Validation of HPLC method for the analysis of methotrexate in bulk drug and pharmaceutical dosage forms

P. Suguna, B. Sathyanarayana, G. Chandrasekhar and B. Narasimhulu

Department of Chemistry, S. V. University, Tirupati, A. P., India

_____

ABSTRACT

A simple, economic, selective, precise, and accurate high performance liquid chromatographic method for the analysis of methotrexate in bulk drug and pharmaceutical formulations was developed and validated in the present study. The mobile phase consists of a mixture of acetonitrile and potassium dihydrogen orthophosphate in the proportion 92:8, a kept at the pH to 6.0 ± 0.05 with sodium hydroxide solution. This was found to give a sharp peak of methotrexate at a retention time of 4.517min. HPLC analysis of methotrexate was carried out at a wavelength of 303 nm with a flow rate of 1.4ml/min. The linear regression analysis data for the calibration curve showed a good linear relationship with a regression coefficient of 0.999 in the concentration range of 50 µg ml⁻¹ to 150 µg ml⁻¹. The linear regression equation was y =37.771X - 413.07. The developed method was employed with a high degree of precision and accuracy for the analysis of ethotrexate. The developed method was validated for accuracy, precision, robustness, detection and quantification limits in accordance with as per the ICH guidelines. The wide linearity range, accuracy, sensitivity, short retention time and composition of the mobile phase indicated that this method is better for the quantification of methotrexate.

Keywords: Methotrexate, HPLC, Validation.
_____

INTRODUCTION

A survey of the literature revealed that different analytical techniques for the assay of MTX have been reported. HPLC with fluorimetric[1-13] and UV[14-19] detection methods have been reported. Using the former methods, the derivatization reactions include; photo-oxidative irradiation at 254 nm in the presence of hydrogen peroxide to yield 2,4-diaminopteridine-6-carboxylic acid[6,11-13], oxidation with permanganate in presence of acetate buffer (pH- 4) to give 2-amino-4-hydroxypteridine-6-carboxylic acid[10] , and oxidative cleavage using either phosphate buffer containing 0.2 of 30% hydrogen peroxide[4,8&9] or pre-column of cerium (IV) trihydroxyhydroperoxide[5,7] to get 2,4-diaminopteridine-6-carboxylic acid. The latter reaction has been applied in flow injection technique[20].

Early analysis of methotrexate in Human plasma by HPLC with fluorescence detection[45] , HPLC determination of methotrexate polyglutamates after Low-Dose methotrexate therapy in patients with Rheumatoid arthritis[31,]Quality control of methotrexate by HPLC[46] and Polarographic and voltammetric methods[26-29] for the quantitation of MTX in pharmaceuticals and plasma samples have been published.

There is however no reported HPLC method for the analysis of methotrexate in its technical grade and formulations. This is describes a validated HPLC method for the quantitative determination of methotrexate. The empirical formula for methotrexate is $C_{20}H_{22}N_8O_5$ and the molecular weight is 454.4 grams. It has the following structure.
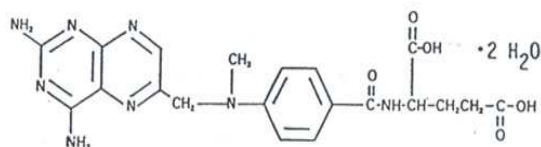
The HPLC method described here is simple, sensitive and reproducible for methotrexate determination in formulations with low background interference. An attempt has been made to develop and validate to ensure their accuracy, precision and other analytical method validation parameters as mentioned in various gradients. One method reported for the HPLC determination for   developed based on the use of a C-18 column with a suitable mobile phase, without the use of any internal standard. For pharmaceutical formulation the proposed method is suitable for their analysis with virtually no interference of the usual additives presented in pharmaceutical formulations

EXPERIMENTAL SECTION

1. Instrumentation
HPLC Analytical column Chromolith RP-C18, 100 mm x 10 µ (C-098&C-099)
Chromatographic conditions for Methotrexate.

Stationary phase	Mobile phase	Flow rate (ml min^{-1})	Run time (min)	Column Temp (0^c)	Volume of injection loop (µl)	Detection wavelength (nm)	Retention time (min)
C18, 100mm x 6mm x 5µ	Buffer, and Acetonitrile 92:8	1.4	10	25	20	303	4.517

2. ANALYTICAL METHODOLOGY
1. Preparation of mobile phase
For isocratic system a mixture of buffer and acetonitrile in the proportion 92:8 respectively was prepared. The PH of the mixture (mobile phase) was adjusted pH to 6.0 ± 0.05 with sodium hydroxide pellets. Then it was filtered through 0.2 µ nylon membrane filter paper and degas prior to use.

2. Preparation of Buffer
Buffer solution was prepared by dissolving accurately weighed 27.22 g of Potassium dihydrogen orthophosphatein distilled water in 1000 ml volumetric flask and made up to mark with distilled water.

3. Chromatographic conditions
Separation was performed on C -18, 100mm x 6mm x 5µ column. dimethyle sulfoxide used as a diluent and mobile phase consists of mixture of buffer, and acetonitrile in the proportion92:8. Injection volume of 20 µl was used. Mobile phase was filtered before use through 0.5 µm Nylon membrane filter paper and degassed with helium purge for 10 min. The components of the mobile phase were pumped from solvent reservoir to the column at flow rate 1.4 ml min^{-1} and wavelength was set to   303 nm. The column temperature was set at 25°C.

4. Preparation of methotrexate standard solution: (pure sample)
Weighed accurately about 100 mg of methotrexate working standard and transferred to a 100 ml volumetric flack. 10 ml of diluents was added and sonicated to dissolve. volume of solution was made up to the mark with diluents. i.e. 1000 µg ml^{-1} (Stock solution A)

From the above stock solution A 10 ml of solution was pipette out into 100 ml volumetric flask and the volume was made up to the mark with dimethyle sulfoxide to obtain the final concentration of 100 µg ml^{-1} (Stock solution B). From the stock solution B ranging from 5-15 ml were transferred into a series of 10 ml volumetric flasks to provide final concentration range of 50-150 µg ml^{-1} and each flask made up to the mark with diluents.

5. Preparation of test solution :(formulation)
Twenty tablets containing methotrexate were weighed and finely powered. An accurately weighed portion of the powder equivalent to 100 mg of methotrexate was transferred into a 100 ml volumetric flask. 10 ml of diluents added and shaken for   20 min by manually and further sonicated for 30 min. and then diluted up to the mark with diluents. This solution was centrifuged at 8000 rpm for 10 min. The supernatant solution was decanted into another test tube (i.e.1000 µg ml^{-1}) and 10 ml of supernatant solution was transferred into another 100 ml volumetric flask and made up the volume with diluents (100 µg ml^{-1}).Further transfer 5-15 ml of solution was transferred   into

another 10 ml volumetric flask and made up the volume with diluents. The solution was filtered through 0.45 µm Nylon membrane filter paper. (50-150 µg ml^{-1}).

6. Assay procedure:

The column was equilibrated for at least 30 minutes with mobile phase flowing through the system with a flow rate of 1.0 ml min^{-1}. Detector was set at a wavelength of 254 nm. Five sets of the drug solutions were prepared in diluents containing methotrexate at a concentration range of 50 - 150 µg ml^{-1}. Then 20 µl of each standard and sample solution was injected for five times separately. The retention time for methotrexate was found to be 4.517 min (Fig – 1.1). The peak areas of the Drug concentrations were calculated. The regression of the drug concentration over the amount of drug in formulation was evaluated.

7. System suitability solution:

Use methotrexate standard working solution as system suitability solution.

8. Procedure:

Equal volumes of blank the replicate injections of systems suitability solution i.e methotrexate standard working solution were injected separately. Then two injections of test solution were injected and chromatograms were recorded. .percentage of RSD of five replicate injections of system suitability solution (methotrexate standard working solution) was calculated.

Figure-1.1: Chromatogram of methotrexate
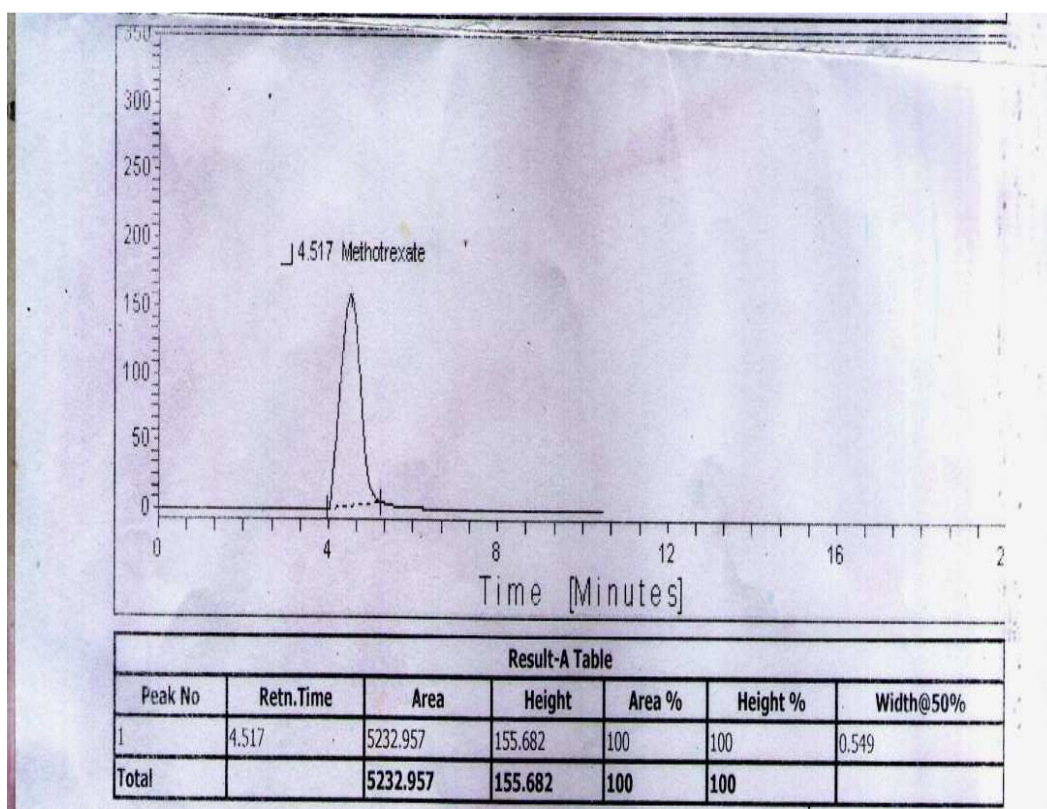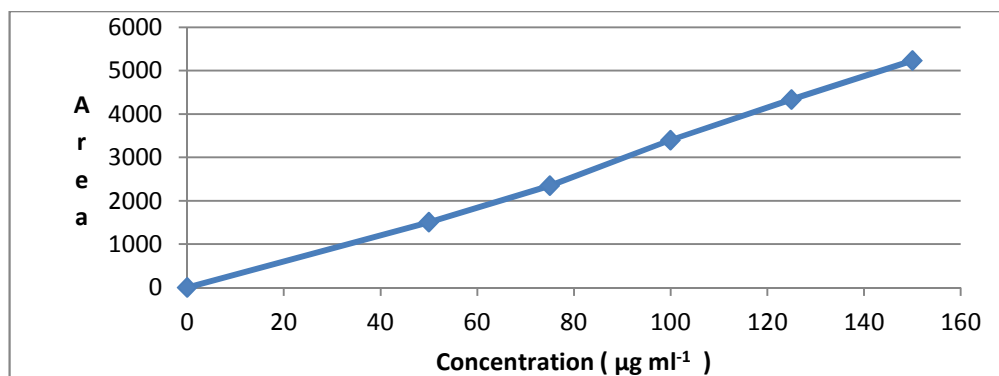

Result-A Table						
Peak No	Retn.Time	Area	Height	Area %	Height %	Width@50%
1	4.517	5232.957	155.682	100	100	0.549
Total		5232.957	155.682	100	100	

Figure -1.2: Linearity of methotrexate standard

RESULTS AND DISCUSSION

Table -1.1: Performance calculations, detection characteristics precision and accuracy of the proposed method for methotrexate

Parameter	HPLC Method
Wavelength (nm)	303
Retention time (t) min	4.517
Linearity range (μg ml^{-1})	50-150
LOD(μg ml^{-1})	1.5789
LOQ(μg ml^{-1})	5.2630
Regression equation (y=bc+a)	
Slope (b)	37.771
Intercept (a)	413.07
Standard deviation (SD)	19.879
Correlation coefficient(r^2)	0.9995
Relative standard deviation (%RSD)*	0.5909
Intermediate precision (%RSD)	0.56
Range of errors	
Confidence limits with 0.05 level	17.4165
Confidence limits with 0.01 level	22.8891

*RSD of five independent determinations

Table – 1.2: System suitability – Selectivity

Sr. No.	Area of methotrexate	Tailing factor	Theoretical plates
1	4710.67		
2	4602.76		
Mean	4656.72	1.11	4231
Standard Deviation ($\pm$)	76.30		
(%) Relative standard deviation	1.64		

Table-13: System suitability - linearity of standard

Sr. No.	Area of methotrexate	Tailing factor	Theoretical plates
1	3205.65		
2	3307.93		
3	3274.33		
4	3284.61	1.17	4052
5	3345.25		
Mean	3283.55		
Standard deviation ($\pm$)	51.37		
(%) Relative standard deviation	1.56		

Table 1.4: Results of linearity of standard

Linearity Level	Sample concentration (in μg ml^{-1})	Average area (n = 2)	Correlation coefficient
Level – 1	50	1505.98	
Level – 2	75	2346.65	
Level – 3	100	3399.13	**0.999**
Level – 4	125	4335.45	
Level – 5	150	5232.96	

Table 1.5: Results of linearity of sample

Linearity level	Sample concentration (in µg ml^{-1})	Average area (n = 2)	Correlation coefficient
Level – 1	50	1531.44	
Level – 2	75	2305.28	
Level – 3	100	3065.38	**0.999**
Level – 4	125	3873.13	
Level – 5	150	4699.30	

Table- 1.6: Results of linearity of standard in presence of placebo

Linearity level	Standard concentration (in mg)	Placebo added to the standard solution (mg)	Average area (n = 1)	Correlation coefficient
Level – 1	50	522	1510.69	
Level – 2	75	522	2244.80	
Level – 3	100	522	3048.23	**0.999**
Level – 4	125	522	3845.84	
Level – 5	150	522	4683.59	

Table -1.7: System precision

Sr. No.	Area of methotrexate
1	3941.08
2	3965.30
3	3969.81
4	3961.52
5	3953.10
6	4017.11
7	3960.08
8	3944.87
9	3959.22
10	3948.69
Mean	3962.08
Standard deviation (±)	21.36
(%) Relative standard deviation	0.54
Tailing factor	1.12
Theoretical plates	4456

Table – 1.8: Results of method precision

Test solution	% Assay of
1	99.76
2	99.66
3	99.44
4	99.87
5	99.17
6	99.24
Mean	99.52
Standard deviation (±)	0.29
(%) Relative standard deviation	0.29

Table – 1.9: Results of intermediate precision

Test Solution	% Assay of methotrexate
1	101.55
2	100.56
3	101.33
4	100.01
5	100.73
6	101.14
Mean	100.89
Standard deviation (±)	0.57
(%) Relative standard deviation	0.56

Table –1.10: Results of twelve test solutions of methotrexate in FOLITRAX - 5
(Six of method precision & six of intermediate precision)

Analysis performed during method precision study By analyst 1 on system 1 and on column 1 on day 1	
Same column	**% Assay of methotrexate**
1	99.76
2	99.66
3	99.44
4	99.87
5	99.17
6	99.24
Analysis performed during intermediate precision study By Analyst 2 on system 2 and on column 2 on day 2	
Column sr. no.	**015337030136 01**
Test solution	**% Assay of methotrexate**
7	101.55
8	100.56
9	101.33
10	100.01
11	100.73
12	101.14
Mean of twelve samples	100.21
Standard deviation (±)	0.83
(%) Relative standard deviation	0.83

Table-1`.11: Determination of accuracy of methotrexate

Amount of MET in formulation (mg)	Amount of Standard MET added (mg)	Total amount found (mg)	% Recovery
99.98	100	199.96	99.97
99.97	100	199.94	99.96
99.99	100	199.98	99.98
99.99	150	249.97	99.98
99.94	150	249.85	99.93
99.89	150	247.22	98.87
99.99	200	299.91	99.96
99.88	200	299.64	99.87
99.57	200	298.71	99.56

Table-1.12: Statistical data for accuracy determination

Total amount found (mean)	Standard deviation	% RSD
199.96	0.02	0.0100
249.03	1.5542	0.6241
299.42	0.6295	0.2102

The results are the mean of five readings at each level of recovered

Table – 1.13: Results for Change in Column Lot

Flow rate →	Same column	Diff column
Sample	**% Assay**	
Test solution	100.44	100.35
Average assay result from method precision	99.52	99.52
Mean	99.98	99.94
Standard deviation (±)	0.65	0.59
(%) Relative standard deviation	0.65	0.59

Table – 1.14: Results for change in flow rate

Flow rate →	1.2mL/minute	1.6 mL/minute
Sample	**% Assay**	
Test solution	100.94	99.79
Average assay result from method precision	99.52	99.52
Mean	100.23	99.66
Standard deviation (±)	1.00	0.19
(%) Relative standard deviation	1.00	0.19

_____

Table – 1.15: Results for change in wavelength

Wavelength →	301nm	305 nm
Sample	**% Assay**	
Test solution	100.85	100.91
Average assay result from method precision	99.52	99.52
Mean	100.19	100.22
Standard deviation (±)	0.94	0.98
(%) Relative standard deviation	0.94	0.98

Table – 1.16: Results for change in pH of mobile phase

pH	5.8	6.2
Sample	**% Assay**	
Test solution	100.50	101.07
Average assay result from method precision	99.52	99.52
Mean	100.01	100.30
Standard deviation (±)	0.69	1.10
(%) Relative standard deviation	0.69	1.09

Table – 1.17: Results for solution stability

% Assay results calculated against the freshly prepared system suitability standard	
Sample	**% Assay of Methotrexate**
0th hr	99.99
12th hr	99.81
24 hr	101.79
36 hr	98.13
48 hr	101.13
Mean	100.17
Standard Deviation (±)	1.40
(%) Relative Standard Deviation	1.40

The appropriate wavelength in UV- visible region has been selected for the measuring of active ingredient in the proposed method. This method was validated by linear fit curve and all the parameters were calculated.

1. Parameters fixation
In developing methods, systematic study of the effects of various parameters was undertaken by varying one parameter at a time controlling all other parameters. The following studies were conducted for this purpose.

2. Mobile phase characteristics
In order to get sharp peaks and baseline separation of the components, carried out number of experiments were conducted by varying different components like percentage of organic phase in the mobile phase, total p^H of the selected mobile phase and flow rate by changing one at a time and keeping all other parameters constant. The optimum conditions obtained were included in the procedure proposed.

3. Detection characteristics
To test whether methotrexate had been linearly eluted from the column, different amounts of methotrexate were taken and analyzed by the above mentioned procedures. The peak area ratios of component areas were calculated and the values are graphically represented in Fig1.1. The linear fit of the system was illustrated graphically. Least square regression analysis for the method was carried out for the slope, Intercepts and correlation coefficient were calculated. The results were presented in Table -1.1.

4. Performance calculations
To ascertain the system suitability for the proposed method, a number of statistical values have been calculated with the observed readings and the results were recorded in   Table-1.1.

5. Method validations
The UV absorption maximum for methotrexate was fixed at 254. As the final detection was made by the UV - absorption spectrum, each method was validated by linear fit curve.

6. Precision
The precision of the method was ascertained separately from the peak area ratios obtained by actual determination of a fixed amount of drug. The percent of relative standard deviation calculated for methotrexate and are presented in

_____

Tables1.7, 1.8, 1.9, 1.10. The precision of the assays was also determined in terms of intra and inter-day variation in the peak areas for a set of Drug solution was calculated in terms of coefficient of variation (CV)

7. Accuracy
To determine the accuracy of the proposed methods, different technical grade samples of methotrexate within the linearity limits were taken and analyzed by the proposed methods. The results (percent error) were recorded in Table-1.11.

8. Interference studies
The effect of wide range of excipients and other additives usually present in the formulations of methotrexate in the determinations under optimum conditions were investigated. The common excipients such as colloidal silicon dioxide, ethyl cellulose, hydroxyl propyl methyl cellulose, magnesium state, microcrystalline cellulose provide have been added to the sample solutions and injected. They have not disturbed the elution or quantification of drug. In fact many have no absorption at this λ_{max}

9. Analysis of Formulation
To find out the stability of the proposed methods for the assay of formulations containing methotrexate was analyzed by the proposed and reference methods. The proposed method does not differ significantly in precision and accuracy from reference method. The results were recorded in Table-1.12.

10. Ruggedness and Robustness
Ruggedness of the proposed method was determined by carrying out the analysis by two different analysts using similar operational i.e. Robustness with Change in Column Lot, change in flow rate, change in wavelength and change in p^H of the Mobile phase . The results were indicated by % CV in Tables – 1.13, 1.14, 1.15, 1.6. Robustness of the method was determined by carrying out the analysis at two different wavelengths i.e. at 252 nm and 256 nm and the results were indicated by % CV in Table -1.15.

11. Recovery Studies
Recovery studies were conducted by analyzing each formulation in the first instance for the active ingredient by the proposed methods known amounts of pure Drug was then added to each of the previously analyzed formulations and the total amount of the Drug was once again determined by the proposed methods after bringing the active ingredient concentration within the linearity limits. The results were recorded in Table -1.12.

12. Solution stability
The stability of the solutions under study was established by keeping the solution at room temperature for 48 Hours. The results indicate no significant change in assay values indicating stability of Drug in the solvent used during analysis. The results are recorded in Table -1.17.

CONCLUSION

The method was found to be accurate and precise, as indicated by recovery studies close to 100 and % RSD is not more than 2. The summery of validation parameters of proposed HPLC method is given in tables.

The simple, accurate and precise HPLC method for the determination of methotrexate as bulk and in tablet dosage form has been developed. The method may be recommended for routine and quality control analysis of the investigated drug in bulk and pharmaceutical formulations. The analytical solution is found to be stable up to 48 Hrs at room temperature. Hence, it is concluded that the analytical method is validated and can be used for routine analysis and for stability study.

REFERENCES

[1].K.Foulmann, J.P. Guastalla, N. Caminet, V. Trillet-Lenoir, D. Raudrant, F. Golfier, A.M. Schott, *Gynecologic Oncology* , **2006,** 103–110.
[2.].N.M.Annest, M.J. VanBeek, C.J. Arpey, D.C. *J. American Academy of Dermatology,* **2007,** 56, 989–993.
[3].T.Shingaki, D. Inoue, T. Furubayashi, T. Sakane, H. Katsumi, A. Yamamoto, S. Yamashita, *Molecular Pharmaceutic,* **2010,**1561–1568.
[5].M.Dabrowska, M. Skoneczny, W. Rode, ,*Tumor Biology* , **2011** , 965–976.
[6].R.Paliwal, S.R. Paliwal, N. Mishra, A. Mehta, S.P. Vyas, *International, Pharmaceutics.* **2009,** 181-188.
[7]. M.Moniruzzaman, N. Kamiya, M. Goto, , *J. Colloid and Interface Science,* **2010,** 136–142.
[8]. S.Natali,J.Mijovic, , *Macromolecule*s, **2010**, 3011–3017

[9]. L.M.Kaminskas, B.D. Kelly, V.M. McLeod, G. Sberna, B.J. Boyd, D.J. Owen,C.J.H.Porter, *Molecular Pharmaceutics* **2010,** 338–349.

[10].L.Ouyang, L.F. Ma, B. Jiang,Y.H. Li, D.S. He, L.Guo, *J. Medicinal Chemistry* , **2010,** 2705–2711.

[11].L.M. Kaminskas, B.D. Kelly, V.M. McLeod, B.J. Boyd, G.Y. Krippner, E.D. Williams, C.J.H. Porter, *Molecular Pharmaceutics,* **2009,** 1190–1204.

[12].R.S.Dhanikula, A. Argaw, J.F. Bouchard, *P. Molecular   Pharmaceutics* , **2008,** 105–116.

[13].Y.Wang, X. Yang, J. Yang, Y. Wang, R. Chen, J. Wu, Y. Liu, N. Zhang, *Molecular Pharmaceutics* , **2011**, 1665–1670.

[14].M.R.Saboktakin, R.M.Tabatabaie, A.Maharramov, M.A.Ramazanov, *International BiologicalMacromolecules* , **2011**, 747–751.

[15].Y.H.Chen, C.Y. Tsai, P.Y. Huang, M.Y. Chang, P.C. Cheng, C.H. Chou, D.H. Chen, C.R.Wang,A.L. Shiau,C.L.Wu, *Molecular Pharmaceutics* , **2007,** 713–722.

[16].N.Kohler, C.Sun, J.Wang, M.Q.Zhang, Langmuir *Molecular Pharmaceutics,* **2005,** 8858–8864.

[17]. Y.Kawashima, , *Advanced Drug Delivery Reviews,* **2001**, 1–2.