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Original Article

Quantification of Polyethylene Glycol Esters of Methotrexate and Determination of Their Partition Coefficients by Validated HPLC Methods

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Abstract

Conjugation of methotrexate (MTX) (MW 454) with different molecular weights of polyethylene glycol (PEG) including methoxy-peg (mpeg) 750 D and 5000 D and diol-peg 35000 D led to compounds that are physicochemically highly different from the parent compound, MTX. In this study, an HPLC system consisting of C8 column and UV detector (λ=342 nm), using a mixture of 30:70 v/v phosphate-citrate buffer (pH 4): methanol mobile phase, was validated for quantification of the esters. Three other HPLC methods using three mixture of buffer phosphate-citrate (pH 6): methanol at 30:70, 40:60 and 50:50 v/v, respectively, was set for estimation of partition coefficients the esters. Eight reference standard materials were selected from literature covering the retention times lower and higher than esters. An identical log P (4.3) was obtained for all three esters, despite their different molecular weights (i.e. 1200, 5500 and 35500 D theoretically). In addition the log P obtained differs from that of the parent drug (-1.4). This high difference comes probably from different ability of drug and esters in ionization of carboxylic acid groups.

Keywords: Partition coefficient; Methotrexate; HPLC; Validation; Pegylation; Polymer conjugation.

Introduction

"Pegylation" is a novel method of drug delivery that has been based on exceptional physicochemical properties of synthetic polymer, polyethylene glycol (PEG). Since pegylation is performed to change the properties of a drug molecule in order to have a more efficient drug delivery, it is necessary to determine the physical and chemical characteristics of new developed conjugates, called "Pegylated forms". The properties like solubility, stability, thermal behavior and partitioning are the parameters that influence invitro and invivo fate of drug-polymer conjugates (1). The appropriate solubility and stability facilitate the stable formulation of conjugates and have a profound impact on invitro stability. Other properties like partition coefficient along with solubility are of high importance in in-vivo fate of these conjugates. The partition coefficient of a compound can determine the extent of protein bonding, the ability for crossing the biological barriers and the how of interaction with cell membranes and

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targets of action. All of these agents determine the biodistribution and pharmacokinetic or pharmacodynamic behavior of the conjugates (2-5). The basic experimental method for determination of lipophilicity is based on the partitioning of a molecule in a system of two immiscible phases (aqueous and organic ones). Practically, this is performed using traditional shake-flask procedure with subsequent determination of the concentrations of the compound in both phases. Although different solvents were investigated for this purpose, octanol-water system remains the most popular model (6). Using this approach lipophilicity of a compound is expressed as a logarithm of partitioning coefficient. However, this method has some disadvantages such as being time-consuming and labor intensive, need to highly pure substances and some limitations including solubility and stability problems and limited use to substances with log P -2 to 4. Hence, nowadays this method is nearly being substituted by modern chromatographic techniques (7-12). Among them, HPLC is the leading and the most frequently used chromatographic method for the routine lipophilicity determination, since it enables rapid, accurate and highly reproducible analysis of relatively large sets of samples. Also, it covers the substances with a range of 0 to 6 of log P (OECD Guidelines, 1989). These experiments are usually performed on reverse phase systems (C18 and C8), where the chromatographic retention behavior of an analyte is directly related to its lipophilicity. Employing HPLC, the lipophilicity of a compound is expressed as a logarithm of retention factor (log k). Recently, many studies have used HPLC method for determination of lipophilicity of different series of substances like antituberculin agents (13), cyclopropylnitrones (9) and terpenoeids (14). In current study, as part of our investigation on pegylation of MTX, the shake-flask method was used to determine partition coefficients of the esters. 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. Thus, a series of HPLC method were developed and validated for quantification of the esters and applied for determination of their log P.

Experimental

Materials

MTX-PEG 750, 5000 D and 35000 D were synthesized in laboratory. The reference standard substances were purchased from Merck–Schuchard company. Solvents and other reagents were HPLC grade and obtained from Merck company.

Methods

HPLC method for quantification of the esters

For analytical HPLC a Merck Hitachi system was used consisting of a model L-7100 pump and a model L-7420 UV-Vis detector equipped with an interface model D-7000. The injector system was fitted with a Rheodyne 50 µl loop. Separation was performed on a Knauer MZ-analytical column (150×4.6 mm) packed with RP-8 perfectsil (5 µm particle size). The mobile phase was a mixture of phosphate-citrate buffer (pH 4): methanol (30:70). The λ_{max} was set at 342 nm and flow rate was 0.8 ml/min. For each ester, three series of standard concentrations were prepared and the linearity and LOQ were obtained in a wide range of concentrations. This process was repeated at one intervals (day 1, day 7 and day 14) and intra-day/inter-day variations at low, medium and high concentrations were calculated for each ester.

Determination of partition coefficient of MTX-PEGs by HPLC

In this study a RP-8 column and three mobile phases with combinations of phosphate-citrate buffer (pH 5) : methanol at 30:70, 40:60 and 50:50 v/v ratios (combinations I, II and III, respectively) were used. The final pH of each these mobile phases was adjusted to 6, which is isoelectric pH of the esters and has obtained from pH-solubility profiles Six reference standard were chosen from literature covering the range of log P 2.7 to 4.2 (Table 1). Each standard was individually dissolved in mobile phase combination I and its absorbance was scanned over a range of 200-400 nm by UV spectrophotometer. Then, samples were injected to the HPLC system using the combination I

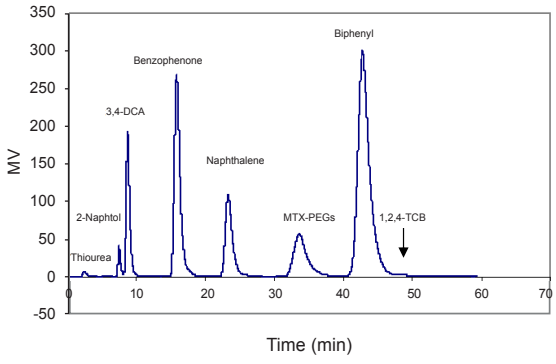

Figure 1. The chromatogram obtained in 40:60 of buffer: methanol as mobile phase. The arrow shows the position of 1,2,4 trichloro benzene that has a very small peak intensity because of low UV absorptivity.

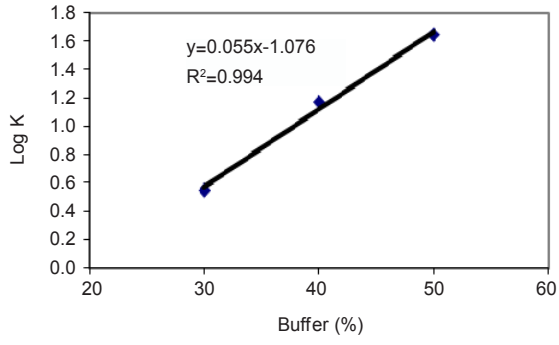

Figure 2. Relationship between buffer (%) and log K for extrapolation to log $K_{100\%}$.

as mobile phase and the retention times were recorded. Then, a mixture of all standards was prepared and injected in the same manner. This procedure was repeated for the standards using combination II and III as mobile phases.

Results and Discussion

HPLC method for quantification of esters

The results of validation assessment, given in Table 2, showed that the response is linear in a wide range of concentration (R^2>0.99). The LOQ values showed that method is more sensitive for determination of MTX-PEG750 rather than others. The results shown in Table 3 indicated that the method is reproducible and accurate at low, medium and high concentrations of all esters but more reproducible and accurate for MTX-PEG35000 with 4.3%, 5.6% and 103.1% for average of intra-day, inter-day and accuracy, respectively.

Determination of MTX-PEGs partition coefficient by HPLC method

The UV spectra showed that all the standards and esters have a λ_{max} near 254 nm. Hence this wavelength was considered for monitoring the compounds by HPLC. The capacity factors, k for all compounds were calculated according to following equation:

$$k = \frac{t_r - t_o}{t_o}$$

Table 1. The reference substances used for log P determination of esters and their log P.

Reference substances	Log P
2-naphtol	2.7
3, 4-DCA	2.8
Chlorobenzene	2.8
Bromobenzene	3.0
Benzophenone	3.2
Naphthalene	3.6
Biphenyl	4.0
1, 2, 4-TCB	4.2

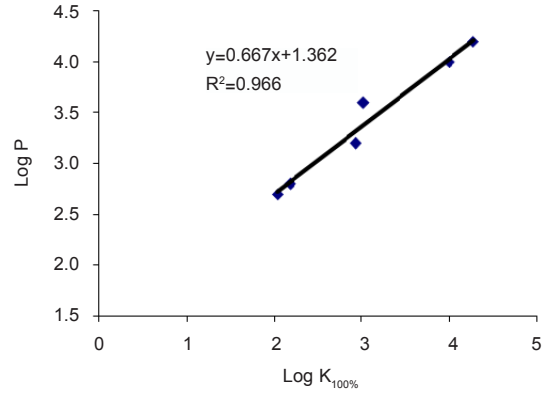

Figure 3. Relationship between log $K_{100\%}$ and log P of reference standards.

Table 2. Linearity, LOQ and range of validated HPLC methods of esters.

	MTX-PEG750	MTX-PEG5000	MTX-PEG35000
R^2	0.999	0.997	0.999
LOQ (mg/ml)	0.001	0.0025	0.05
Range (mg/ml)	0.001 - 1	0.0025 - 1	0.05 - 5

in which, t_r is retention time of the test or reference substances, and t_o is average time of a highly hydrophilic substance (thiourea in this study) which passes through the column fast (dead-time). Figure 1 demonstrates the chromatogram of all references and esters, where the the mobile phase was a combination II mixture (i.e. buffer and methanol at 40: 60 v/v, respectively). All esters are eluted simultaneously and hence have the same K. The capacity factors of standards and esters for three mobile phases, depicted as $K_{30\%}$, $K_{40\%}$ and $K_{50\%}$ were calculated and $K_{100\%}$ was obtained from extrapolation (Table 4). Figure 2 shows log K for each esters against percentage of the buffer in mobile phase, used for extrapolation and estimation of log $K_{100\%}$. Finally the standard curve was prepared by plotting log $K_{100\%B}$ versus log P of standards and the log P of esters was calculated using the standard curve (Figure 3). The log P obtained from standard curve was 4.31 which was the same for all esters. These results showed that the esters have high tendencies to the lipophilic phase compared to the hydrophilic phase (P>10000). The difference between log P of MTX and MTX-PEGs is high and this is the reason for elution of the free drug on RP-18 HPLC column by 20% methanol in mobile phase, whereas the conjugates elute on more polar RP-8 column only by 70% methanol mixture. This profound difference is probably due to ionizion nature of carboxylic group of the free drug and can be explained by the fact that in esters, the carboxylic acid group of MTX is esterified with hydroxy group of polymer and cannot participate in ionization. Conversely, the same group in free drug is ionized in mobile phase and has more tendency to the aqueous phase (log P -1.4 by shake-flask method). Another remarkable result is that partitioning is not dependent on moleculat weight of the polymer and all esters have the same log P. This can be explained with the fact that esterifying the carboxylic acid group covers the powerful site of ionization in drug molecule and this effect is dominant to the hydrophilic effect of polymer chains.

In conclusion, in this study HPLC method was pound suitable for determining log P of methotrexate-polyethylene glycol esters. The results showed that the esters were more lipophilic than the parent drug (log P 4.3 versus -1.4) and therefore, shake flask method couldnot determine the partition coefficients accurately. In addition, the similar values obtained for log P showed that the ionization status of drug is more effective than polymer chain length in lipophilicity of conjugates.

Table 3. Reproducibility parameters and accuracy of validated HPLC methods of esters at diffent level of concentrations.

	MTX-PEG750			MTX-PEG5000			MTX-PEG35000		
	%CV		Accuracy %	%CV		Accuracy%	%CV		Accuracy%
	Intra-day	Inter-day		Intra-day	Inter-day		Intra-day	Inter-day	
Low	2.98	6.02	117.67	2.36	9.77	91.62	5.34	4.49	99.34
Medium	7.30	11.14	113.62	7.85	10.09	89.44	3.78	7.89	98.47
High	1.97	13.47	109.62	5.02	4.00	83.78	3.86	3.85	111.52

Table 4. The HPLC method data for determination of the esters partition coefficients.

Compounds	log $k_{30\%B}$ [a]	log $k_{40\%B}$	log $k_{50\%B}$	log $k_{100\%B}$(ext.)[b]
2-naphtol	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-PEG750	0.55	1.17	1.65	4.42
MTX-PEG5000	0.55	1.17	1.65	4.42
MTX-PEG35000	0.55	1.17	1.65	4.42

[a] The logarithm of capacity factor when mobile phase consists 30%buffer.
[b] The log K obtained by extrapolation to 100% buffer as mobile phase.
[c] 3,4-Dichloro aniline
[d] 1,2,4- Trichloro benzene

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