Acta Poloniae Pharmaceutica – Drug Research, Vol. 69 No. 6 pp. 1342–1346, 2012 ISSN 0001-6837
Polish Pharmaceutical Society

METHODS FOR METHOTREXATE DETERMINATION IN MACROMOLECULAR CONJUGATES DRUG CARRIER

JAROSŁAW CIEKOT[1]*, TOMASZ GOSZCZYŃSKI[1] and JANUSZ BORATYŃSKI[1,2]

[1]Polish Academy of Sciences, Institute of Immunology and Experimental Therapy (IITD), Laboratory of Biomedical Chemistry "Neolek" Rudolfa Weigla 12, Wrocław 53-114, Poland
[2]Jan Dlugosz Academy, Al. Armii Krajowej 13/15, Częstochowa 42-201, Poland

Abstract: In this paper, two simple, cost-effective and fast methods for quantification of methotrexate (MTX) in the macromolecular conjugates are presented. The method for analysis of total MTX in preparations was based on absorption spectrophotometry. Validation was performed by measuring absorbance at 372 nm of the sodium bicarbonate solution. Curve describing drug concentration against absorption had a linear character in the range of 1.204–40.13 µM. The reproducibility and precision of method was 0.1558 to 3.086%. The recovery of the method was between 99.56 and 104.7%. The limit of quantitation method was 1.050 µM. The method for free MTX determination was based on size exclusion chromatography and UV-VIS detection at the wavelength of 302 nm. Superdex® Peptide column (150 × 4.6 mm) and a mobile phase 0.1 M sodium bicarbonate with a flow rate of 0.4 mL/min was used. In the free drug determination method, the curve had a linear character in the range of 2.006–200.6 µM. The reproducibility and precision of method was 0.3761 to 2.452%. The recovery of the method was between 93.18 and 104.5%. The limit of quantitation method was 0.9203 µM.

Keywords: HPLC, spectrophotometric, conjugate, drug carrier, methotrexate

Methotrexate [(2S)-2-[(4-{[(2,4-diaminopteridin-6-yl)methyl](methyl)amino}benzoyl)amino] pentanedioic acid] (MTX) is one of the oldest and still used drugs (Fig. 1). It is an antimetabolite of folic acid, and a specific inhibitor of dihydrofolate reductase (DHFR), having an indirect and direct effect on other molecular targets, influencing DNA replication and cell proliferation (1). MTX has a wide use in the treatment of tumor and autoimmunological diseases (2). However, this drug demonstrates a range of disadvantages, characteristic for low molecular compounds such as fast metabolism and fast excretion from an organism, as well as adverse biodistribution and a low selectivity of therapeutic use. Solving these problems involves the copuling MTX with macromolecular carriers, which results in enhancement of the delivery, selectivity and improvement in pharmacological properties of MTX (3). Research on MTX conjugates with natural and synthetic polymers such as dextrans, albumin, fibrinogen, polyethylene glycol, and others, is in progress (4–7). In order to study for potential

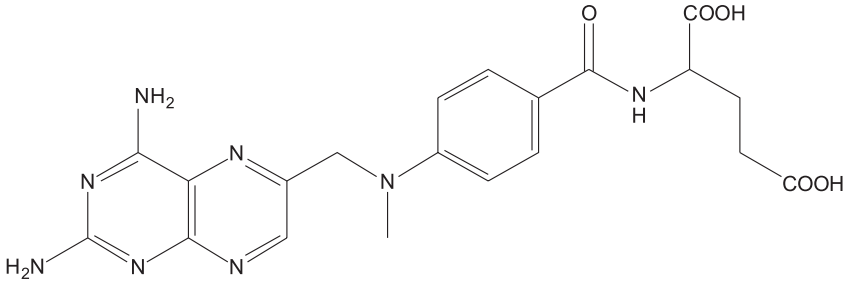

Figure 1. Structure of methotrexate

* Corresponding author: e-mail: jaroslaw.ciekot@iitd.pan.wroc.pl

MTX carrier, a fast and precise method is needed to determine the total amount of the drug bound and free in investigated preparation.

EXPERIMENTAL

Materials

MTX was purchased from Ebewe Pharma (Austria). Sodium bicarbonate (POCH Poland) was of analytical grade.

Method for analysis of total MTX in preparations

Instrumentation

UV-VIS spectrophotometric method was performed on double beam UV-VIS spectrophotometer (Analityk Jena Specord 250, Germany) equipped with the quartz cells with 1-cm light path.

Chemicals and reagents

Sodium bicarbonate (0.1 M) was used throughout UV-VIS spectrophotometric method. Dilution of MTX stock solution (199.5 mM) was performed with sodium bicarbonate to prepare different concentrations of MTX.

Linearity and range

The linearity of the method was determined by measuring nine independent concentrations of calibration curve in the range of 1.204–40.13 μM. Each concentration was measured in the wavelength range of 220–500 nm. Validation was performed by using absorbance at 372 nm. The calibration curve of absorbance at 372 nm *vs*. concentration was plotted and regression parameters were determined.

Accuracy

Accuracy was determined by comparing a known concentration of the drug in the sample relative to the concentration calculated by the method for analysis of total MTX in preparations used. The result was presented as a percent of recovery.

Reproducibility

The reproducibility of the method was calculated by comparing the signals for three independent measurements of one MTX concentration. The result was presented as coefficient of variations.

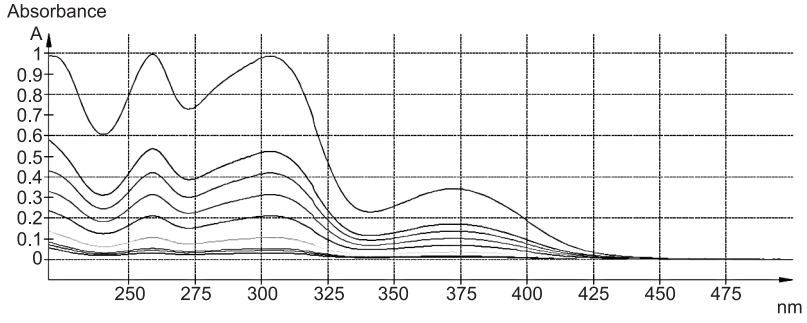

Figure 2. Overlay spectra of methotrexate by UV spectroscopy in 0,1 M sodium bicarbonate

Table 1. Chromatographic conditions.

Stationary phase	Superdex Peptide (150 × 4.6 mm, 5 μm)
Mobile phase	0.1 M sodium bicarbonate
Mode	Isocratic flow rate 0.4 mL/min
Column temperature	22°C
Detector	Diode array detector
Detection wavelength	302 nm
Injection volume	10 μL
Run time	20 min

Table 2. Summary of validation parameters for analysis of total methotrexate in preparations.

Parameters	Results
λ_{max}	372 nm
Molar absorptivity (ε)	8571
Linearity	1.204–40.13 µM
Regression line equation	y = 0.008571x
Correlation coefficient (R^2)	0.9999
Reproducibility (% coefficient of variation)	0.1558–3.086%
Accuracy (% recovery)	99.56–104.7%
LOD	0.3150 µM
LOQ	1.050 µM

Table 3. Summary of validation parameters of free methotrexate determination.

Parameters	Results
Linearity	2.006–200.6 µM
Regression line equation	Y = 0.5850x – 0.6730
Correlation coefficient (R^2)	0.9993
Reproducibility (% coefficient of variation)	0.3761–2.452%
Accuracy (% recovery)	93.18–104.5%
LOD	0.2761 µM
LOQ	0.9203 µM

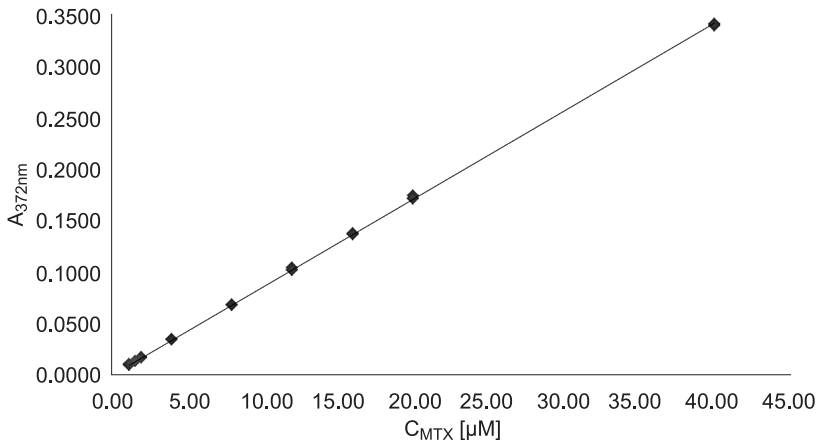

Figure 3. Calibration curve of total drug determination

Limit of detection (LOD) and limit of quantitation (LOQ)

The LOD was based on signal-to-noise ratio of 3:1. The LOQ was based on signal-to-noise ratio of 10:1.

Method of free MTX determination

Instrumentation

A Dionex Ultimate 3000 (USA) liquid chromatography system equipped with DAD 3000 detector and auto sampler was used. The chromatography

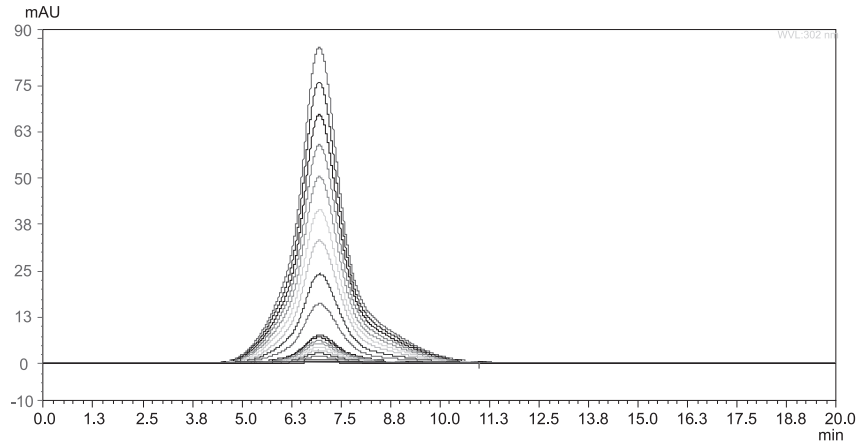

Figure 4. Overlay HPLC chromatogram of methotrexate

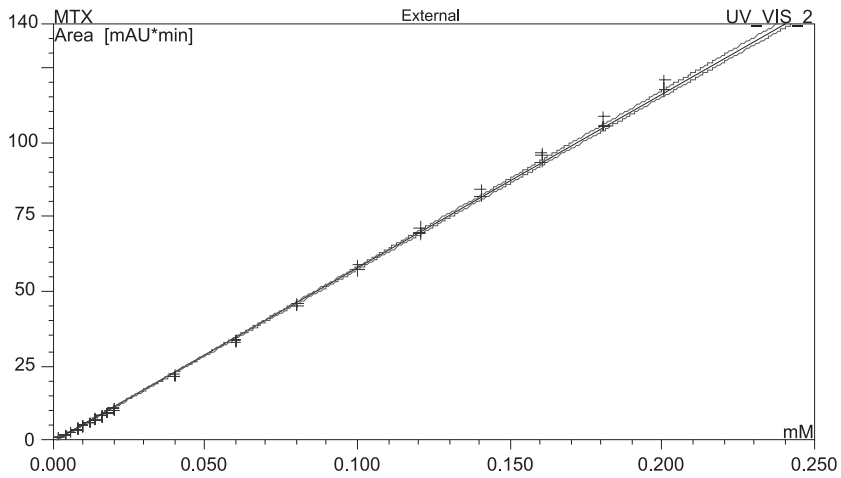

Figure 5. Calibration curve of free drug determination

column was a 150 × 4.6 (i.d.) millimeter Superdex Peptide. Chromatographic conditions are shown in Table 1.

Solutions of external standards

Dilution of MTX stock solution (199.5 mM) was done with sodium bicarbonate to prepare different concentrations of MTX.

Linearity

The linearity of the method was determined by measuring of 19 independent concentrations of calibration curve in the range of 2.006–200.6 µM. Ten microliters of each concentration of drug were injected separately into high performance liquid chromatography (HPLC) column and peak area was measured at 302 nm. The calibration curve of peak area *vs*. concentration was plotted and regression parameters were determined.

Accuracy

The accuracy was determined by comparing a known concentration of the drug in the sample relative to the concentration calculated by the method of free MTX determination used. The result was presented as a percent of recovery.

Reproducibility

The reproducibility of the method was calculated by comparing the signals for independent

measurements of one methotrexate concentration. The result was presented as coefficient of variations.

Limit of Detection (LOD) and Limit of Quantitation (LOQ)

The LOD was based on signal-to-noise ratio of 3:1. The LOQ was based on signal-to-noise ratio of 10:1.

RESULTS

Method for analysis of total MTX in preparations

The results of the method validation are presented in Table 2.

Linearity: The linearity of the method was found to exist in a concentration range of 1.204–40.13 μM with a correlation coefficient of 0.9999. Figure 2 shows the absorbance spectrum of MTX in sodium bicarbonate with the wavelength range 220–500 nm. Calibration curve is shown in Figure 3.

Accuracy: % recoveries of the method were found to be 99.56–104.7%.

Reproducibility:% coefficient of variation was found to be 0.1558–3.086%.

Limit of detection and limit of quantitation: LOD of this method was found to be 0.3150 μM. LOQ was found to be 1.050 μM.

Method of free MTX determination

The results of the method validation are presented in Table 3.

Linearity: The linearity of the method was found to exist in a concentration range of 2.006–200.6 μM with a correlation coefficient of 0.9993. Figure 4 shows the chromatogram of MTX in sodium bicarbonate. Calibration curve is shown in Figure 5. Retention time was 6.955 ± 0.016 min**.**

Accuracy: % recoveries of the method were found to be 93.18–104.5%.

Reproducibility: % coefficient of variations were found to be 0.3761–2.452%.

Limit of detection and limit of quantitation: LOD of this method was found to be 0.2761 μM. LOQ was found to be 0.9203 μM.

DISCUSSION AND CONCLUSION

Several methods of determination of MTX in biological fluids have been described thus far. All of these methods are based on reverse-phase HPLC (8–13). Determination of free MTX in a reverse-phase chromatography is not recommended, because it may co-elute with the conjugate drug-carrier. These problems arose the need to develop a new method for MTX determination in a conjugate. A specific, accurate, reproducible isocratic HPLC method has been developed for the determination of the free drug in conjugate. Chromatography method with sample preparation took about 20 minutes for each analysis. A simple, economic, specific, accurate UV spectrophotometric method has been developed for the determination of total drug in the conjugate. The proposed methods can be used for the drug analysis in any type of conjugate.

Acknowledgments

This project is co-financed by the European Union as part of the European Social Fund. This project was supported by National Science Centre, Poland (N N302 098434).

REFERENCES

1. Fairbanks L.D., Rückemann K., Qiu Y., Hawrylowicz C.M., Richards D.F., Swaminathan R., Kirschbaum B., Simmonds H.A.: Biochem. J. 342, 143 (1999).
2. Benson M.D.: Allergy. in Medicine: Essentials of Clinical Practice, 2nd edn., p. 575, Wilkins R.W., Levinsky N.G. Eds., Little Brown and Company, Boston 1978.
3. Bertino J.R.: J.Clin.Oncol. 11, 5 (1993).
4. Nevozhay D., Budzynska R., Kanska U., Jagiello M., Omar M.S., Boratynski J., Opolski A.: Anticancer Res. 26, 1135 (2006).
5. Boratyński J., Opolski A., Wietrzyk J., Górski A., Radzikowski C.: Cancer Lett. 148, 189 (2000).
6. Yousefi G., Foroutan S.M., Zarghi A., Shafaati A.: Chem. Pharm. Bull. 58, 147 (2010).
7. Taheri A., Dinarvand R., Atyabi F., Ahadi F., Nouri F.S., Ghahremani M.H., Ostad S.N. et al.: Int. J. Mol. Sci. 12, 4591 (2011).
8. Lawson G.J., Dixon P.F.: J. Chromatogr. 223, 225 (1981).
9. Breithaupt H., Kuenzelen E., Goebel G.: Anal. Biochem. 127, 103 (1982).
10. Salamoun J., Frantsiek J.: J. Chromatogr. 378, 173 (1986).
11. Brimmed P.A., Sams D.J.:. J. Chromatogr. 413, 320 (1987).
12. Najjar T.A., Matar K.M., Alfawaz I.M.: Ther. Drug Monit. 14, 142 (1991).
13. Assadullahi T.P., Dalgi E., Warner J.O.: J. Chromatogr. 565, 349 (1991).
14. Cosolo W., Drummer O.H., Christophidis N.: J. Chromatogr. 223, 225 (1981).
15. Farid Y.Z., Watson I.D., Stewart M.J.: J. Pharm. Biomed. Anal. 1: 55 (1983).