Regular Article

ANALYTICAL
AND BIOANALYTICAL
CHEMISTRY
RESEARCH

Published by the
Iranian Chemical Society

***Anal. Bioanal. Chem. Res.**, Vol. 2, No. 1, 42-51, June 2015*

Simultaneous Spectrophotometric Determination of Mycophenolate Mofetil and Its Active Metabolite in Human Plasma Using Chemometrics Methods

T. Madrakian[a,*], M. Madadi-Shad[b] and M. Soleimani[a]

[a]*Faculty of Chemistry, Bu-Ali Sina University, Hamedan, Iran*

[b]*Department of Chemistry, Saveh Branch, Islamic Azad University, Saveh, Iran*

(Received 7 February 2015, Accepted 17 April 2015)

A spectrophotometric method for selective complexation reaction and simultaneous determination of mycophenolate mofetil (MPM), and mycophenolic acid (MPA) using three multivariate chemometric methods, *i.e.* partial least squares regression, principal component regression and principal component artificial neural networks, is proposed. The method is based on the complexation reaction of MPM and MPA with Fe(III) ion in the solution. A nonionic surfactant, Triton X-100, was used for dissolving the complexes and intensifying the signals. The linear determination ranges for the determination of MPA and MPM were 5.0-215.0 mg l^{-1}, and 10.0-1000.0 mg l^{-1}, respectively. The detection limit for MPA and MPM was obtained as 0.3 mg l^{-1} and 1.1 mg l^{-1}, respectively. Satisfactory results were obtained by the combination of spectrophotometric method and chemometrics techniques. The method was successfully applied to the simultaneous determination of MPM and MPA in serum sample and the results were comparable with HPLC method.

Keywords: Mycophenolate mofetil, Mycophenolic acid, Simultaneous determination, Chemometric methods, Spectrophotometry

INTRODUCTION

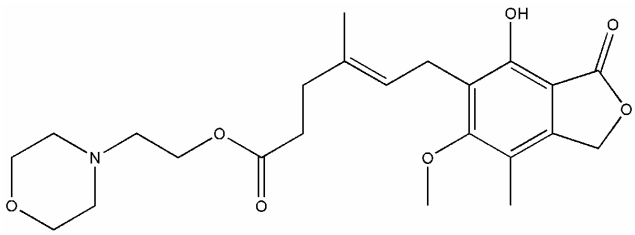
Mycophenolate mofetil, 2-(4-morpholino)ethyl-(E)-6-(1,3-dihydro-4-hydroxy-6-methoxy-7-methyl-3-oxo-5-isobenzo-furanyl)-4-methyl-4-hexenoate, (MPM), an ester prodrug of the immunosuppressant- mycophenolic acid, (4E)-6-(4-Hydroxy-6-methoxy-7-methyl-3-oxo-1,3-dihydro-2-benzo-furan-5-yl)-4-methylhex-4-enoic acid (MPA) has been approved for maintenance immunosuppressive therapy of allergenic graft rejection following solid organ transplantation. MPM is hydrolyzed in order to form free MPA, which is the active metabolite and it is conjugated to form a phenolic glucuronide conjugate [1], which is pharmacologically inactive but may be hydrolyzed *in vivo* to form free MPA. MPA is a potent and specific inhibitor of de novo purine synthesis and blocks proliferation of both T and B lymphocytes [2-4]. Due to the rapid and extensive metabolism of MPM to the active plasma metabolite, pharmacokinetic investigation following the administration of the prodrug-MPM has been based principally on the kinetics of MPA. Since MPM is, at the moment, at a relatively early stage of the drug development process, the full pharmacokinetic characterization in recipients of kidney transplantation, in conjunction in with pharmacokinetics (clinical efficacy) are essential for optimization of drug therapy. To support this pharmacokinetic investigation, establishment of an appropriate analytical method such as sensitivity, selectivity and reproducibility for quantification of MPM in biological fluids is essential [5]. MPM is potentially present in the plasma and immediately follows intravenous infusion. Therefore HPLC methods were developed for the determination of MPM and MPA in plasma and ultra-performance liquid chromatography-electro spray ionization tandem mass spectrometry procedure [6-10].

Several analytical methods namely, HPLC [11-13], LC-MS [14], spectrophotometric [15], and micellar electrokinetic chromatographic [16] methods have been reported for the determination of MPM and MPA in bulk, pharmaceutical formulations, and biological samples. The

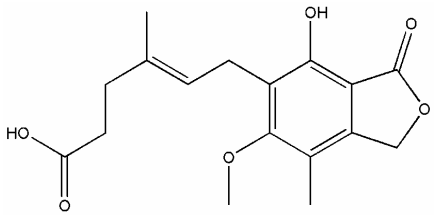
*Corresponding author. E-mail: madrakian@gmail.com

reported chromatographic and spectroscopic methods were found to be time consuming. Recently, we reported an electrochemical method that has been developed for the simultaneous determination of MPM and its active metabolite MPA using the MWCNTs/GCE [17].

In this study a spectrophotometric method for simultaneously determination of MPM and MPA using chemometric methods is described. This method is based on the combination of multivariate calibration methods with direct spectral information and the PC-ANN. Satisfactory sensitivity, accuracy, simplicity, speed and precision were noted in this procedure. To the best of our knowledge, simultaneous spectrophotometric determination of MPM and MPA has not been reported so far.



(a)



(b)

Fig. 1 The chemical structure of the (a) MPM and (b) MPA.

EXPERIMENTAL

Materials

MPM, and MPA, were obtained from Alborz bulk pharmaceutical Company (Saveh, Iran). The purity of the MPM, MPA was over 99%. Figure 1 show the chemical structure of the MPM and MPA. All the chemicals used were obtained from analytical reagent grade or the highest purity available from Merck Company (Darmstadt, Germany). Double distilled water (DDW) was used throughout. A 1000 mg l^{-1} standard solution of both MPM and MPA was prepared in a 1:1 mixture of methanol and DDW in 100 ml calibrated flask. A 100 ml of 0.1 M Fe^{3+} solution was prepared from $FeCl_3.6H_2O$ salt in DDW.

Instrumentation

A Metrohm model 817 pH meter was used for pH measurements. A centrifuge model Hettich Rotofix 32A was used for protein precipitation of human plasma. A photodiode array Multi Spec Agilent with a 1-cm quartz cell was used for absorbance measurements. The spectra were registered in the range of 200-800 nm at 1 nm intervals. A Knauer 1050 HPLC pump and a Knauer 2850 PDA detector were used. For instrumental control, data collection and processing chromgate software was employed. The absorbance data were transformed into Excel files and then the wavelength range 450-700 nm was selected for MATLAB (Version 7.6.0) in windows. The singular value decomposition (SVD) based on principal component analysis (PCA) was written in MATLAB. A back-propagation neural network having three layers was created with a Visual-Basic software package.

Procedure

To a solution containing appropriate amounts of MPM and MPA in a 10 ml volumetric flask, 1 ml of 0.1 M Fe(III) and 200 µl of triton X-100 solution (1% v/v in water) were added. The mixture was diluted to the mark with DWW. Absorption data of this solution were transformed in Excel program and then MATLAB for multivariate and PC-ANN process. The singular value decomposition (SVD) based on principal component analysis (PCA) was written in MATLAB program. A back-propagation neural network having four layers was created with a Win NN32 software package. The number of hidden layers, nods, learning rate and momentum were optimums by experimental design.

RESULTS AND DISCUSSION

Complexation Reaction of Fe(III) with MPA and MPM

Figures 2 and 3 show UV-Vis spectra of MPA and MPM in the absence and presence of Fe^{3+} ion. In the absence of Fe^{3+} the spectra of MPA and MPM overlapped

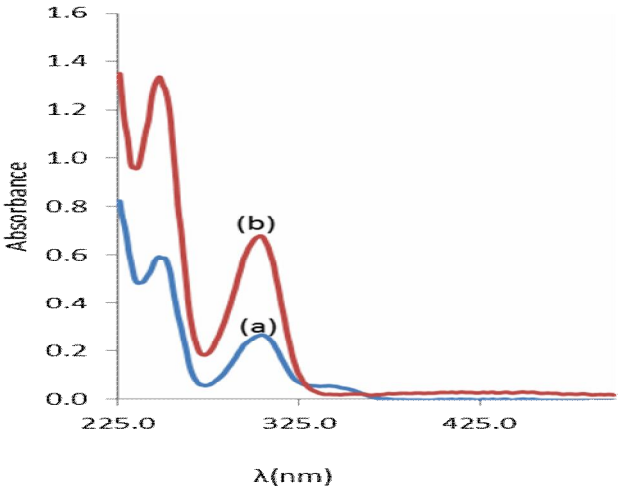


Fig. 2. UV absorption spectra for (a) MPM 217.0 mg l^{-1} (5.0×10^{-4} M) and (b) MPA160.0 mg l^{-1} (5.0×10^{-4} M). Conditions: pH = 2.5 and 0.02% of Triton X-100 in absence of Fe^{3+} ion.

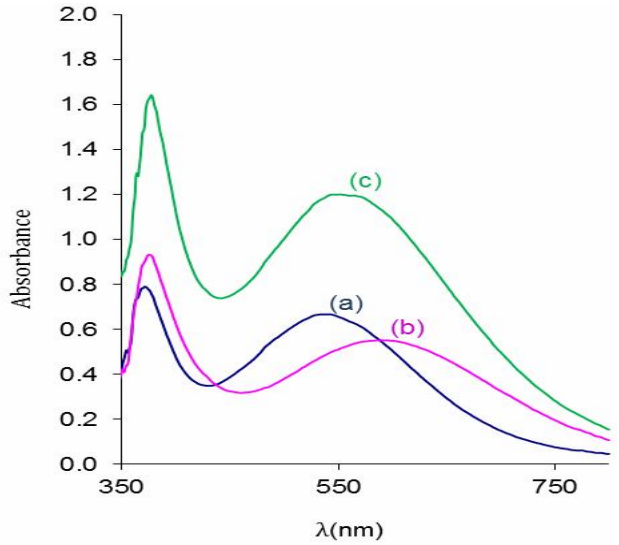


Fig. 3. UV-Vis absorption spectra of the complexes for (a); 161.6 mg l^{-1} (5.0×10^{-4} M) of MPA, (b); 586.0 mg l^{-1} (1.35×10^{-3} M) of MPM and (C); their mixture. Conditions: pH = 2.5 and 0.02% of Triton X-100 in the presence 0.01 Mof Fe^{3+} ion.

completely, as can be seen in Fig. 2. After addition of Fe^{3+} to the solution (Fig. 3) a red shift (about 200 nm) was observed in the spectra of MPA and MPM that confirms formation of complexes with different maximum absorbance wavelengths between the drugs and ferric ion. The complexes were stable. This matter is the base of selective determination of these drugs in the presence of each other by chemometric methods. It is conveniently studied by Job's method of continuous variations [11]. The Job's diagram for the complexation of MPA and MPM with Fe^{3+} was obtained by plotting the absorbance variation of complex *vs.* mole fraction of Fe^{3+} at 550 nm and 580 nm for MPA and MPM, respectively (Fig. 4). Each plot consists of two straight lines intersecting at mole fraction of Fe^{3+} equal to 0.5, as is typically the case when only a 1:1 complex (Fe(III):MPM) is formed, while the maximum at 0.66 mol fraction for MPA in plot indicated the formation of a 2:1(MPA: Fe(III)).

Influence of Chemical Variables

The complexation of Fe^{3+} with MPA and MPM occurred at acidic pHs. It was observed that the absorbance of the complexes decreased in alkaline media. Figure 5 resumes the effect of pH on the absorbance signals. Analysis of spectrophotometric data for MPM and MPM showed that the absorbance intensity increased by increasing pH in the range 2.0-2.5 and decreased at higher pHs. Therefore, pH 2.5 was selected as working pH. A 0.1 M of HCl solution was used to adjust the pH. The decrease in the absorbance intensity at higher pHs can be due to the precipitation of Fe^{3+}.

Several surfactants including anionic (sodium dodecyl sulfate), cationic (cetyltrimethyl ammonium bromide) and nonionic (Triton X-100, Triton X-114 and Tween-80) were tested in order to increase the sensitivity of the method by increasing interaction between drugs and Fe^{3+} for increasing absorbance of the solution. Addition of nonionic surfactants increased the molar absorptivity of complex. Among the nonionic surfactants investigated, Triton X-100 was found as the best. It caused the most sensitivity on the absorbance of the complexes. This can be due to the presence of polyoxyethylene groups and long alkyl chain (higher hydrophobisity and higher viscosity) $(C_{14}H_{22}O(C_2H_4O)_n$, n =

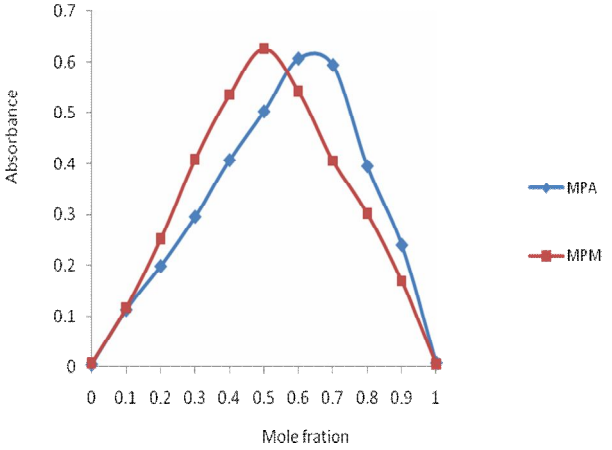


Fig. 4. Job's plot for determination relation concentration of Fe(III) and MPM or MPA. Conditions: pH = 2.5 and 0.02% of Triton X-100 at 590 nm and 540 nm, respectively.

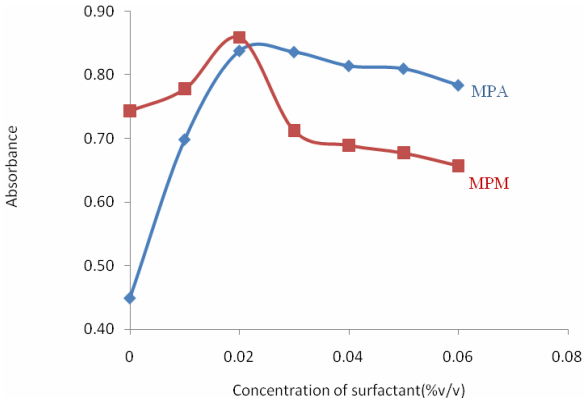


Fig. 6. The influence of concentration Triton X-100 surfactant on the absorbance for 650.0 mg l^{-1} of MPM and 192.0 mg l^{-1} of MPA in the presence of 0.01 M of Fe^{3+} in solution at 590 nm and 540 nm, respectively.

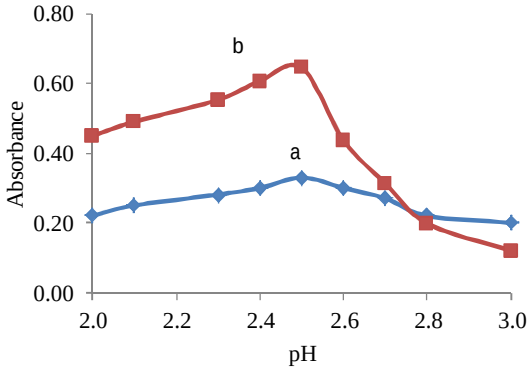


Fig. 5. Influence of pH on the absorbance of a: of 78.5 Mg l^{-1} (2.4×10^{-4} M) MPA and b: 686.7 mg l^{-1} (1.6×10^{-3} M) of MPM in the presence of 0.01 M Fe^{3+} at 540 nm and 590 nm, respectively.

9-10). The effect of Triton X-100 concentration on the absorbance of the MPA-Fe^{3+} or MPM-Fe^{3+} complexes were examined in the range of 0.00-0.06% (v/v) Triton X-100 and for 650.0 mg l^{-1} of MPM and 192.0 mg l^{-1} of MPA (Fig. 6). The results showed that the absorbance of the solutions increased by increasing Triton X-100 concentration in the range 0.0-0.02% (v/v), and then decreased at higher concentrations. Therefore, the suitable concentration surfactant for this study was 0.02%.

Multivariate Calibration

Figure 2 shows that the UV-Vis spectra of MPA and MPM that are completely overlapped. After complexing MPA and MPM with Fe^{3+}, the maximum absorbance of their complexes is at 540 and 590 nm, respectively. From Fig. 2 it is obvious that the analysis of the target drugs mixtures is not possible by spectrophotometry. But as Fig. 3 shows the analysis of their mixtures by spectrophotometry, based on the spectra of their complexes, using chemometric methods is possible. In order to propose a spectrophotometric method for the simultaneous determination of these compounds, three different chemometric approaches were evaluated. Based on a full factorial design [18], a training set with 36 samples at optimum condition were chosen, which are given in Table 1. The spectral region between 450-700 nm was selected as suitable for the analysis, which implies the use of 250 experimental points for each spectrum. Selection of spectral information was made according to the spectra of the pharmaceutical products. The range of the spectrum between 200-400 nm was rejected due to differences

Table 1. Concentration of MPM and MPA with Fe^{3+} (0.01 M) in Different Mixtures Used for Constructing the Training Set

Samples	MPA Concentration (mg l^{-1})	MPM Concentration (mg l^{-1})	Sample	MPA Concentration (mg l^{-1})	MPM Concentration (mg l^{-1})
	5.0	10.0	19	128.0	10.0
2	5.0	87.0	20	128.0	87.0
3	5.0	217.0	21	128.0	217.0
4	5.0	303.0	22	128.0	303.0
5	5.0	390.0	23	128.0	390.0
6	5.0	520.0	24	128.0	520.0
7	64.0	10.0	25	160.0	10.0
8	64.0	87.0	26	160.0	87.0
9	64.0	217.0	27	160.0	217.0
10	64.0	303.0	28	160.0	303.0
11	64.0	390.0	29	160.0	390.0
12	64.0	520.0	30	160.0	520.0
13	96.0	10.0	31	192.0	10.0
14	96.0	87.0	32	192.0	87.0
15	96.0	217.0	33	192.0	217.0
16	96.0	303.0	34	192.0	303.0
17	96.0	390.0	35	192.0	390.0
18	96.0	520.0	36	192.0	520.0

between the artificial mixture spectra and the pharmaceutical spectra products at the same concentration. A validation data set was built using 10 solutions within the concentration range spanned by the calibration data set. The best results in our particular case were for the PC-ANN method.

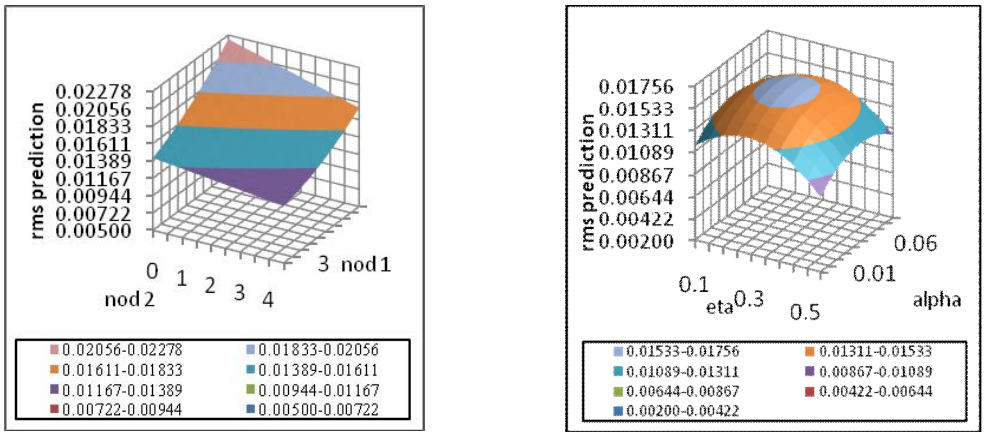
PCA is a traditional multivariate statistical method commonly used to reduce the number of predictive variables and solve the multi co-linearity problem [19] and PCA looks for a few linear combinations of the variables that can be used to summarize the data without losing too much information in the process. This method of dimension reduction is also known as “parsimonious summarization” [20] of the data. The PLS model was developed in the PLS1 mode. In order to select the number of factors, the leave-one-out cross-validation method was used [21]. The criterion of Haaland and Thomas [21,22] was used for selecting the optimum number of factors. From the spectra, the appropriated wavelengths regions were selected. The PC-ANN algorithm which was developed and was used in this study can be summarized as follows: in step 1, 250 experimental data points based on absorption spectra were selected; step 2, PCA was used to assess the intrinsic dimensionality of the problem and to extract a linear combination. In the presented PCA, we started with a 35 × 250 correlation matrix. The dominant principal components (PCs) were used as variables for each sample. In step 3, a feed-forward, back- propagation ANN was constructed to model the absorption-concentration relationship. The input vector was the set of absorption values for each sample in the series, as generated in step 2. The network was configured with two hidden layer of processing elements. The network was trained to reproduce the binding affinities by repetitive presentation of the set of input vectors in random order within each presentation of the entire set.

A back-propagation neural network having four layers

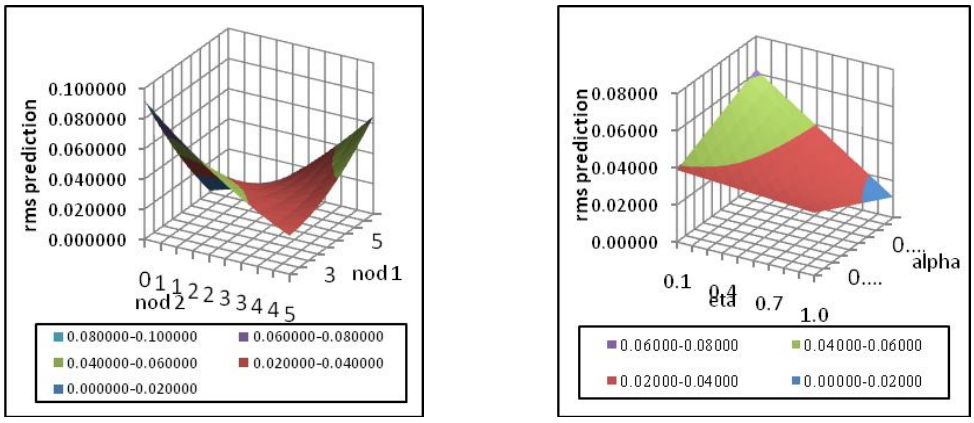
was created white a Win NN32 software package. The number of hidden layers, nods, learning rate and momentum were optimums by experimental design (Fig. 7). Network weights for a processing node which received an output from processing input were initially assigned random values between -1 and +1. A sigmoid transfer function generated the output of a neuron from the weighted sum of inputs belonged to the preceding (input) layer. In order choose the optimum ANN model, different topological networks were conducted with different hidden units. The values of learning rate, momentum coefficient and the original values of weights and biases (Table 2) were tested in order to find the best performance, *i.e.* the quickest convergency between predicted and actual values.

To obtain the number of factors which are required in PLS, PCR and PCA algorithms to avoid over fitting, *i.e.* discarding useless factors, cross validation method (leaving out one sample at a time) and prediction error sum of squares, PRESS, method have been used (Table 3). The predicted concentrations of the compound in each prediction set sample were compared with the true value and PRESS were calculated by each number of factors.

A set of sample solutions with different drugs concentrations was prepared and measurements were



(a)



(b)

Fig. 7. Experimental design for determination optimum parameters ANN model (a) MPA, (b) MPM.

Table 2. Optimum Parameters for ANN Model

parameters	MPM	MPA
Learning rate/η	0.9	0.5
Momentum/α	0.09	0.01
Number of iterations	6040	1175
Transfer function	Sigmoid	Sigmoid
Nodes of hidden layer 1	7	4
Nodes of hidden layer 2	2	4

Table 3. The Statistical Parameters of Prediction for PLS-1, PCR and PC-ANN Models Obtained at Optimum Experimental Condition

Parameters	Method					
	PLS-1		PCR		PC-ANNs	
	MPM	MPA	MPM	MPA	MPM	MPA
Factor number	3	3	3	3	3	3
PRESS	1.1592	0.1429	1.4438	0.1500	0.2961	0.0467
RMSD	0.3405	0.1195	0.3800	0.1225	0.1814	0.0720
r^2	0.9904	0.9943	0.9880	0.9940	0.9973	0.9981
REP (%)	6.1904	3.4154	6.9087	3.4991	3.5490	1.8523

carried out under the optimum conditions. The calibration curves of two analytes were linear in the ranges 5.0-215.0 mg l^{-1} (1.5×10^{-5}-6.7×10^{-4} M) and 10.0-1000.0 mg l^{-1} (2.3×10^{-5}-2.3×10^{-3} M) for MPA and MPM, respectively. The triplicate signals demonstrated good reproducibility. Equations for calibration graphs were obtained as: A = 1232.6 C + 0.018 for MPA and A = 387.6 C + 0.026 for MPM where A is the Absorbance and C is the concentration of analyte in mg l^{-1}. Correlation coefficients for MPA and MPM were 0.997 and 0.998, respectively. The limit of detection (LOD) and limit of quantification (LOQ) were defined as the compound concentration that produced a signal-to-noise ratio of 3 and 10, respectively. Based on these criteria LOD values were found to be 1.1 mg l^{-1} and 0.3 mg l^{-1} and LOQ values were found to be 3.8 mg l^{-1} and 0.9 mg l^{-1} for MPM and MPA, respectively.

A training set of 36 standard samples (26 samples as calibration set and 10 samples as prediction set) in aqueous media was taken from different mixtures of MPM and MPA (Table 1). Each concentration was varied between 10.0-520.0 mg l^{-1} for MPM and 5.0-192.0 mg l^{-1} for MPA through the calibration and prediction matrices. The correlation between the different calibration samples has to be avoided because colinear components in the training set data will tend to cause under-fitting in the PLS models. The obtained model was validated with a 10 synthetic mixture set containing the considered drugs in different proportion. Table 2 shows that the statistical parameters for PLS-1, PCR and PC-ANN models obtained at optimum experimental condition. The root mean squares difference

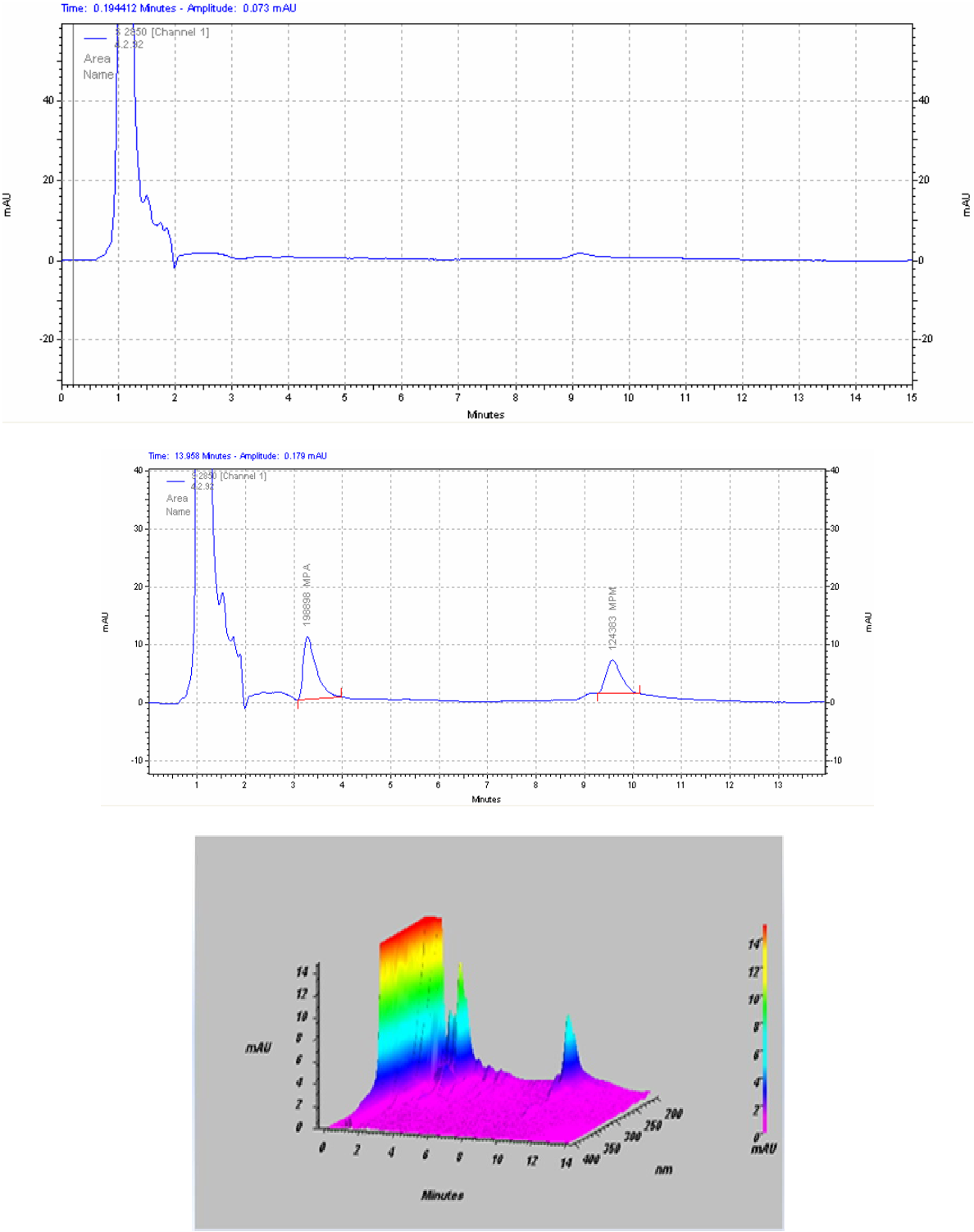


Fig. 8. Chromatograms of extract of (a): drug-free plasma, (b): spiked plasma sample with MPA (10.0 mg l^{-1}) and MPM (10.0 mg l^{-1}) (c):3D Chromatogram for spiked plasma sample with MPA (10.0 mg l^{-1}) and MPM (10.0 mg l^{-1}).

Table 4. The Results Obtained Using PC-ANN Method and HPLC Method

Plasma samples	Spiked (mg l^{-1})		Found (mg l^{-1}) PC-ANN		Found (mg l^{-1}) HPLC		t-Test[a]	
	MPM	MPA	MPM	MPA	MPM	MPA	MPM	MPA
1	85.0	10.0	83.1	9.2	84.2	9.5	2.46	0.53
2	220.0	130.0	210.6	125.3	216.8	127.9	2.55	1.48
3	270.0	160.0	256.3	155.4	261.2	158.7	2.64	2.61

[a]Tabulated value at 95% confidence limit was 2.78, N = 3.

(RMSD), which is an indication of the average error in the analysis of each component and the square of the correlation coefficient (r^2), which is an indication of the quality of fitting of all data points to a line. The predictive ability of each method and each component can also be described in terms of the relative error of prediction (REP).The results from r^2 and REP efficiency show that using (PC-ANNs) model for this purpose is more suitable than using PLS and PCR.

Real Sample Analysis

Different volumes of MPM and MPA solutions were added to one ml of plasma sample. For protein precipitation of plasma 3 ml acetonitrile as a solvent was added. After centrifugation at 4000 rpm for 10 min, the clear layer was transferred in to a beaker. The sample was then analyzed by the PC-ANN and HPLC as the standard method. For HPLC analysis, a 20 µl of clear sample was injected to the HPLC column, (Machereynagel column C_8, 150 mm × 2.5 mm, particle size 4 µm).The mobile phase consisted of a mixture of 51:49 (v/v) acetonitrile and potassium dihydrogen phosphate-phosphoric acid buffer (pH was adjusted at 3) and was pumped at flow rate of 1.5 ml min^{-1}. Chromatograms of drug-free plasma samples and spiked plasma are shown in Fig. 8. The results are given in Table 4 indicate that the accuracies were satisfactory in all cases. There is also a good agreement between the results obtained from the proposed method with those obtained from HPLC method. Application of the t-test showed that there were no statistically significant differences between the results obtained from the PC-ANN method and HPLC method at the 95% confidence level.

CONCLUSIONS

MPM is treated with Fe(III) to form complex that has a blue color and MPA is treated with Fe(III) to form a complex that has a violet color. The complex can be used to the spectrophotometric determination of MPM and MPA after pH control and addition of Trito*n X-100*. The results show that using PC-ANNs model for this purpose is more suitable than using PLS and PCR. This method is rapid, sensitive and inexpensive. It is appropriate for dosage control of pharmaceutical preparations. The PC-ANN model was applied to determination of two analytes in biological samples. The results obtained using PC-ANN methods were also compared with those obtained using HPLC method. There was a good agreement between the results. Although the HPLC method is more specific than the spectrophotometric methods, HPLC method need expensive equipment and materials such as columns and HPLC grad solvents [23,24].

ACKNOWLEDGEMENTS

The authors acknowledge the R&D Alborz Bulk Pharmaceutical Company for providing support to this work.

REFERENCES

[1] G. Bahrami, B. Mohammadi, Clin. Chim. Acta 370 (2006) 185.

[2] A.C. Allison, E.M. Eugui, Clin. Transplant. 7(1993) 96.

[3] R.E. Morris, J. Heart Lung Transplant. 12 (1993) S275.

[4] C.J. Young, H.W. Sollinger, Transplant. Proc. 26 (1994) 3144.

[5] K. Na-Bangchang, O. Supasyndh, T. Supaporn, V. Banmairuroi, J. Karbwang, J. Chromatogr. B 738 (2000) 169.

[6] A. Elbarbry Fawzy, S. Shoker Ahmed, J. Chromatogr. B 859 (2001) 276.

[7] X. Delavenne, L. Juthier, B. Pons, C. Mariat, T. Basset, Clin. Chim. Acta 412 (2011) 59.

[8] J. Kuhn, C.G. Ting, K. Kleesiek, Talanta 80 (2010) 1894.

[9] G. Absalan, M. Solemani, Anal. Sci. 20 (2004) 879.

[10] T. Irene, K. Martin, J. Chromatogr. B 681(1996) 347.

[11] N. Sugioka, H. Odani, T. Ohta, H. Kishimoto, T. Yasumura, K. Takada, J. Chromatogr. B 654 (1994) 249.

[12] U.D. Renner, C. Thiede, M. Bornhauser, G. Ehninger, H.M. Thiede, Anal. Chem. 73 (2001) 41.

[13] I. Tsina, M. Kaloostian, R. Lee, T. Tarnowski, B. Wong, J. Chromatogr. B 681 (1996) 347.

[14] M. Plätzer, K. Jahn, J. Wohlrab, R.H.H. Neubert, J. Chromatogr. B 755 (2001) 355.

[15] S. Verma, H. Gupta, O. Alam, P. Mullick, N. Siddiqui, S.A. Khan, J. Appl. Spect. 76 (2009) 876.

[16] V.P. Tripodi, S.E. Lucangioli, C.L. Barbara, V.G. Rodríguez, C.N. Carducci, Chromatographia 54 (2001) 93.

[17] T. Madrakian, M. Soleimani, A. Afkhami, Mater. Sci. Eng. C 42 (2014) 38.

[18] E. Schuetz, M. Shipkova, V.W. Armstrong, M. Oellerich, Clin. Chem. 45 (1999) 419.

[19] B. Eric, T. Hastie, P. Debashis, R. Tibshirani, J. Amer. Statis. Assoc. 101 (2006) 119.

[20] R. Roman, N. Krämer, Lect. Notes Comput. Sci. 3940 (2006) 34.

[21] D.M. Haaland, E.V. Thomas, Anal. Chem. 60 (1988) 1193.

[22] D.M. Haaland, E.V. Thomas, Anal. Chem. 60 (1988) 1202.

[23] E. Dinc, D. Baleanu, F. Onur, J. Pharm. Biomed. Anal. 26 (2001) 949.

[24] N.H. Al-shaalan, J. Saudi Chem. Soc. 14 (2010) 15.