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Research Article

Development and Validation of Stability Indicating LC-PDA Method for Mycophenolate Mofetil in Presence of Mycophenolic Acid and Its Application for Degradation Kinetics and pH Profile Study

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Factorial design tool applied for development of isocratic reversed-phase stability-indicating HPLC method for the analysis of mycophenolate mofetil (MMF) and its degradation products. MMF stress degradation products mycophenolate acid (MPA) and DP3 (USP impurity H) were isolated and used for quantitation. Separation achieved on a Symmetry C18 (250 mm × 4.6 mm, 5.0 μ) column using a methanol: acetate buffer (75 : 25 v/v), pH 6.0 (adjusted with acetic acid), at 0.5 mL flow rate, column maintained at 55°C, and data integrated at 251 nm. MMF is subjected to hydrolysis, oxidation, heat degradation, and so forth; under all these conditions degraded products are well separated. The method validation characteristics included accuracy, precision, linearity, range, specificity, and sensitivity. Robustness testing is conducted to evaluate the effect of minor changes to the chromatographic conditions and to establish appropriate system suitability parameters. The proposed method is used to investigate kinetics of acid, alkali hydrolysis and oxidation process. Major degradation products MPA and DP3 were isolated and quantitated. Characterization of MPA by NMR and LC-MS/MS and other degraded products by LC-MS/MS is attempted successfully. The method is used successfully for the quality assessment of three MMF drug commercial formations and its acid, alkali, and oxidative degradation kinetics study.

1. Introduction

Mycophenolate mofetil (MMF) is chemically 2-(morpholin-4-yl) ethyl (4*E*)-6-(4-hydroxy-6-methoxy-7-methyl-3-oxo-1,3-dihydroisobenzofuran-5-yl)-4-methylhex-4-enoate. It is inhibitor of nucleic acid synthesis. It is the ester moiety of mycophenolic acid (MPA). It is a white or almost white crystalline powder and is used as immunosuppressive [1]. HPLC and HPTLC analytical methods for dissolution study of MMF in capsules, for its assay in bulk and dosage forms, [2–6] are reported. Bioanalytical and immunoassay methods are available for its determination individually or in presence of MPA [7–9] and one UV dissolution method is reported [10]. Its degradation products in aqueous solution are studied [11]. Stability-indicating HPLC and LC-MS/MS methods [12, 13] are reported where structural elucidation of degraded product and separation of the stress degraded product is lacking. MMF is included in BP, EP, and USP and assayed by potentiometry [14, 15]. Isocratic LC method was described in proposed USP monograph [16, 17] for MMF tablet and capsule assay using mobile phase acetonitrile : water containing 0.3% triethylamine (30 : 20 v/v), pH 5.3 (adjusted with phosphoric acid) with run time of 10 min. Proposed isocratic RP-HPLC-PDA method has many advantages, which includes mass spectrometry compatible and simple methanol and acetate buffer mixture used as mobile phase and the method

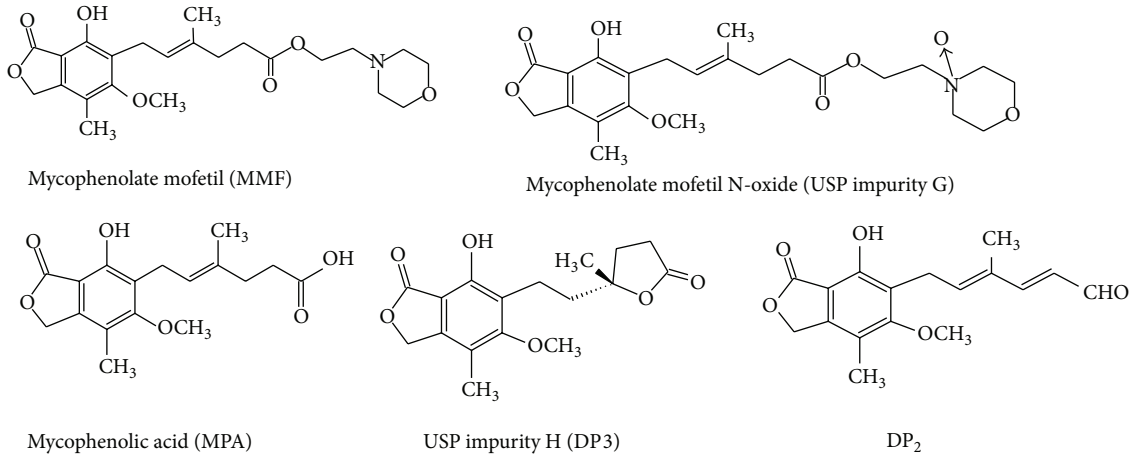

Figure 1: Structure of MMF and its major degraded products.

is stability indicating and highly sensitive. Quantitation of MMF, MPA, and DP3 (USP reported impurity H) is achieved (Figure 1); proposed method is capable of separating all the stress degraded products with short run time. Accordingly, the aim of the present study was to establish inherent stability of MMF through stress studies under a variety of ICH recommended test conditions [18] and to develop a sensitive stability-indicating assay method with MS compatible and simple mobile phase and structural elucidation of the stress degraded products.

2. Experimental

2.1. Reagent and Chemicals. HPLC grade methanol was purchased from Merck (Mumbai, India). Analytical grade acetic acid and ammonium acetate were purchased from Loba Chemie Pvt. Ltd., Mumbai. Tablet used for analysis was Mycophen 250 (manufactured by Micro Labs Ltd., Hosur, India, batch number MYLH0001) and capsules used were Cellcept (imported and disturbed by Taksal Pharma Pvt. Ltd., New Delhi, batch number m1201) and Mycept (manufactured by Panacea Biotech Ltd., Solan, batch number 4669502), each containing 250 mg of MMF. Pure drug sample of MMF (% purity 99.6) and standard MPA (% purity 99.8) were kindly supplied as a gift sample by Panacea Biotech Ltd., Solan, and standard hydrochlorothiazide (HTZ) (% purity 99.78) was gift by Cipla Ltd., Mumbai.

2.2. Chromatographic Conditions and Software. The HPLC system is equipped with binary pump (model Waters 515 HPLC pump), autosampler (model 717 plus), column oven (model-Waters CHM), and PDA detector (Waters 2998). Data collection and analysis were performed using Empower-version 2 software. Separation was achieved on Symmetry C18 (250 mm × 4.6 mm, 5.0 μ) columns maintained at 55°C. The column was supported with Waters symmetry C18 (3.9 × 20 mm, 5.0 μ) guard column. Mobile phase composed of methanol : acetate buffer (25 mM) (75 : 25% v/v), pH adjusted to 6.0 with acetic acid. The flow rate was 0.5 mL/min and the PDA detector was set at 251 nm; the injection volume was 20 μL. Trial version of Minitab 15.1.2 software was used for optimization study.

2.3. Preparation of Standard and Sample Solutions and Calibration Curve. A stock solution of MMF, MPA, and DP3 (USP impurity H) containing 1000 μg/mL was prepared, separately, in methanol. From these stock solutions working solutions of MMF 0.1–120, MPA 0.08–16, and DP3 0.06–12 μg/mL were prepared in mobile phase. For preparation of calibration curve mixed standard solutions of MMF, MPA, and DP3 from stock solutions were prepared and injected onto the column and calibration curve was prepared by using concentration and area of chromatographic peaks. System suitability test (SST) standard mixture containing MMF, MPA, and DP3, 60, 8, and 6 μg/mL, respectively, was prepared by mixing and diluting stock solutions with mobile phase. For preparation of sample, solution powder from twenty tablets/capsules equivalent to 100 mg of MMF was transferred to a flask containing 80 mL of methanol, sonicated for 5 min, and filtered. Filter was rinsed with methanol; filtrate was transferred to flask and volume was made to 100 mL with methanol; it was diluted suitably with mobile phase to contain 60 μg/mL of MMF, filtered through 0.45 μm Nylon 66-membrane filter, and used for analysis.

2.4. Method Validation. The HPLC method was validated in terms of precision, accuracy, specificity, sensitivity, robustness, and linearity according to ICH guidelines. Assay method precision (interday and intraday) was determined using nine-independent test solutions and precision of repeatability was performed by six-time injection of the solution. Assay method was evaluated with the recovery of the standards from excipients by spiking standards at three levels (50%, 100%, and 150%) to preanalyzed formulation. Values of limit of detection (LOD) and limit of quantification (LOQ) were calculated by using σ (standard deviation of response) and b (slope of the calibration curve) and by using equations, LOD = $(3.3 \times \sigma)/b$ and LOQ = $(10 \times \sigma)/b$.

Calculated values of LOD and LOQ were confirmed by repeated injections of samples containing amounts of analyte in the range of LOD and LOQ. To determine the robustness of the method, the final experimental conditions were purposely altered and the results were examined. The flow rate was varied by (±)5%. Column temperature was varied by (±)2°C and effect of column from different suppliers was studied. Measurement wavelength was varied by (±)1 nm, injection volume was changed (±)2 μL, % organic was changed by (±)5%, and buffer strength was changed by (±)5 mM. Change in one parameter was considered for study at a time. Each parameter was studied by triplicate injection of the sample containing HTZ, MMF, MPA, and DP3. Robustness was evaluated by studying the effect of parameter change on assay values and resolution of the analytes injected and its effect on other system suitability parameters (retention time, number of theoretical plates, and peak symmetry). Long term (4°C for 5, 10, 15, and 30 days) and autosampler stability (6, 12, and 24 hrs in autosampler) of the drug in solution were studied.

2.5. Procedure for Forced Degradation Study. Forced degradation of the drug product was carried out under thermolytic, photolytic, acid/base hydrolytic, and oxidative stress conditions. For photolytic stress, drug product in the solid state was irradiated with UV radiation with peak intensities at 254 and 366 nm. The UV dose from the lamp of peak intensity at 366 nm was measured by use of a quinine monohydrochloride (2% solution in water) chemical actinometer as mentioned in the ICH guidelines [18]. Minimum desired exposure (200 Wh/m^2) was observed after irradiation for 26 h. Sample solution containing 1000 μg/mL MMF was subjected to selected stressed conditions, neutralized, appropriately diluted, and injected onto column. Samples except for photo oxidation were protected from light. For acid, base, and water induced degradation, solutions containing 1000 μg/mL of the drug were prepared in 0.1 N HCl, 0.05 N NaOH, and water and analysed after 5, 6, and 12 hrs exposure, respectively. For oxidative degradation, solution was prepared in water containing 10% v/v of H_2O_2 and analysed after 8 hrs. During dry heat study sample was exposed to 60°C for 8 hrs in oven and analysed.

2.6. Degradation Kinetics. MMF solution containing 1000 μg/mL was used for acid (0.1 N HCl), alkali (0.05 N NaOH), and oxidative (10% H_2O_2) degradation; kinetics were studied at 60, 70, 80, and 90°C.

2.7. Preparation and Isolation of Alkali/Acid Induced Degraded Products, MPA and DP3. MMF, 1 g, was dissolved in 50 mL methanol and refluxed with 100 mL 0.5 M NaOH/HCl at 100°C for 15 min. Subsequently, pH of the solution was adjusted to 2.5 with 5 M HCl to precipitate MPA. The precipitate was filtered, dried under vacuum, and used. Degraded product DP3 is observed during acid, alkali, oxidative degradation condition, which was isolated using TLC plates. DP3 was isolated from chloroform extract of degraded solution by using laboratory prepared silica gel G TLC plates.

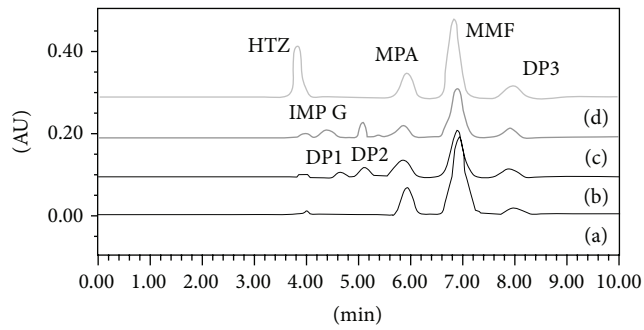

Figure 2: Chromatograms of stress degraded products of MMF with (a) HCl, (b) NaOH, (c) H_2O_2, and (d) system suitability chromatogram showing peaks of HTZ (internal standard) and analytes MPA, MMF, and DP3.

Mobile phase used for the TLC separation of DP3 was chloroform : toluene : methanol : acetic acid (7 : 3 : 0.2 : 0.1 v/v) with development time of 20 min, and it was eluted with R_f 0.7. Purity of isolated DP3 was confirmed by HPLC using PDA detector.

2.8. LC-MS/MS and NMR Study. The MS experiments were performed on LC-ESI-MS-MS/MS 410 prostar binary LC with 500 MS IT detector (Varian, USA) at IIT, Powai, Mumbai. LC unit consisted of prostar 210 binary pumps with prostar 410 autosampler. Previously described chromatographic conditions were used. MS was operated at enhanced scan mode, capillary voltage was 80, and mass range was 50–600 m/z. Prom (version 3.01.01.00) software was used for data acquisition and processing. For ^{1}HNMR and ^{13}CNMR, measurements of MMF and MPA were recorded on Mercury 300 NMR (Varian, USA) instrument at University of Pune, Pune, and CDCl3 was used as solvent.

3. Results and Discussion

3.1. Method Development. Factorial design was used as tool for optimization of the chromatographic conditions to develop a stability-indicating method. Various pilot trials were carried out to investigate different stationary phases, for example, C18 column (Symmetry and Kromasil), different mobile phases containing organic modifiers (methanol, acetonitrile, and THF), different buffers of different pH (3–6.5), and temperature (30 to 65°C). Our objectives were to achieve a peak tailing factor <1.2, retention times (t_R) from 4 to 9 min, and MMF and MPA resolution >2. From this experiment pH 6.0 was selected as most appropriate because at lower pH there is increase in baseline disturbances and peaks were not symmetric. Column was maintained at 55°C and methanol as organic modifier was selected to be the most appropriate. Effect of mobile phase flow rate and % of methanol was studied by full factorial design (Table 1(a)) and 2^2 full factorial design experiments were carried out to study the effect on t_R of MMF and MPA (Table 1(b)). By using full factorial design data linear equations obtained for MMF and MPA were $Y = 7.113 - 0.4343x_1 + 1.0478x_2 - 0.0528x_1x_2$ and $= 6.1515 - 0.5265x_1 + 0.9135x_2 - 0.008x_1x_2$, respectively. From the data

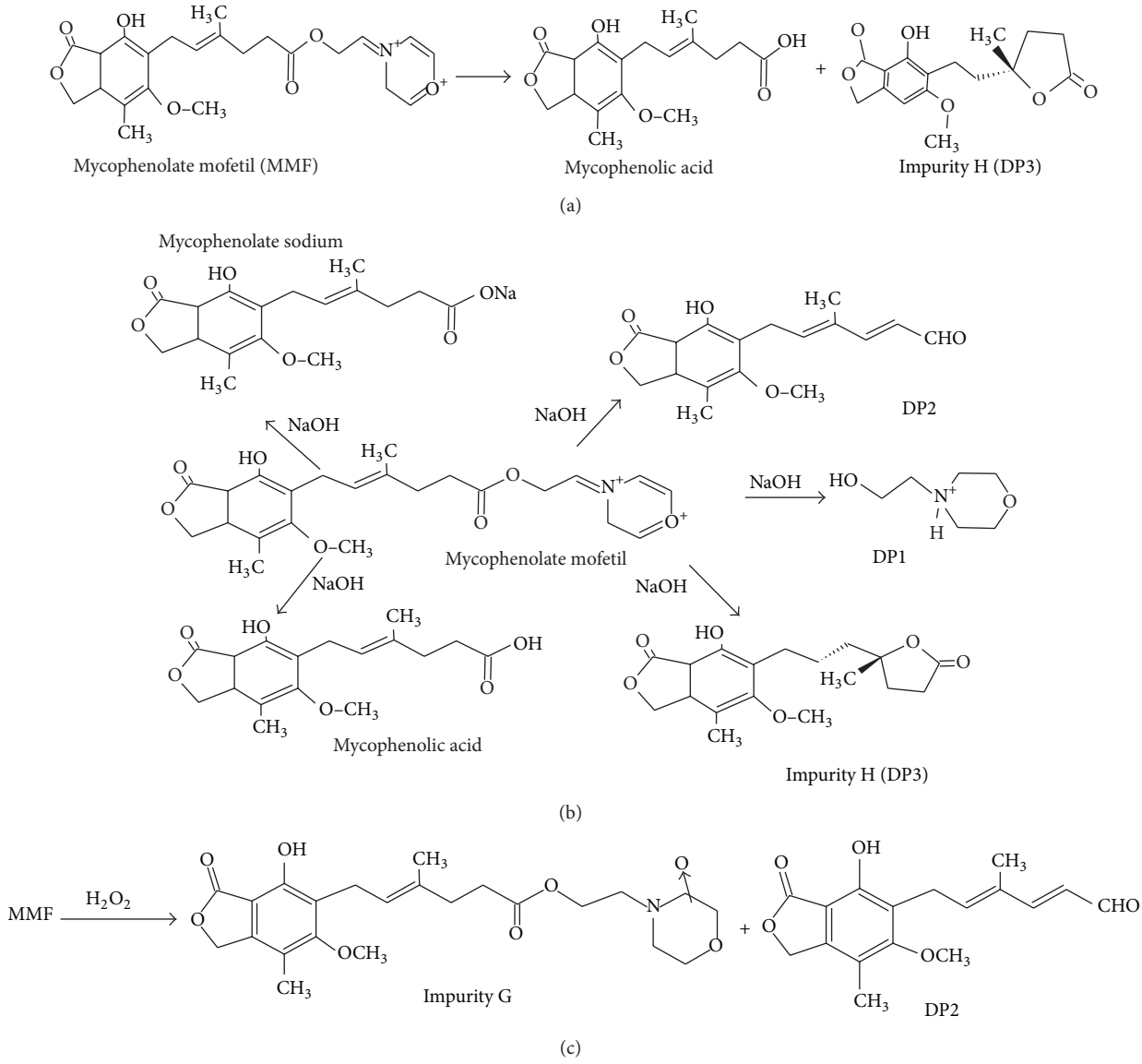

Figure 3: Proposed mechanism of (a) acid, (b) alkali, and (c) oxidative stress degradation of MMF.

obtained (Table 1(c)), it can be concluded that there is no interaction between the variables and flow rate does not play an important role. Methanol % affects t_R and is considered as main effect. Target values of t_R for MPA (6.0) and MMF (7.0) were fixed and weight = 1 was assigned to it to obtain optimum conditions. HPLC runs were carried out by varying the % of methanol in mobile phase and final chromatographic conditions selected were Symmetry C18 (250 mm × 4.6 mm, 5.0 μ) column maintained at 55°C using column oven. Mobile phase composed of methanol: acetate buffer (pH 6.0) (75 : 25 v/v). The flow rate was 0.5 mL/minutes, the PDA detector was set at 251 nm, and injection volume was 20 μL.

3.2. Method Validation. The method was validated according to ICH guidelines. The following validation characteristics were addressed: linearity, range, accuracy, precision, specificity, sensitivity (LOQ and LOD), and robustness. Among drotaverine, HTZ, diclofenac sodium, etoricoxib, and atenolol, hydrochlorothiazide (HTZ) was observed as the most appropriate internal standard (IS) with values of peak symmetry, theoretical plates, and resolution well within limit. Assay value determined for three formulations was in the range of 100 ± 1.33% with %RSD <1.2. Specificity of the method was determined by analyzing samples containing a mixture of the drug product excipients, samples containing MMF, MPA, and DP3, and stress degraded samples. All chromatograms were examined to determine if MMF, MPA, and DP3 coeluted with each other or with any excipient peak. Linear least square regression and LOD and LOQ data summarized in Table 2 indicate linearity and sensitivity of the method. Accuracy and precision were established for MMF, MPA, and DP3. Results for the accuracy of analytes tested in drug products by

Table 1: Results of method optimization.

(a) Factors and their low (−), high (+), and zero (0) levels

Factors	Factors level		
	(−)	(+)	(0)
Methanol % (x_1)	70	80	75
Flow rate (mL/min) (x_2)	0.60	0.70	0.65

(b) Obtained results for t_R

Factors		Retention factors	
x_1	x_2	MMF	MPA
−	−	8.64	7.67
−	+	6.44	5.68
+	−	7.69	6.46
+	+	5.70	4.79

(c) Effect of variables on t_R of MMF and MPA

Variable	% effect on t_R of	
	MMF	MMF
x_1 (% methanol)	13.94	38.51
x_2 (flow rate)	85.84	61.04
x_1x_2 (interaction)	0.22	0.45

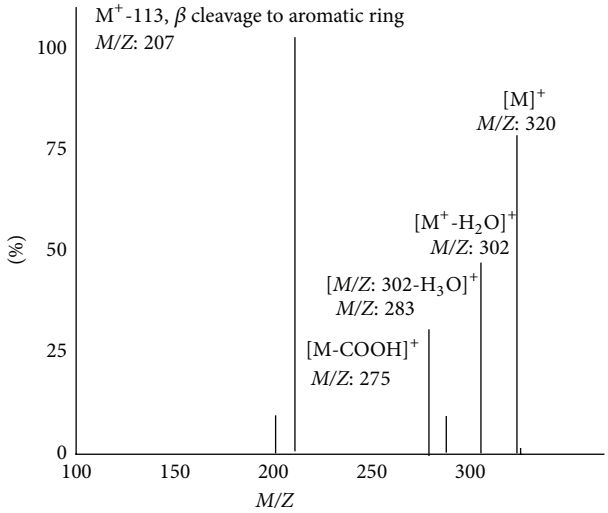

Figure 4: Mass fragmentation pattern of proposed impurity H (Enantiomer, DP3).

the technique of standard addition ranged from 98.87 to 101.6%. Results of precision, SST, and recovery are summarized in Table 2. Robustness parameters studied were as described in the method validation section. During robustness study, resolution between MMF, MPA, and DP3 was >2.2 and assay variability of the method for MMF, MPA, and DP3 was always within 100 ± 1.5%. Values of other system suitability parameters were always within the limits indicating robustness of the method. The %RSD of assay values of MMF and MPA during solution and autosampler stability experiments were within 1.6 and no significant changes were observed.

3.3. NMR Study. The PMR spectrum of MPA in deuterated chloroform showed protons signals δ_H at 1.804 ppm (singlet, 2H), 2.146 ppm (singlet, 3H), 2.277–2.325 ppm (triplet, 1H) 2.378–2.460 (quartet, 2H), 3.373–3.395 (doublet, 2H), 3.620 (singlet, 1H), 3.757 (singlet, 3H), 5.193 (singlet, 3H), 5.228–5.270 (triplet, 2H), and 7.840 (broad singlet 1H). These signals were compared with ^{1}HNMR of standard sample and found at the same position, intensity, and multiplicity. ^{13}C NMR signals, for MMF and MPA, were observed at shift value and carbon numbers are δ_C 11.23(C-20), 15.86(C-17), 22.42(C-18), 34.42(C-13), 60.79(C-12), 69.69(C-5), 106.17(C-6), 116.41(C-15), 121.95(C-7), 122.67(C-9), 133.66(C-16), 143.84(C-4), 153.35(C-3), 163.33(C-8), 172.47(C-19), and 172.96(C-2); additional signals were observed for mofetil moiety of MMF at 53.69(C-23), 56.79(C-22), 61.48(C-24), and 66.59(C-25). The ^{1}H NMR and ^{13}C NMR data confirmed that the isolated compound is mycophenolic acid.

3.4. Stress Testing of MMF. Under the optimized conditions, MMF and its degradation products were well separated and are shown in Figures 2(a)–2(d). During the initial forced degradation experiments, it was observed that the MMF is very sensitive to alkaline stress condition and is extensively degraded by alkaline hydrolysis, and five degraded products (MPA, mycophenolate sodium, DP1, DP2, and DP3) were formed. During acid hydrolysis degraded products MPA and DP3 were formed. One of the oxidative stress degradation products of MMF, which is eluted at t_R 4.5 min was found to be USP impurity G based on LC-MS data. Stress degraded products MPA and DP3 were isolated and used for study. Proposed chemical degradation schemes under acid, alkali, and H_2O_2 stress conditions are shown in Figures 3(a)–3(c),

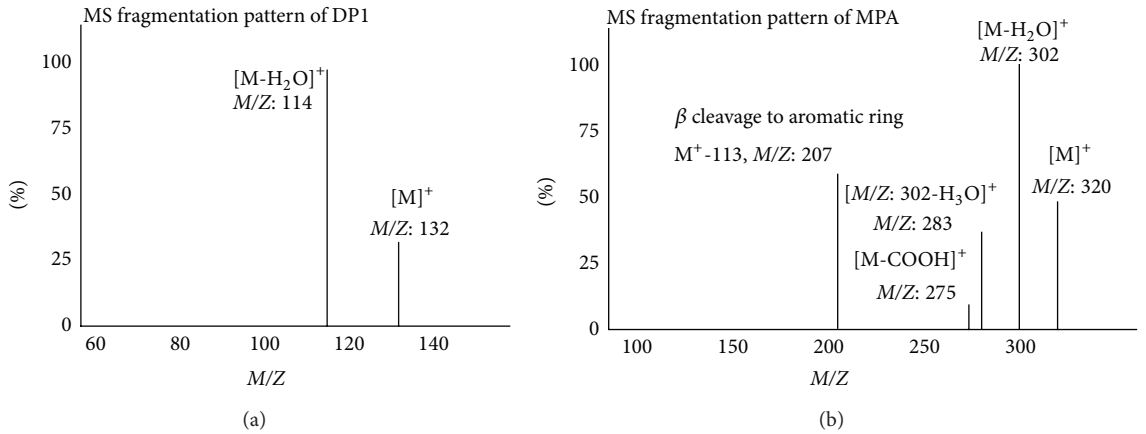

Figure 5: Mass fragmentation pattern of (a) DP1 and (b) mycophenolic acid.

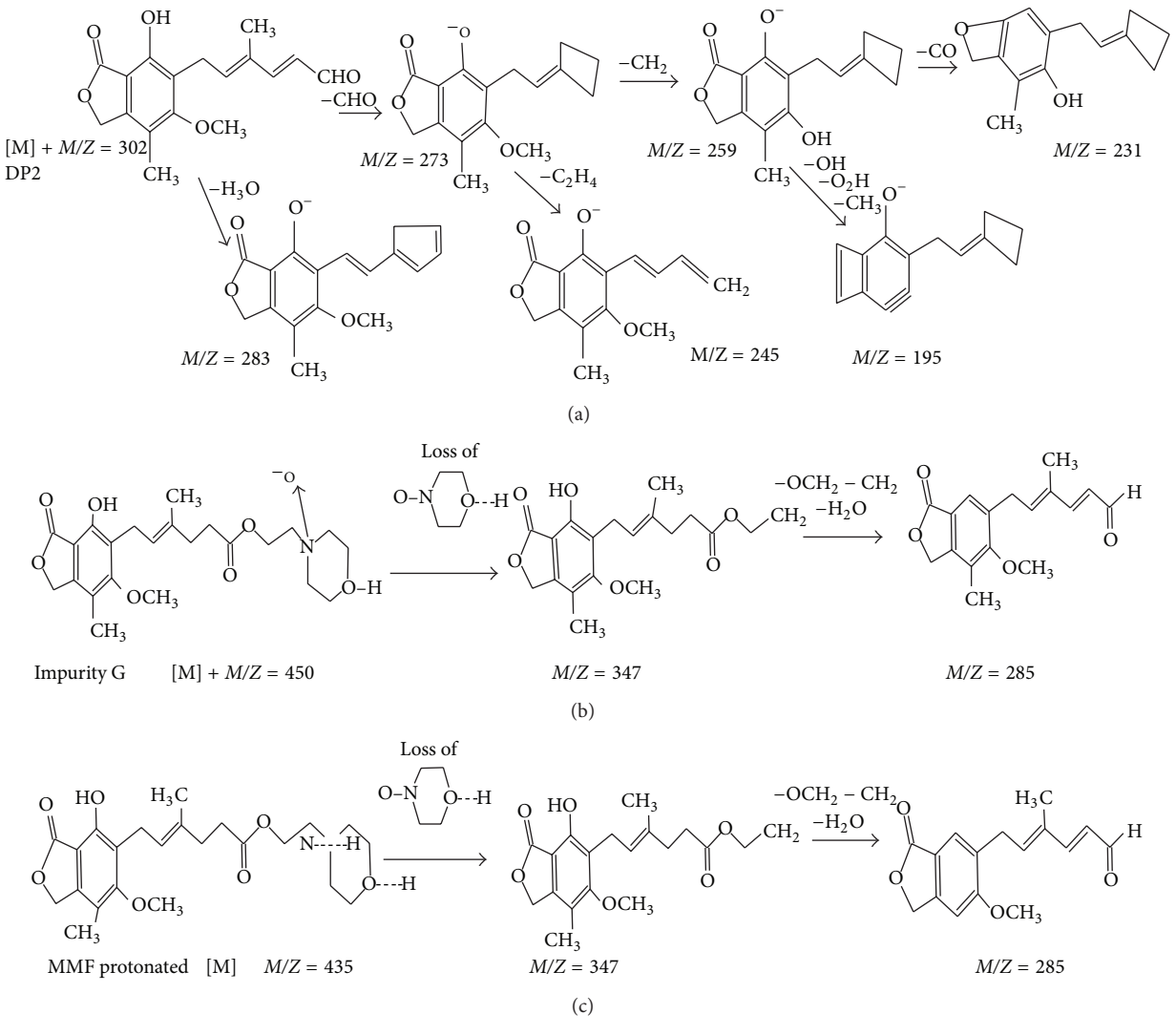

Figure 6: Mass fragmentation pattern of (a) proposed impurity DP2, (b) impurity G, and (c) mycophenolate mofetil.

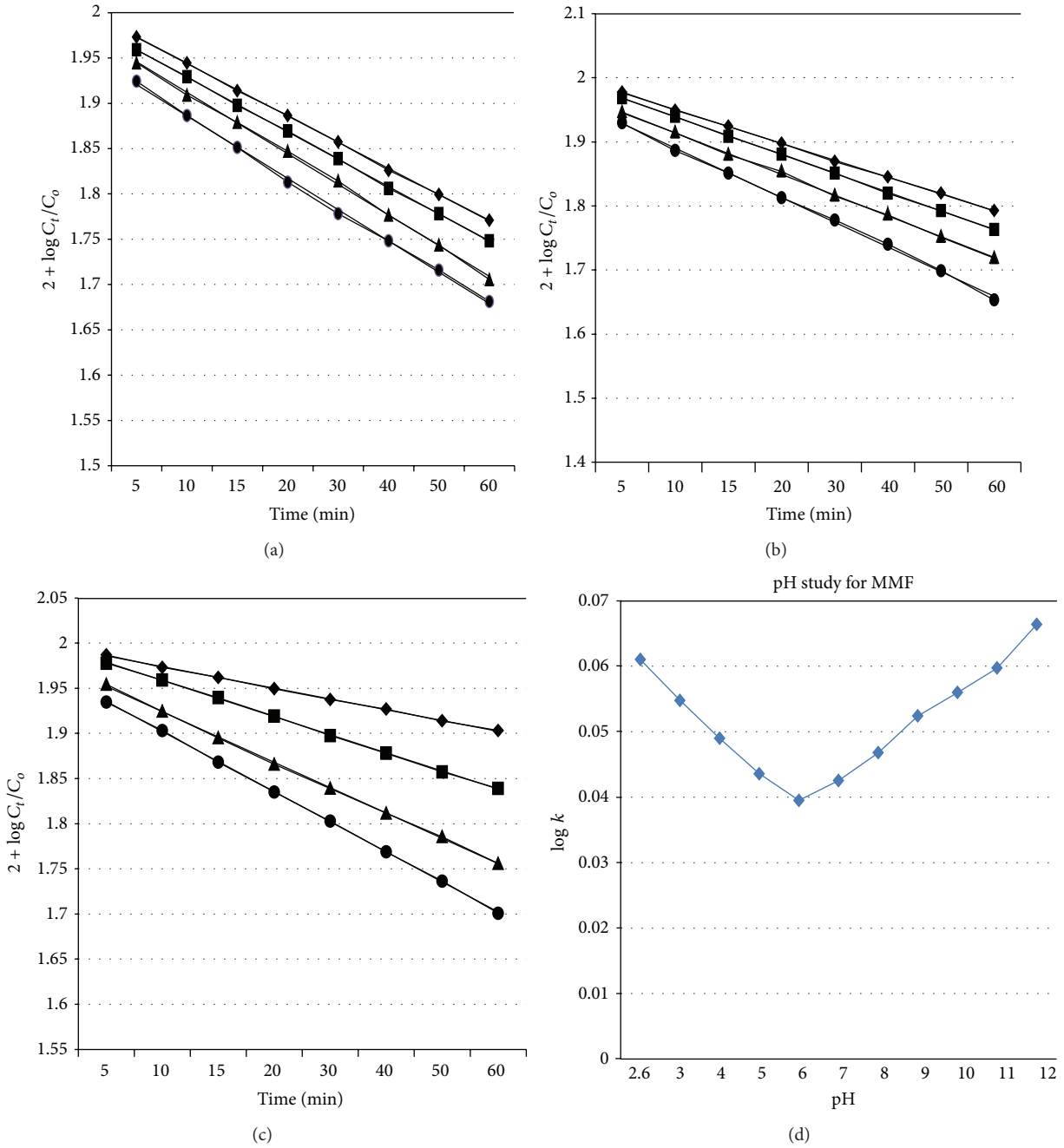

FIGURE 7: Degraded kinetics plot for MMF in (a) 0.1 N HCL, (b) 0.05 N NaOH, (c) 10% v/v H_2O_2 at 60°C (♦), 70°C (■), 80°C (▲), and 90°C (●), and (d) pH profile of MMF in Britton-Robinson buffer.

respectively. During various stress conditions, extent of degradation, peak purity, recovery of MMF, and mass balance (always >98.58) were calculated. Peak purity of stressed samples of MMF was checked by using PDA detector. The purity angle within the purity threshold limit obtained in all stress degraded samples demonstrated analyte peak homogeneity. Results of stress degradation, peak purity, and mass balance study are presented in Table 3. MPA, MMF degradation product, was isolated and identified by NMR and LC-MS/MS study and by comparing t_R and UV spectrum of standard and degraded products. DP3 formed under all the stress conditions was isolated; its structure was determined by LC/MS study and found to be USP impurity H and is eluted at t_R 7.9. It has MS fragmentation pattern like MPA except intensities of the MS peaks. It is less polar than MPA and was retained on C18 column for more time; its proposed structure by MS-MS study is shown in Figure 4. Mass fragmentation pattern and proposed structures of MPA and DP1 are shown in Figures 5(a) and 5(b). Mass fragmentation patterns of proposed impurity DP2, impurity G, and mycophenolate

Table 2: Results of method sensitivity, linearity, precision, system suitability, and recovery of analytes.

Parameter/analytes (t_R)	MPA (5.9)	MMF (6.8)	DP3 (7.9)
Method sensitivity			
Limit of detection (LOD, μg/mL)	0.026	0.121	0.02
Limit of detection (LOQ, μg/mL)	0.078	0.366	0.06
Concentration range (μg/mL)	0.08–16	0.4–120	0.06–12
Regression equation ($Y = b \times$ Concentration $\pm a$) data			
Intercept (a)	1975.0	−2289.8	−2253.7
Slope (b)	70583.2	44428.2	86070.0
[a]$S_a \pm {}^*tS_a$ (error in intercept)	4786 ± 9299.2	6789.6 ± 12628.6	1731.9 ± 3281.9
[b]$S_b \pm {}^*tS_b$ (error in slope)	734 ± 1426.3	118.3 ± 286.4	286.4 ± 542.8
Correlation coefficient (r)	0.999	0.999	0.999
Precision data, % RSD			
Repeatability, $n = 6$	0.29	0.15	0.34
Intraday precision, $n = 3 \times 3$ times	0.4	0.35	0.43
Interday precision, $n = 3 \times 5$ days	0.45	0.42	0.52
System suitability data (USP-NF 29 section 621, pp. 2135)			
Number of theoretical plates (±SD)	3450 ± 32	4255 ± 26	3865 ± 50
USP tailing factor (±SD)	1.01 ± 0.01	1.01 ± 0.01	1.00 ± 0.042
USP resolution	5.9[c]	2.2	2.25
Recovery study at the level (% recovery, % RSD), $n = 3$			
50%,	99.35, 0.77	101.6, 0.59	99.56, 1.23
100%	100.5, 0.73	100.3, 0.45	98.87, 0.76
150%	100.8, 0.89	99.98, 0.34	100.2, 0.91

*t_{tab} = 1.86, 1.895, 1.943 for MMF, DP1, and MPA, respectively ($P = 0.05$; df = 8), [a]standard deviation (SD) of intercept, [b]SD of slope, and [c]resolution with respect to IS HTZ peak.

Table 3: Result of stress degradation study, peak purity, and mass balance, $n = 3$.

Stress condition	Degraded products reported at t_R	MMF % recovery	Peak purity*		Mass balance
			Peak angle	Peak threshold	
1 mL of 0.1 N HCl, 5 h	5.9 (MPA), 5.9 (DP3)	89.45	0.247	0.415	99.96
1 mL of 0.05 N NaOH, 6 h	4.61 (DP1), 5.9 (MPA), 5.9 (DP3)	80.26	0.278	0.389	98.67
2 mL of 30% H_2O_2, 8 h	4.23 (IMP G), 4.61 (DP1), 5.9 (MPA), 7.9 (DP3)	86.89	0.403	0.638	98.18
Short UV-254 nm, 26 h	5.9 (MPA), 7.9 (DP3)	93.43	0.189	0.278	99.57
Long UV-366 nm, 26 h	5.9 (MPA), 7.9 (DP3)	95.56	0.289	0.356	100.2
Wet heat, 12 h	5.9 (MPA), 7.9 (DP3)	96.45	0.137	0.267	99.78
Dry heat-60°C, 8 h	5.9 (MPA), 7.9 (DP3)	92.87	0.265	0.315	99.98

*Values of peak angle which are less than peak threshold indicate homogenous peak.

mofetil are shown in Figures 6(a)–6(c), respectively. The formation of MPA due to thermal degradation at various pH reported in literature [11] was confirmed. Peroxide catalyzed formation of MPA and N-oxide of MMF (USP impurity G) was supported by the experimental findings during proposed study.

3.5. Degradation Kinetics. Forced degradation of MMF by HCl, NaOH, and H_2O_2 resulted in decrease in peak area by time and it was found to be temperature dependent. Apparent first order degradation constant (Figure 7) and half-life at each temperature and Arrhenius equations were calculated. Apparent first order degradation constant ($2 + \log C_t/C_o \pm$ standard deviation) for HCl, NaOH, and H_2O_2 degradation at 60°C was found to be 1.799 ± 0.08455, 1.884 ± 0.0645, and 1.944 ± 0.0292, respectively. Half-life ($t_{1/2}$, day^{-1}) for HCl, NaOH, and H_2O_2 degradation at 60°C was found to be 0.7256, 0.7117, and 0.7084, respectively. Calculated Arrhenius equations at each of the selected conditions were $\text{Log}\,K = 1.223 - (1.192 \times 10^{-3})/T$ (for 0.1 N HCl), $\text{Log}\,K = 1.300 - (1.630143 \times 10^{-3})/T$ (for 0.05 N NaOH), and $\text{Log}\,K = 1.363 - (1.906 \times 10^{-3})/T$ (for 10% H_2O_2).

4. Conclusion

A simple and efficient stability-indicating reverse-phase HPLC method was developed and was found to be accurate,

precise, and linear across the analytical range and is reported for the first time. The method is simple, fast, sensitive, and specific for the determination and quantification of MMF, MPA, and DP3 with 9 min run time. The method can be used to assess the quality of commercially available MMF drug products and to study the kinetics of the MMF and MPA under different conditions. Mobile phase used is MS compatible; therefore, method can be used for LC-MS study of MMF and MPA and other degraded products. Hydrochlorothiazide can be used successfully as IS. Stress degraded products MPA and DP3 were isolated and structurally determined by ^{1}H, ^{13}C NMR, and LC/MS study, and quantitation of the degraded products was archived.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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