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Development and validation of HPTLC method for quantification of mycophenolic acid

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Abstract

Mycophenolic Acid (MPA) is an immunosuppressant; produced by *Penicillium Sp.* In present study an analytical method is developed for analysis of MPA in fermentation broth by High Performance Thin Layer Chromatography (HPTLC), which has been a routine analytical technique by virtue of its advantages of low operating cost, high sample throughput and need for minimum sample clean-up. The method was carried out in TLC precoated silica gel on aluminium plate 60 F_{254}. Various mobile phases were screened, out of which chloroform: methanol: water (9.2:0.7:0.1) gave the best separation at 220 nm by densitometric evaluation. The linearity was found to be in the range of 100-500ng with R^2 value 0.997, % RSD of intraday and interday precision was found to be in the range of 1.22-2.03 and 1.23-2.81 respectively. Limit of Detection (LOD) and Limit of Quantification (LOQ) of mycophenolic acid was found to be 23.13ng/band and 77.09 ng/band respectively. Recovery of MPA obtained was found to be 98.76%; which suggest that the method is simple, accurate and reproducible. The experimental data was statistically analysed by Analysis of Variance (ANOVA) which proves that, the developed chromatographic method can be used for routine analysis of MPA.

Keywords: Mycophenolic acid, HPTLC, method development, validation.

Introduction

Secondary metabolites are compounds with varied and sophisticated chemical structures, produced by strains of certain microbial species and some by plants. Although antibiotics are the best known secondary metabolites, there are other such metabolites with an enormous range of biological activities, hence acquiring actual or potential industrial importance (Barrios-Gonzalez et al. 2005).

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Mycophenolic acid (6-(4-hydroxy-6-methoxy-7-methyl-3-oxophthalanyl)-4-methyl-4-hexenic acid, MPA) is a secondary metabolite of *Penicillium brevicompactum* with diverse biological properties and has been extensively studied for its antineoplastic, immunosuppressive, anti-inflammatory, antiviral, antipsoriasis and antifungal activities (Xu and Yang 2006). MPA is known to exhibit multiple activities. Its antibiotic activity against *Staphyloccoccus aureus* led to its discovery by Gosio (Abraham 1945). MPA is an immunosuppressive drug inhibiting T- and B-lymphocyte-mediated immune responses with a profile of activities different from other immunosuppressives (Eugut et al. 1991). Derivatives of MPA show antitumor activity also (Ohsugi et al. 1976). Morpholino-ethyl ester derivative of MPA was introduced by Roche-Syntex into the therapy as an immunosuppressive drug in the transplantation of kidney (Jekkel et al. 2001). Mycophenolate mofetil (MMF) is an ester of MPA that inhibits the *de novo* synthesis of guanine nucleotides. Following oral administration, MMF is hydrolyzed by esterase in the intestine and blood to release MPA. MPA exerts a more potent cytostatic effect on lymphocytes than on other cell types, such that its effect on lymphocytes is the principle mechanism of its immunosuppressive activity (Iwasaki 2004). MPA could be produced by several species of *Penicillium* in submerged cultures. Among them *P. brevicompactum* showed a relative high ability of producing MPA (Ardestani et al. 2010). Filamentous fungi were often most suited to solid state fermentation (SSF) for the production of several valuable metabolites since these conditions were similar to their natural habitat. Also the isolation and purification processes were simple. MPA can also be produced in solid substrate fermentation. SSF proves to be a better method since the agricultural wastes used as supports make the production of MPA cost effective (Sadhukhan et al. 1999). HPTLC has been used for quantification of mycophenolate mofetil in bulk and pharmaceutical formulation (Kathirvel et al. 2012); mycophenolate mofetil is the ester prodrug of mycophenolic acid (MPA), however analysis of MPA in crude form or in fermentation media with strong statistical analysis of the data has not been considered. Pharmacokinetic studies of MPA have been done by HPLC (Kagaya et al. 2006; Zeng et al.

2009). Other methods of detection included capillary zone electrophoresis and LC-MS method was also developed (Ohyama et al. 2008; Carlucci et al. 2007; Mendonza et al. 2006). A concern in the analysis of MPA by electrospray ionization MS is the reported susceptibility of MPAG to in-source fragmentation, which can lead to loss of the glucuronide moiety and the production of MPA (Vogeser et al. 2001). Hence, chromatographic separation of MPA proves to be cost effective, easy and accurate. It also facilitates repeated detection of chromatogram with the same or different parameters. A further major advantage of HPTLC is that several samples can be run simultaneously using a small quantity of mobile phase, unlike HPLC, thus lowering analysis time and cost per analysis (Abou-Donia et al. 2008; Patel et al. 2010). Also it has many advantages of high sample throughput at low operating cost, easy sample preparation, short analysis time and analytical assurance (Dhalwal et al. 2010). Few reports suggest the quantification as well as detection of MPA from fermentation broths using HPTLC; hence the present study illustrates a simple and reproducible method for detection of MPA by High Performance Thin Layer Chromatography (HPTLC).

Materials and Methods

Apparatus

HPTLC system consisted of CAMAG LINOMAT V applicator, CAMAG TLC Scanner III for densitometric analysis of MPA, Hamilton syringe (100µl), WINCATS software version 1.4.3.6336 and CAMAG twin trough chamber (20X10cm). Pre-coated silica gel 60 F_{254} TLC plates, layer thickness 0.2 mm (20X10cm), purchased from Merck Chemicals Corporation Ltd, Mumbai and were used as a stationary phase. Beckman's J2-MC centrifuge (rotor JA-20) was used for extraction of sample.

Reagents and Media components

Standard MPA and other chemicals like Chloroform and methanol (HPLC grade) were purchased from Himedia Laboratory, Mumbai, India. Media components such as glucose, glycine, L-methionine, KH_2PO_4, $MgSO_4.7H_2O$, $FeSO_4.7H_2O$, $CuSO_4.5H_2O$, $ZnSO_4.7H_2O$, $MnSO_4.4H_2O$, $KMoO_4$ and potato dextrose agar were procured from Himedia Labs, Mumbai.

Microorganism and culture conditions

Penicillium brevicompactum NRRL 2011 was procured from Agricultural Research service culture collection (NRRL), USA. The culture was grown on potato dextrose broth (PDB) for seven days at 25^0C and maintained on potato dextrose agar (PDA) slants by preparing fresh slants every 2 weeks.

Chromatographic Conditions

Standard preparation

Standard stock solution of MPA was prepared by accurately weighing 10 mg of MPA in 10 ml volumetric flask and dissolving it in 10ml methanol. Further dilution was done to get final concentration of 100µg/ml.

Sample preparation

Penicillium brevicompactum NRRL 2011 was grown in media containing glucose (100g/L), glycine (14g/L), L-methionine (0.5g/L), KH_2PO_4 (2g/L), $MgSO_4.7H_2O$ (1g/L) and trace element solution 1ml/L [$FeSO_4.7H_2O$, $CuSO_4.5H_2O$, $ZnSO_4.7H_2O$, $MnSO_4.4H_2O$ and $KMoO_4$] and incubated on an incubator shaker (180 rpm) at 25 ºC for 240 h. The medium after fermentation was separated from the cells by filtration. pH of the filtrate was adjusted between 10-11 and centrifuged in Beckman J2-MC Centrifuge, USA, (Rotor JA 20) at 12100 x g at 25 ºC for 25 min. Supernatant collected was filtered through 0.22µm and used for detection of MPA.

Sample application

Standard MPA aliquots were spotted on TLC plates at a constant application rate of 150 nL s^{-1}. Band length of the spots was 5mm.

Mobile phase

Various mobile phases were screened for better separation and resolution of MPA. However chloroform: methanol: water in the ratio of 9.2: 0.7:0.1 (v/v/v) gave the best separation with Retardation factor (R_f) value 0.32 ± 0.01. The optimized chamber saturation time for mobile phase was 20 min at 25 ± 2°C. Migration distance was 80 mm.

Detection

After development of plates mentioned in the previous section they were further air dried for 5 min and chromatogram was recorded by scanning at 220nm using Camag scanner III. Spectral scanning was performed to check the wavelength at which MPA shows maximum absorption. Spectral scanning includes multi-wavelength scanning, in which the chromatogram is scanned automatically at all wavelengths from 200 to 700 nm in order to achieve maximum selectivity. This software function is unique in HPTLC. After spectral scan of MPA in sample and standard, maximum absorption of MPA was seen at 220 nm (Figure 1).

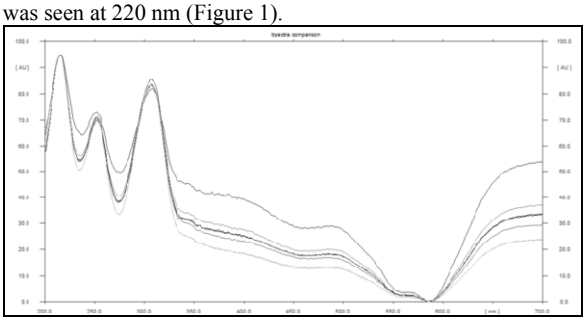

Figure 1: Overlay of spectra of Mycophenolic acid. X axis: wavelength (200-700nm); Y axis: Absorbance (AU)

Validation

The developed HPTLC method was validated for linearity, specificity, sensitivity, accuracy, precision, repeatability and robustness. The experimental data was statistically analysed by analysis of variance (ANOVA) and p values were obtained by using Graphpad Prism 5 software.

Linearity (calibration curve)

Five different concentrations of MPA (100, 200, 300, 400 and 500 ng/band) were applied in triplicates on HPTLC plate. Calibration curve was calculated by plotting peak area *vs* concentration with the help of WINCATS software.

Specificity

Specificity of the method developed was studied by analyzing the sample solution containing MPA and standard MPA. MPA in the sample was confirmed by comparing the R_f value of the spot with that of standard MPA.

Sensitivity

It was studied by calculating Limit of detection (LOD) and limit of quantification (LOQ). Blank spot of methanol was scanned six times to determine noise. Different concentrations of MPA (100-600 ng) were spotted on the plate. LOD was calculated as 3 times the noise level and LOQ was calculated as 10 times the noise level.

Accuracy

Recovery studies of MPA were carried out to check accuracy of the method. Study was carried out at three levels in triplicates. Three different concentrations of standard MPA (200, 400 and 600 ng) were added to preanalyzed samples containing MPA. Data was analyzed and p value was calculated.

Precision

Precision of the method was determined in terms of intraday and interday precisions. Interday precision was calculated by analyzing standard MPA solution in the range of 100-500 ng/band, three times on the same day. Mean, relative standard deviation (RSD) and p values of the peak area were calculated.

Repeatability

Repeatability of sample application was evaluated by spotting standard MPA (500ng/band) six times without changing the position of the plate and analyzing it.

Robustness

To check the robustness of the method, small changes were made in mobile phase composition, chamber saturation time, solvent migration distance and temperature. Study was carried out in triplicates at an MPA concentration of 500ng/band. Mean, % RSD and p value was calculated.

Results and Discussion

HPTLC method for detection of MPA was developed. Various mobile phases were screened for better separation and resolution of MPA. The mobile phase chloroform: methanol: water in the ratio of 9.2:0.7:0.1 (v/v/v) gave the best resolution.

Table 1: Recovery study of Mycophenolic acid (n=3)

MPA in sample (ng)	Standard MPA added (ng)	Total conc.	Conc. Obtained	% Recovery	Mean	ANOVA
106.504	200	306.504	304.601	99.38	98.76	R^2=0.999 P<0.0001
103.380	400	503.38	490.098	97.36		
105.336	600	705.336	702.057	99.53		

Linearity

The linearity of MPA was established by plotting the peak area versus concentration. Linearity was observed in the range of 100-500 ng. The regression equation and correlation coefficient (R^2) of MPA was found to be Y= -29.770 + 7.534*X and 0.9971 respectively, with SD value 3.84% (Figure 2).

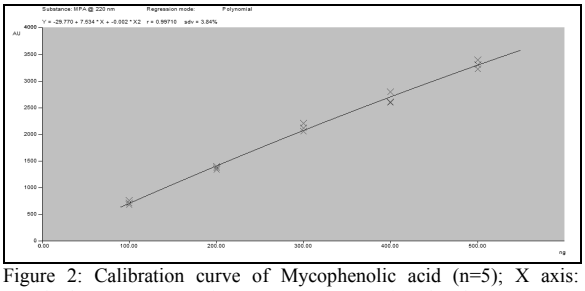

Figure 2: Calibration curve of Mycophenolic acid (n=5); X axis: Standard MPA concentration (100-500ng); Y axis: Absorbance (AU)

Sensitivity

LOD and LOQ were calculated by using the equation LOD= 3 x SE/S and LOQ= 10 x SE/S, where SE is standard error of the peak areas of standard taken as measure for noise and S is slope of calibration curve. The LOD and LOQ of MPS with signal to noise ratio was found to be 23.13 and 77.09 ng/ band respectively.

Precision

Intraday and Interday precision was calculated by taking RSD of three repeated assay of standard MPA at three different concentration levels (100, 300 and 500 ng/band). The % RSD of intraday and interday precision was found to be in the range of 1.22-2.03 and 1.23-2.81 respectively. ANOVA indicates R^2 value of 0.999 and p value is less than 0.0001, which suggests that results are statistically significant.

Accuracy

Percentage recovery of MPA obtained was found to be 98.76 with R^2 0.999 and p value less than 0.0001, which suggest that the developed method is statistically significant and accurate for MPA detection (Table 1).

Specificity, Repeatability and Robustness

Analysis of standard MPA (500ng) (Figure 3) and MPA in sample solution (Figure 4) was done. Rf values were same, which confirmed the presence of MPA in sample. Specificity study of the method showed that excipient present in the sample did not interfere with MPA peak which was obtained at Rf value 0.32 ± 0.01. %RSD for repeatability was found to be 1.9. Robustness of the method was studied by carrying out each experiment in triplicates and the results for the same are shown in Table 2. % RSD values based on peak measurement was found to be less than 1, ANOVA indicates R^2 value of 0.999 and p value less than 0.0001. Hence the method was found to be precise.

Conclusions

HPTLC method was developed for analysis of MPA in

fermentation broth. Linearity, sensitivity, precision, accuracy, specificity, repeatability and robustness of the analytical method have been validated. ANOVA of the statistical data suggests that the proposed method is significant, accurate, robust and specific which can be used for routine analysis of MPA.

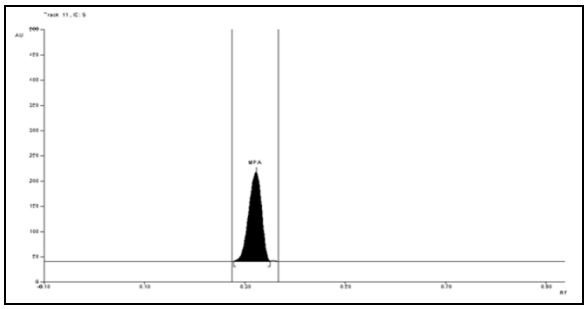

Figure 3: HPTLC chromatogram of standard Mycophenolic acid. X axis: Rf value; Y axis: wavelength (AU)

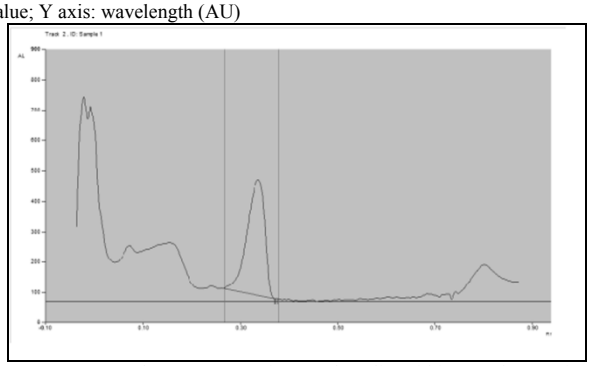

Figure 4: HPTLC chromatogram of Mycophenolic acid in sample. X axis: Rf value; Y axis: Absorbance (AU).

Table 2: Studies to check robustness of the developed method (n=3)

Parameters	SD	% RSD	ANOVA
Mobile phase composition (Chloroform: methanol: water) (8.5:1.3:0.2/9.7:0.3:0.1/9.2:0.8:0.1)	2.84	0.12	R^2=0.999 P<0.0001
Chamber saturation time (10, 20, 30 mins)	3.98	0.15	
Mobile phase volume in ml (10, 12 and 14)	2.54	0.09	
Temperature (25°C, RT[a] and 37°C)	4.96	0.19	

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