac{v_\mathrm{w}}{v_\mathrm{o}}$$

where c_i and c_w represent solute concentration in the aqueous phase before and after distribution respectively, v_w and v_o represent volume of the aqueous and octanol phases, respectively. According to the guideline,[30] this method is useful when log P of compounds are in the range of −2 to 4. In the case of MTX, the average P and log P of three experiments were 0.04 and −1.4, respectively. Due to higher tendencies of MTX esters to the organic phase, their concentrations in aqueous phase varied and were not detectable under the experimental conditions. It can only be concluded that log P of the esters are too high to be determined by shake-flask method.

HPLC Method: The chromatographic method can give an exact estimatation of partition coefficient in range of 0 to 6, especially when the compounds are very lipophilic (log P≥4) and shake-flask method is not applicable. This method benefits from a reversed-phase column (RP-8 or 18) and is based on the capacity factor; k is calculated according to the following equation:

$$k = \frac{t_\mathrm{r} - t_\mathrm{o}}{t_\mathrm{o}}$$

in which, t_r is retention time of the test or reference substances, and t_o is average time of thiourea (a highly hydrophilic substance) which passes through the column with no retention (dead-time). Figure 5 shows the chromatogram in

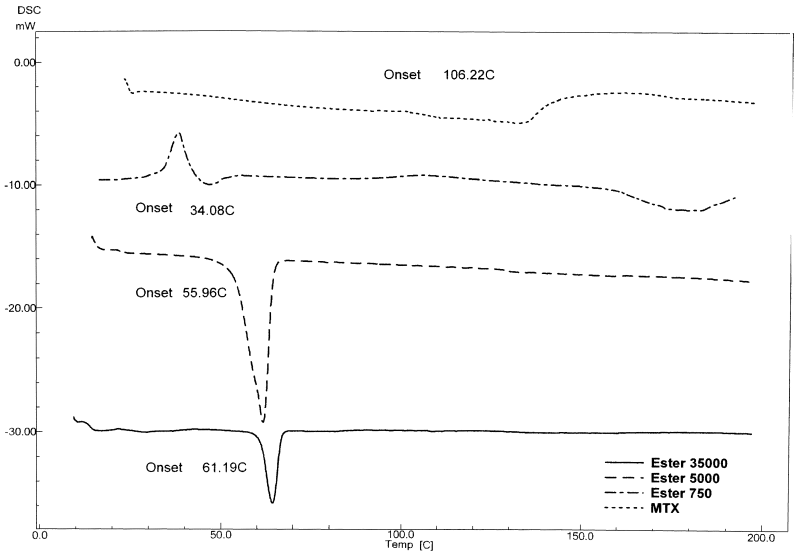

Fig. 3. The DSC Thermal Behavior of MTX and MTX-PEGs

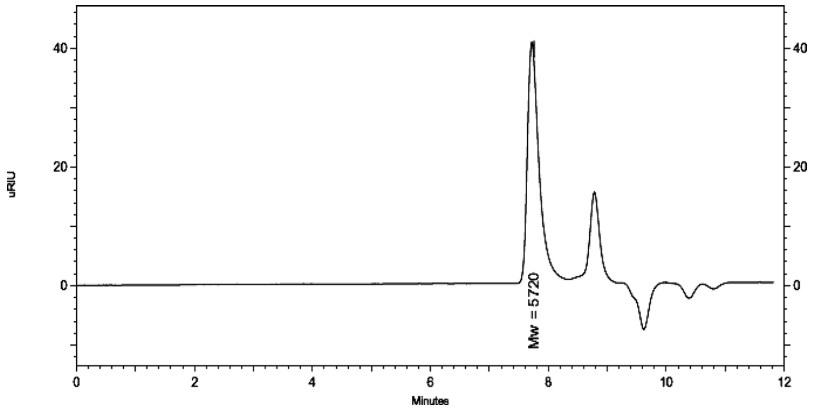

Fig. 4. GPC Profile of MTX-PEG5000

The large peak with MW 5720 is related to the conjugate.

40 : 60 of buffer: methanol mobile phase. The log k of standards and esters in a mobile phase containing 100% buffer solution was obtained by extrapolation and the standard curve was obtained by plotting $\log k_{100\%B}$ *versus* log P. Finally the log P of esters was calculated using the standard curve. Table 2 shows the HPLC data for determination of partition coefficients. The log P obtained from standard curve is 4.31 and identical for all conjugates. These results show that the conjugates have very high tendency to lipophilic phase compared to the aqueous phase ($p>10000$).

Study on MTX-PEGs Stability Stability studies on MTX-PEGs showed that the esters stability was pseudo first order in all pH values (log % remaining *versus t* was linear). It was obsvered a sigmoidal pH-stability curve with a minimum at pH 3—4 (maximum stability) and a maximum at pH 5—6 (minimum stability) (Fig. 6). The results showed that MTX-PEG5000 is the most stable ester (Table 3).

Determination of MTX and MTX-PEGs Solubility Insilico Methods: Water solubility (S_w) was calculated using the following equations (Table 4):

$$\text{Log } S_w = -\log P - 0.01mp + 1.05 \quad (1)$$

$$\text{Log } S_w = 1.17 - 1.38 \log P \quad (2)$$

$$\text{Log } S_w = 0.54 - \log P - 1.11\Delta Sf(mp-2)/1364 \quad [\Delta Sf = 13.5 + 2.5(n-5)] \quad (3)$$

where "mp" reperesents melting point, "log P" is logarithm of partition coefficient and "n" stands for the number of non-hydrogen atoms in the polymer chains.

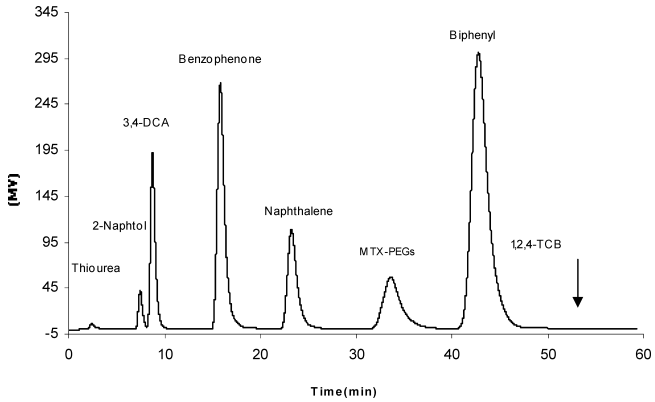

Fig. 5. The Chromatogram Obtained in a Mixture of 40 : 60 v/v Buffer-Methanol as Mobile Phase

The arrow shows position of 1,2,4-trichloro benzene which has a very small peak intensity due to poor UV absorptivity.

Table 2. Partition Coefficient Data of the Esters Obtained by the HPLC Method

Compounds	$\log k_{30\%B}$ [a]	$\log k_{40\%B}$	$\log k_{50\%B}$	$\log k_{100\%B}$ (ext.) [b]
2-Naphthol	0.09	0.34	0.65	2.04
3,4-DCA [c]	0.17	0.43	0.75	2.19
Benzophenone	0.42	0.76	1.14	2.93
Naphthalene	0.61	0.96	1.30	3.03
Biphenyl	0.84	1.24	1.75	4.01
1,2,4-TCB [d]	0.91	1.31	1.88	4.28
MTX-PEGs	0.55	1.17	1.65	4.42

a) The logarithm of capacity factor when mobile phase contains 30% buffer. *b*) The log of k obtained by extrapolation to 100% buffer as mobile phase. *c*) 3,4-Dichloro aniline. *d*) 1,2,4-Trichloro benze.

Experimental Method: Solubility of MTX and MTX-PEGs in pH range of 2 to 8 was shown in Table 5. All the compounds had a wide parabolic profile. The MTX solubility increased dramatically when pH changes from 6 to 8. However, the increase in solubility was not profound for MTX-PEGs.

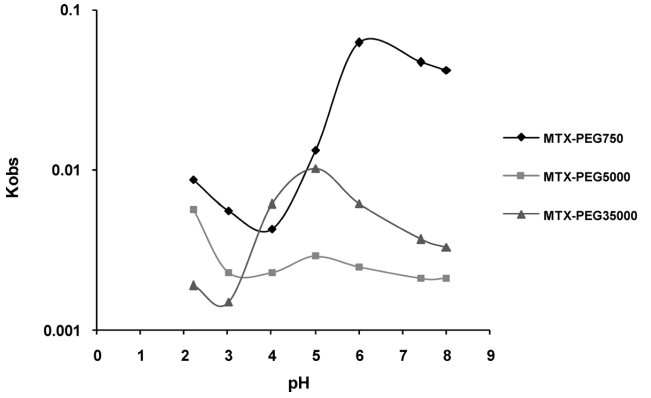

Fig. 6. pH Profile for the Hydrolysis of MTX-PEGs at Room Temperature

Table 3. Observed K_{obs} for the Hydrolysis of MTX-PEGs in Room Temperature

pH	K (obs) ± S.D.		
	MTX-PEG750	MTX-PEG5000	MTX-PEG35000
2.2	0.0087±0.00150	0.0041±0.00016	0.0019±0.00037
3	0.0056±0.00053	0.0023±0.00023	0.0015±0.00005
4	0.0043±0.00037	0.0017±0.00023	0.0061±0.00134
5	0.0133±0.00197	0.0040±0.00040	0.0102±0.00271
6	0.0621±0.00115	0.0031±0.00021	0.0062±0.00216
7.4	0.0468±0.00220	0.0024±0.00005	0.0037±0.00023
8	0.0418±0.00076	0.0020±0.00023	0.0033±0.00067

Table 4. Water Solubility of MTX-PEGs Calculated by Different Equations

	Log S_w		
	Equation 1	Equation 2	Equation 3
MTX-PEG750	−3.95	−4.78	−3.77
MTX-PEG5000	−3.82	−4.78	17.64
MTX-PEG35000	−3.87	−4.78	170.97

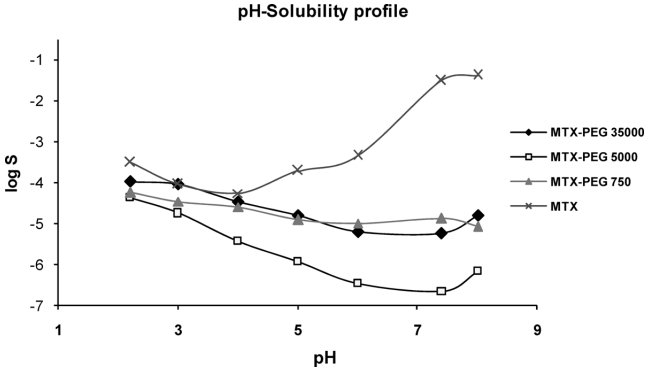

Fig. 7. The Solubility Profiles of MTX and MTX-PEGs Determined in Different pH Values at 37 °C

Table 5. Solubility of MTX and MTX-PEGs in Different pH Values

pH	MTX		MTX-PEG750		MTX-PEG5000		MTX-PEG35000	
	S (mg/ml)	log S (mol/l)	S (mg/ml)	log S (mol/l)	S (mg/ml)	log S (mol/l)	S (mg/ml)	log S (mol/l)
2.2	0.43	−3.02	0.221	−3.73	0.746	−3.86	11.797	−3.48
3	0.13	−3.55	0.131	−3.96	0.310	−4.25	10.167	−3.54
4	0.08	−3.76	0.096	−4.10	0.064	−4.93	3.895	−3.96
5	0.27	−3.22	0.047	−4.41	0.020	−5.43	1.752	−4.31
6	0.63	−2.86	0.039	−4.49	0.006	−5.96	0.699	−4.71
7.4	43.64	−1.01	0.051	−4.37	0.004	−6.15	0.635	−4.75
8	58.17	−0.89	0.032	−4.58	0.012	−5.65	1.778	−4.30

Quantities are expressed as mg/ml and mol/l.

Conversely, the MTX-PEGs solubilities increased dramatically when pH changes from 6 to 2. In Fig. 7, the solubility profiles of MTX and MTX-PEG esters were demonstrated for comparison. Among polymers, the PEG 35000D showed the highest deviation from MTX solubility (Table 5).

Discussion

Synthesis and Purification of MTX-PEG Esters The results showed that esterification with DCC and TEA is a suitable method for preparation of MTX-PEG esters. Preactivation of MTX by DCC in cold temperature was an easy and effective way for increasing the efficiency of reaction (from 15 to 30%). Because of MTX has two aliphatic carboxylic groups (α and γ) for pegylation several esterified products including mono (α or γ) or disubstituted (α and γ) esters can be obtained. However, the mono substituted product was separated by preparative TLC. Although at equimolar ratio, α carboxylic group is preferred to be esterified[9] but according to our data it is not possible to assign the position of mono pegylation. However, with respect to the integral ratio of hydrogens in ^{1}H-NMR and obtained molecular weight of purified product by GPC, the mono substituted MTX-PEG is confirmed. Also, preparative TLC system, specifically developed in this study, was easy and efficient for purifying MTX-PEG esters. Methanol was necessary in TLC mobile phase mixture to displace the esters from original spot on the plate, as the esters have ability to make hydrogen bonding with silanol groups of silicagel. Regarding the ratio of MTX and PEG in conjugation reaction, it should be noted that the different ratio of PEG (2 : 1, 3 : 1, 5 : 1) have been studied for preparation of MTX-PEG, but there was no significant increasing in the yield of the reaction was observed. Moreover at low amounts of PEG, the workup of reaction was easier and therefore pure product was obtained. This equimolar ratio is more important for PEG35000 due to presence of two positions (diol) for disubstitution. Accordingly, the risk of formation of disubstituted conjugates was significantly reduced by 1 : 1 ratio of the reactants (as revealed by TLC monitoring).

Spectroscopic Studies The shift observed in UV absorption maximum of MTX from 302 to 342 nm may be explained by the increase in electron conjugation system of MTX aromatic ring due to their interaction with paired electrons of oxygen on ethylene oxide groups of PEGs. In studying IR spectra of MTX-PEGs, two strong absorption observed at 1100 and 2800 cm^{-1}, which relate to stretching of multiple C–O and C–H bonds of the polymers, indicated the formation of esters of MTX. Also, it should be mentioned that these two absorptions were so strong that they masked the absorption of ester moiety of the MTX-PEGs. Again, the strong peaks in ^{1}H-NMR spectra of the esters, observed at 3.52—3.78 ppm, were related to protons of the ethylene oxide groups of PEG which confirm formation of the chemical link between the drug and the polymer.

Differential Scanning Calorimetry The sharp and dominant endothermic peak which were observed for MTX-PEGs, are similar to the peaks of pure polymers and proved the presence of polymer chain moiety in the esters. Also, the thermal behavior of the drug and its polymeric esters in DSC profile proved distinct differences in physicochemical properties of MTX and MTX-PEGs which sounds a promising possibility of novel systems for delivery of the drug.

Determination of the Molecular Weight of Esters by Gel Permeation Chromatography Gel permeation chromatography (GPC) has extensively been used for the determination of molecular weight of polymer conjugates.[35] The molecular weights obtained for MTX-PEG750 and 5000 (1108 and 5720, respectively) indicated that the reaction between the drug and PEGs were on a 1 : 1 ratio which means only one carboxylic group of MTX was esterified with one mPEG molecule. The same results were also obtained for MTX-PEG35000 by GPC method.

Determination of Partition Coefficients of MTX and MTX-PEGs The shake-flask method was able to accurately determine log P of MTX (the mean value for three experiments was −1.4). MTX has two aliphatic COOH groups; α and γ; of which α is more active and has the main role in drug ionization and water solubility.[7] After conjugation, this carboxylic group was esterified with OH group of the polymer and thus, MTX lost one of its strong ionization group. Therefore, the change in ionization properties of the molecule, transformed the compound to a highly lipophilic ester which is freely soluble in organic solvents. Additionally, in these polymer conjugates, there is a threshold in polymer length (MW 750 D) that partitioning doesn't change more because the polymer is long enough to cover the surface of drug molecule, entirely and longer chains of polymer are not effective more.

Study of MTX-PEG Esters pH Stability The results indicated a significant difference among K_{obs} of different MTX-PEGs in a certain pH and among different pHs of a certain MTX-PEG ester. These results are in contrast to the indomethacine[36] and hydrocortisone-PEG esters[37,38] that hydrolysis rate does not depend on the PEG molecular weight.

The results showed a threshold in PEG chain length (MW 5000 D) that is optimum for maximum stability of MTX-PEG esters.

Determination of MTX and MTX-PEGs Solubility The results of insilico methods calculated different values for solubility of esters (Table 4). The calculated values are closer to experimental values in acidic pH values (pH 2—4) (Table 5). Equations 1 and 2 led to similar results for three esters because the log P and melting point were similar for the esters. Equation 3 resulted to false positive outputs for MTX-PEG5000 and 35000 because this equation includes "n"; the number of nonhydrogen atoms in polymer chains; which is too large in these esters. The experimental method showed A parabolic pH-solubility profile for drug and esters with higher solubility of the drug in alkaline aqueous solutions (pH>5) and higher solubility of esters in acidic aqueous solutions (pH<5). This difference in solubility between MTX and its esters is due to the ionization of aromatic amine groups which are prominent in the absence of the α carboxylic group. These findings are in accordance with pK_a values of compounds (3.5 and 4.5 for MTX and 6.0 and 7.4 for PEG esters). This is noticeable that many researchers have reported the solubility increases with pegylation. These reseachers have used a weakly ionizable OH, SH or NH_2 group of drugs such as paclitaxel, podophyllotoxin and 6-mercaptopurine for esterifying with a highly water soluble PEG-COOH that can efficiently increase the aqueous solubility.[13)]

Conclusion

The synthesis and purification methods used in this study were suitable for preparing equimolar (1 : 1) drug–polymer conjugates and the characterization methods including UV, IR, NMR, DSC and GPC were successfully used to determine the physical and chemical properties of the produced conjugates. The partition coefficients of the resulting esters, obtained by chromatography method were found to be independent of the molecular weight of the polymers and or the length of their chains. Methotrexate and its conjugates showed a dramatic difference in their solubilities in different pH aqueous solutions. These results indicated that physicochemical properties of the pegylated esters of MTX, are profoundly different from the parent compound that can influence on pharmacokinetic behavior and biodistribution of the compounds. The stability experiments showed an optimum polymer molecular weight of 5000 D and a pH value of 3—4 as the most stable conditions. These properties facilitate the formulation of a stable solution for *in-vivo* studies of esters, especially MTX-PEG5000 and present a promising approach to improve delivery of the drug to human body in the future.

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