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Quantification of Pomalidomide Using Conventional and Eco-Friendly Stability-Indicating HPTLC Assays: A Contrast of Validation Parameters

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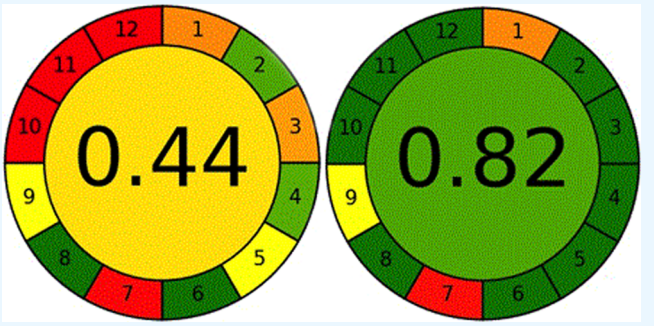
ABSTRACT: High-performance thin-layer chromatographic (HPTLC) assays for pomalidomide (PMD) measurement are lacking in the published database. Furthermore, eco-friendly stability-indicating analytical assays for PMD measurement are also lacking in the published database. In order to detect PMD in commercial products more accurately and sustainably than the conventional normal-phase HPTLC (NP-HPTLC) assay, an effort was made to design and verify a sensitive and eco-friendly reversed-phase HPTLC (RP-HPTLC) assay. The silica gel 60 NP-18F254S and 60 RP-18F254S plates were used as the stationary phases for NP-HPTLC and RP-HPTLC methods, respectively. The solvent system for NP-HPTLC was chloroform−methanol (90:10 v/v). However, the solvent system for RP-HPTLC was ethanol−water (75:25 v/v). The greenness scores for both assays were measured by AGREE approach. PMD measurement was performed for both assays at 372 nm. In the 50−600 and 20−1000 ng/band ranges, the NP-HPTLC and RP-HPTLC methods were linear for PMD measurement. The RP-HPTLC assay was superior to the NP-HPTLC method for measuring PMD in terms of sensitivity, accuracy, precision, and robustness. The ability of both methods to identify PMD in the presence of its degradation products suggests that both methods have stability-indicating features. When employing the NP-HPTLC and RP-HPTLC assays, respectively, the assay for PMD in commercial capsules was 88.68 and 98.83%. The AGREE scores for NP-HPTLC and RP-HPTLC assays were calculated to be 0.44 and 0.82, respectively, suggesting an outstanding greenness characteristic of the RP-HPTLC method than the NP-HPTLC method. The RP-HPTLC method was found to be superior to the NP-HPTLC method based on these findings. Therefore, the RP-HPTLC method could be successfully applied for the determination of PMD in pharmaceutical products.



1. INTRODUCTION

Pomalidomide (PMD) is a highly potent immunomodulatory medicine for anticancer therapy.[1,2] It exists in two different enantiomers i.e., (+)-*R*-enantiomer and (−)-*S*-enantiomer (Figure 1).[3] It has shown significant anticancer effects in treating multiple myeloma (MM) patients with disease refractory to lenalidomide and bortezomib.[4,5] It has been found highly potent than lenalidomide and thalidomide.[6,7] It is used either alone or in combination with dexamethasone in patients with MM.[4,5] It has also been studied in the treatment of some other solid tumors, such as prostate cancer, small cell lung cancer, pancreatic cancer, and Waldenstrom's macroglobulinemia.[8] Its oral absorption is good with an oral bioavailability of more than 70%.[9] Under the brand name Imnovid, PMD is marketed and sold on the market as 1, 2, 3, and 4 mg capsules. The measurement of PMD both qualitatively and quantitatively is crucial for its commercial products.

The literature analysis revealed numerous analytical methods of PMD measurement in commercial products and biological samples, but most of them are associated with biological samples. Numerous high-performance liquid chromatographic (HPLC) methods are reported for the detection of PMD in its bulk drug and capsule formulations.[10−13] Identifying and characterizing the related substances in PMD has also been done by a liquid chromatography−mass spectrometry (LC-MS) method.[14] A validated capillary electrophoretic method has also been reported for the chiral separation of uncharged

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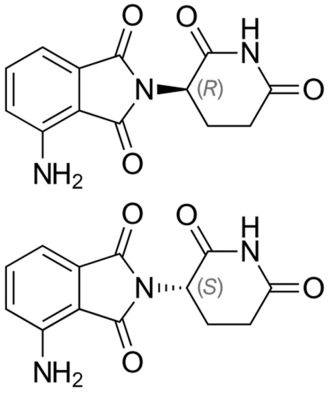

Figure 1. Molecular structures of (*R*) and (*S*) enantiomers of pomalidomide (PMD).

PMD.[3] PMD has been identified using LC-MS/MS methods in mouse plasma, mouse brain tissues, and human plasma samples.[15−17] The detection of PMD in rat and human plasma samples has also been done using certain ultraperformance LC-MS/MS (UPLC-MS/MS) assays.[18−20] A rapid and selective electrochemical detection of PMD in pharmaceuticals and human plasma samples has also been performed.[21] A simple and rapid spectrofluorometric determination of PMD in spiked human plasma and urine samples has also been carried out.[22] Numerous methods of PMD measurement were found in the published database. However, none of the literature assays' greenness scores were measured. Furthermore, no PMD measurement was performed by high-performance thin-layer chromatographic (HPTLC) assays. The greenness indices of analytical procedures have been measured using a variety of quantitative and qualitative methods.[23−30] Only the "Analytical GREENness (AGREE)" approach takes into account all 12 principles of "green analytical chemistry (GAC)" for the prediction of greenness score.[28,31] The greenness scores of the current HPTLC experiments were therefore calculated by considering all 12 GAC principles using the AGREE method.[28,31] In order to confirm the greenness results by AGREE method, the chloroform-oriented toxicity estimation Scale (ChlorTox scale) method was applied additionally to measure the greenness of the present methods.[32] The objective of the current work was to develop and validate a reversed-phase HPTLC (RP-HPTLC) method for measuring PMD in commercial capsules that was more precise, accurate, sensitive, robust, and eco-friendly than the conventional normal-phase HPTLC (NP-HPTLC) method. Following "The International Council for Harmonization (ICH)" Q2-R1 protocols, both assays for PMD measurement were verified.[33]

2. RESULTS AND DISCUSSION

2.1. Method Development and Optimization. The system appropriateness parameters for NP-HPTLC and RP-HPTLC assays are summarized in Table 1. For PMD measurement, the "retardation factor (R_f), asymmetry factor (A_s), and theoretical plates number/meter (N/m)" for the NP-HPTLC method were found to be reliable. For PMD measurement, the "R_f, A_s, and N/m" for the RP-HPTLC method were also acceptable.

The TLC plates for both assays were developed using chamber saturation conditions (Figure 2). For the PMD measurement by the NP-HPTLC assay, numerous chloroform ($CHCl_3$)/methanol (MeOH) mixtures within the 40−90% $CHCl_3$ range were studied as the conventional eluent systems. The combinations of conventional eluent systems and numerous chromatography responses are presented in Table 1. The results showed that a well-eluted and sharp chromatography signal for PMD at R_f = 0.61 ± 0.01 (Figure 3A) was provided by the conventional eluent system $CHCl_3$/MeOH (90:10 v/v). Additionally, it was found that PMD has A_s values of 1.05 ± 0.02, which are appropriate for PMD assessment. Accordingly, $CHCl_3$/MeOH (90:10 v/v) was chosen as the final solvent system for PMD assessment using the NP-HPTLC method.

Table 1. Optimization of the Eluent Systems and Chromatography Parameters of Pomalidomide (PMD) Measurement for the NP-HPTLC and RP-HPTLC Assays (mean ± SD, n = 3)[a]

NP-HPTLC			
eluent system	As	N/m	R_f
$CHCl_3$/MeOH (40:60 v/v)	1.27 ± 0.04	1891 ± 2.41	0.66 ± 0.02
$CHCl_3$/MeOH (50:50 v/v)	1.25 ± 0.04	2172 ± 291	0.65 ± 0.02
$CHCl_3$/MeOH (60:40 v/v)	1.23 ± 0.03	2684 ± 3.94	0.64 ± 0.02
$CHCl_3$/MeOH (70:30 v/v)	1.18 ± 0.03	3012 ± 4.21	0.63 ± 0.01
$CHCl_3$/MeOH (80:20 v/v)	1.13 ± 0.02	3422 ± 4.56	0.62 ± 0.01
$CHCl_3$/MeOH (90:10 v/v)	1.05 ± 0.02	4571 ± 5.81	0.61 ± 0.01
RP-HPTLC			
EtOH/H_2O (45:55 v/v)	1.35 ± 0.02	1345 ± 1.53	0.78 ± 0.03
EtOH/H_2O (50:50 v/v)	1.34 ± 0.03	1461 ± 1.91	0.77 ± 0.02
EtOH/H_2O (55:45 v/v)	1.32 ± 0.03	1984 ± 2.12	0.76 ± 0.02
EtOH/H_2O (60:40 v/v)	1.29 ± 0.03	2341 ± 3.11	0.75 ± 0.02
EtOH/H_2O (65:35 v/v)	1.26 ± 0.03	2878 ± 3.43	0.74 ± 0.01
EtOH/H_2O (70:30 v/v)	1.24 ± 0.02	3161 ± 3.86	0.73 ± 0.01
EtOH/H_2O (75:25 v/v)	1.09 ± 0.02	4712 ± 4.17	0.72 ± 0.01
EtOH/H_2O (80:20 v/v)	1.18 ± 0.03	4431 ± 3.97	0.72 ± 0.01
EtOH/H_2O (85:15 v/v)	1.20 ± 0.03	4051 ± 3.88	0.70 ± 0.01
EtOH/H_2O (90:10 v/v)	1.23 ± 0.03	3612 ± 3.76	0.69 ± 0.02

[a]$CHCl_3$: chloroform; MeOH: methanol; EtOH: ethanol; H_2O: water; R_f: retardation factor; A_s: asymmetry factor; N/m: theoretical plates number per meter.

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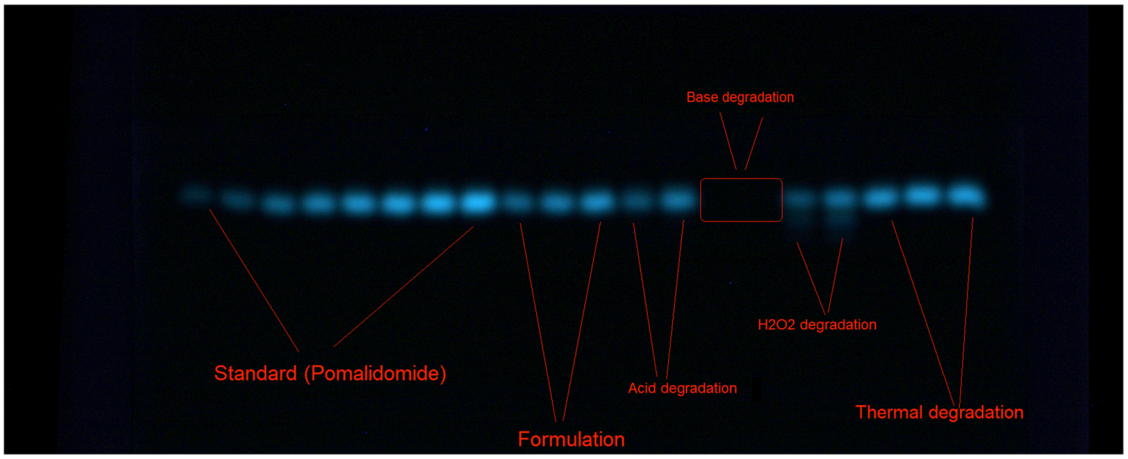

Figure 2. Representative TLC image of standard PMD, formulation, and forced-degradation samples obtained using eco-friendly EtOH-H_2O (75:25 v/v) solvent system for the RP-HPTLC method.

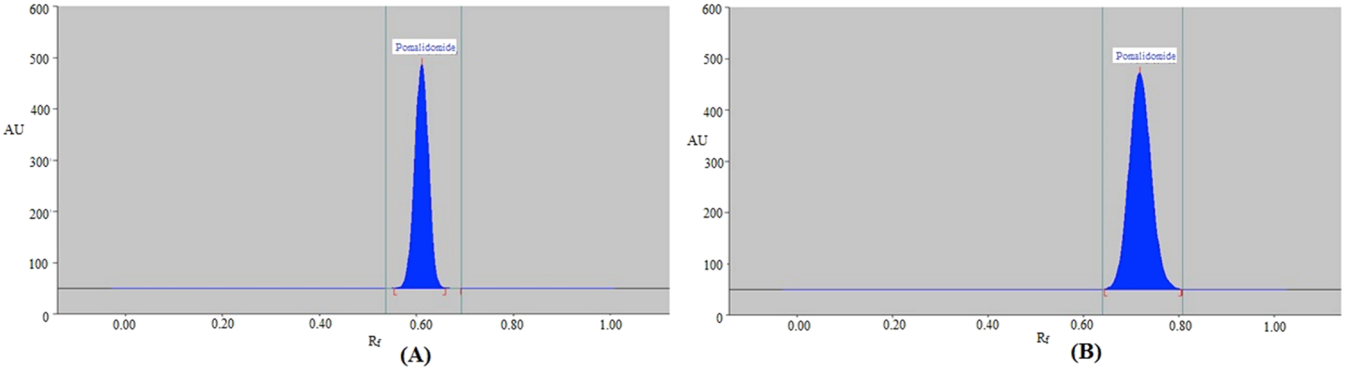

Figure 3. Representative spectrodensitograms of standard PMD (300 ng/band concentration) derived by (A) NP-HPTLC and (B) RP-HPTLC assays.

For the PMD measurement using the RP-HPTLC method, numerous ethanol (EtOH)/water (H_2O) combinations within the 45−90% EtOH range were studied as eco-friendly eluent systems. We have also tried other green solvents to develop RP-HPTLC procedures, but chromatographic responses were not acceptable. The mixture of EtOH and H_2O provides acceptable chromatographic responses. In addition, the combination of EtOH and H_2O has been studied as green solvent systems extensively in the literature.[34−36] As a result, different combination of EtOH and H_2O was investigated to develop RP-HPTLC procedures. The combinations of the eco-friendly eluent systems and numerous chromatographic parameters for the RP-HPTLC method are summarized in Table 1. According to the findings, the EtOH/H_2O (75:25 v/v) provided a well-resolved and intact PMD chromatographic signal at R_f = 0.72 ± 0.01 (Figure 3B). Additionally, it was projected that PMD would have A_s value of 1.09 ± 0.02, which was appropriate for PMD assessment. The EtOH/H_2O (75:25 v/v) was chosen as the final eco-friendly mobile phase for PMD assessment utilizing the RP-HPTLC method as a result. The highest TLC response for PMD was found at 372 nm when the spectral bands for PMD were studied in spectrodensitometry mode. As a result, the entire PMD measurement was conducted at 372 nm.

2.2. Validation Studies. The ICH-Q2-R1 procedures were followed to record the numerous PMD validation parameters.[33] Results of the linearity evaluation of PMD calibration plots utilizing both methods are listed in Table 2.

Table 2. Findings of the Linearity Measurement of PMD for the NP-HPTLC and RP-HPTLC Assays (Mean ± SD; n = 6)[a]

parameters	NP-HPTLC	RP-HPTLC
linearity range (ng/band)	50−600	20−1000
regression equation	y = 29.76x + 1084.5	y = 48.674x + 708.9
R^2	0.9930	0.9982
R	0.9964	0.9991
standard error of slope	2.20	3.03
standard error of intercept	22.32	7.28
95% confidence interval of slope	20.25−39.26	35.61−61.72
95% confidence interval of intercept	988.42−1180.57	677.55−740.24
LOD ± SD (ng/band)	0.59 ± 0.05	0.50 ± 0.03
LOQ ± SD (ng/band)	1.79 ± 0.15	1.51 ± 0.09

[a] R^2: determination coefficient; R: regression coefficient; LOD: limit of detection; LOQ: limit of quantification.

The PMD calibration plot for the NP-HPTLC method was linear in the range from 50 to 600 ng/band. The PMD calibration plot for the RP-HPTLC method was linear in the range from 20 to 1000 ng/band. For the RP-HPTLC assay, PMD's determination coefficient (R^2) and regression coefficient (R) were 0.9930 and 0.9964, respectively. PMD's R^2 and R were 0.9982 and 0.9991, respectively for the RP-HPTLC method. The measured spot areas and PMD concentrations

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Table 3. Accuracy Results of PMD for the NP-HPTLC and RP-HPTLC Assays (Mean ± SD; n = 6)

conc. (ng/band)	intraday accuracy			interday accuracy		
	conc. (ng/band)	recovery (%)	CV (%)	conc. (ng/band)	recovery (%)	CV (%)
			NP-HPTLC			
300	321.41 ± 5.61	107.13	1.74	287.42 ± 5.84	95.80	2.03
400	384.58 ± 7.54	96.14	1.96	379.81 ± 7.71	94.95	2.02
500	526.33 ± 8.15	105.26	1.54	476.32 ± 8.86	94.26	186
			RP-HPTLC			
450	456.34 ± 4.14	101.40	0.90	442.13 ± 4.19	98.25	0.94
600	611.51 ± 5.21	101.91	0.85	594.32 ± 5.33	99.05	0.89
750	741.23 ± 6.02	98.83	0.81	761.34 ± 6.36	101.51	0.83

Table 4. Measurement of PMD Precision for NP-HPTLC and RP-HPTLC Assays (Mean ± SD; n = 6)

conc. (ng/band)	intraday precision			interday precision		
	conc. (ng/band)	SE	CV (%)	conc. (ng/band)	SE	CV (%)
			NP-HPTLC			
300	316.71 ± 8.12	3.31	2.56	319.25 ± 8.56	3.49	2.68
400	382.61 ± 8.91	3.63	2.32	371.64 ± 9.63	3.93	2.59
500	519.84 ± 9.94	4.05	1.91	471.98 ± 10.68	4.36	2.26
			RP-HPTLC			
450	445.60 ± 3.56	1.45	0.79	454.32 ± 3.68	1.50	0.81
600	592.82 ± 4.48	1.82	0.75	611.54 ± 4.81	1.96	0.78
750	765.41 ± 5.39	2.20	0.70	738.39 ± 5.45	2.22	0.73

showed a high correlation in these results. All of these results revealed that both methods were reliable for measuring PMD. However, the RP-HPTLC method was more linear than the NP-HPTLC assay.

The accuracy in terms of % of recovery was determined for both assays of PMD measurement. The findings of accuracy measurement for both methods are listed in Table 3. For the NP-HPTLC method, the intra-assay and interassay recoveries of PMD at three distinct quality control (QC) samples were found to be 96.14−173.13 and 94.26−95.80%, respectively. For the RP-HPTLC assay, the intra-assay and interassay recoveries of PMD at three distinct QC samples were measured to be 98.83−101.91 and 98.25−101.51%, respectively. The % recovery of PMD in commercial capsules using an HPLC method has been reported as 91.23−132.59%.[10] The % recovery of PMD by another HPLC method has been reported as 82.90−112.30%.[11] The recorded recoveries of PMD using the present NP-HPTLC and RP-HPTLC methods were superior to reported HPLC methods.[10,11] These outcomes indicated that both methods were accurate for PMD measurement. However, the RP-HPTLC method was more accurate than the NP-HPTLC method in measuring PMD.

For the purpose of measuring PMD, the intra-assay and interassay precision of both methods was assessed, and the data were expressed as a percentage of the coefficient of variance (%CV). The intra-assay and interassay precisions for both methods of PMD measurement are shown in Table 4. For the NP-HPTLC method, the CVs of PMD for the intra-assay ranged from 1.91 to 2.56%. The CVs of PMD for interassay for the NP-HPTLC method ranged from 2.26 to 2.68%. For the RP-HPTLC method, the CVs of PMD for intra-assay ranged from 0.70 to 0.79%. For the RP-HPTLC method, the CVs of PMD for interassay ranged from 0.73 to 0.81%. These measurements showed that both methods were precise for measuring PMD. For PMD measurement, however, the RP-HPTLC assay was more precise than the NP-HPTLC method.

The robustness of both methods for measuring PMD was investigated by adding deliberately planned adjustments to the elements of eluent systems. The outcomes of the robustness measurement for both methods are shown in Table 5. For the

Table 5. Outcomes of Robustness Measurement of PMD for NP-HPTLC and RP-HPTLC Assays (Mean ± SD; n = 6)

conc. (ng/band)	mobile phase combination ($CHCl_3$-MeOH)			results		
	original	used		conc. (ng/band)	CV (%)	R_f
			NP-HPTLC			
		92:8	+2.0	374.58 ± 9.87	2.63	0.60
400	90:10	90:10	0.0	387.52 ± 10.78	2.78	0.61
		88:18	−2.0	412.31 ± 11.12	2.69	0.62
			RP-HPTLC			
		mobile phase composition ($EtOH$-H_2O)				
		77:23	+2.0	581.42 ± 4.94	0.84	0.71
600	75:25	75:25	0.0	593.61 ± 5.18	0.87	0.72
		73:27	−2.0	603.25 ± 5.48	0.90	0.73

NP-HPTLC method, the CVs for PMD were 2.63−2.78%. For the NP-HPTLC method, PMD R_f values were found to be 0.60−0.62. The RP-HPTLC method's PMD CVs ranged from 0.84 to 0.90%. PMD R_f values for the RP-HPTLC method were calculated to be between 0.71 and 0.73. These measurements showed that both methods were robust for PMD measurement. However, the RP-HPTLC method outperformed the NP-HPTLC method in terms of PMD measurements.

The sensitivity of both methods of PMD measurement was evaluated in terms of "limit of detection (LOD) and limit of quantification (LOQ)". The derived values of "LOD and

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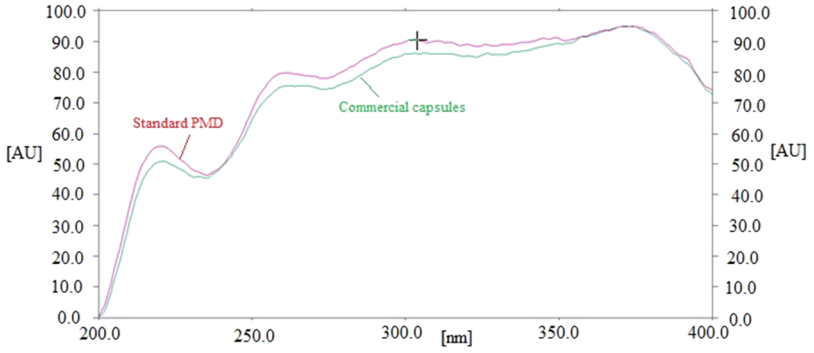

Figure 4. UV absorption spectra of standard PMD and marketed products, superimposed.

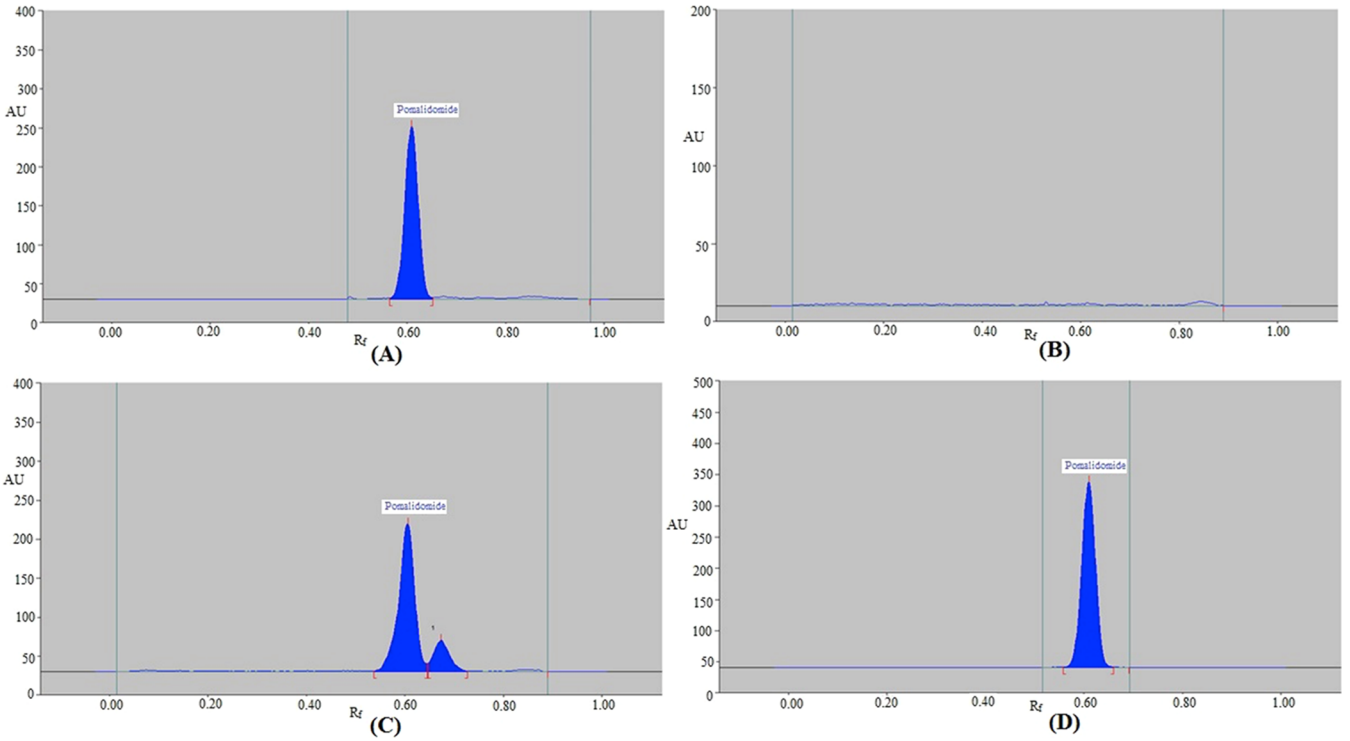

Figure 5. Spectrodensitograms of PMB recorded under (A) acid, (B) base, (C) oxidative, and (D) thermal degradations of PMD by NP-HPTLC assay.

LOQ" for PMD for both methods are mentioned in Table 2. For the NP-HPTLC assay, the "LOD and LOQ" for PMD were derived to be 0.59 ± 0.05 and 1.79 ± 0.215 ng/band, respectively. For the RP-HPTLC assay, the "LOD and LOQ" for PMD were derived to be 0.50 ± 0.03 and 1.51 ± 0.15 ng/band, respectively. Based on these findings, it was shown that both methods were quite sensitive for measuring PMD. The RP-HPTLC assay, however, was more sensitive in measuring PMD than the NP-HPTLC assay.

We were able to evaluate the selectivity of the suggested assay for PMD measurement by contrasting the R_f values and overlaid UV absorption spectra of PMD in procured capsules with those of bulk PMD. Figure 4 presents the superimposed UV absorption spectra of bulk PMD and PMD in procured capsules. At a wavelength of 372 nm, the greatest response of PMD in commercial capsules and standard PMD was discovered. Additional proof of the suggested HPTLC assay's selectivity for PMD detection came from the comparable UV absorption spectra, R_f values, and wavelengths of PMD in bulk and procured capsules.

2.3. Forced-Degradation Evaluation. The forced degradation of NP-HPTLC and RP-HPTLC assays was studied using numerous stress conditions. Figure 5 and Table 6 contain the findings of the NP-HPTLC assay. PMD peak was well separated at numerous stress settings (Figure 5). The degradant was not quantified/identified in this work. The amount of PMD was quantified after degradation. From the amount of PMD remained, the amount of degradation was

Table 6. Outcomes of Forced-Degradation Experiments of PMD for the NP-HPTLC Assay under Varied Stress Conditions (Mean ± SD; n = 3)

degradation setting	degradation products (R_f)	PMD R_f	PMD remained (ng/band)	PMD recovered (%)
1M HCl	0	0.61	400.00	100.00 ± 0.00
1M NaOH	ND	ND	0.00	00.00 ± 0.00
30% H_2O_2	1 (0.67)	0.61	329.36	82.34 ± 2.13
thermal	0	0.61	400.00	100.00 ± 0.00

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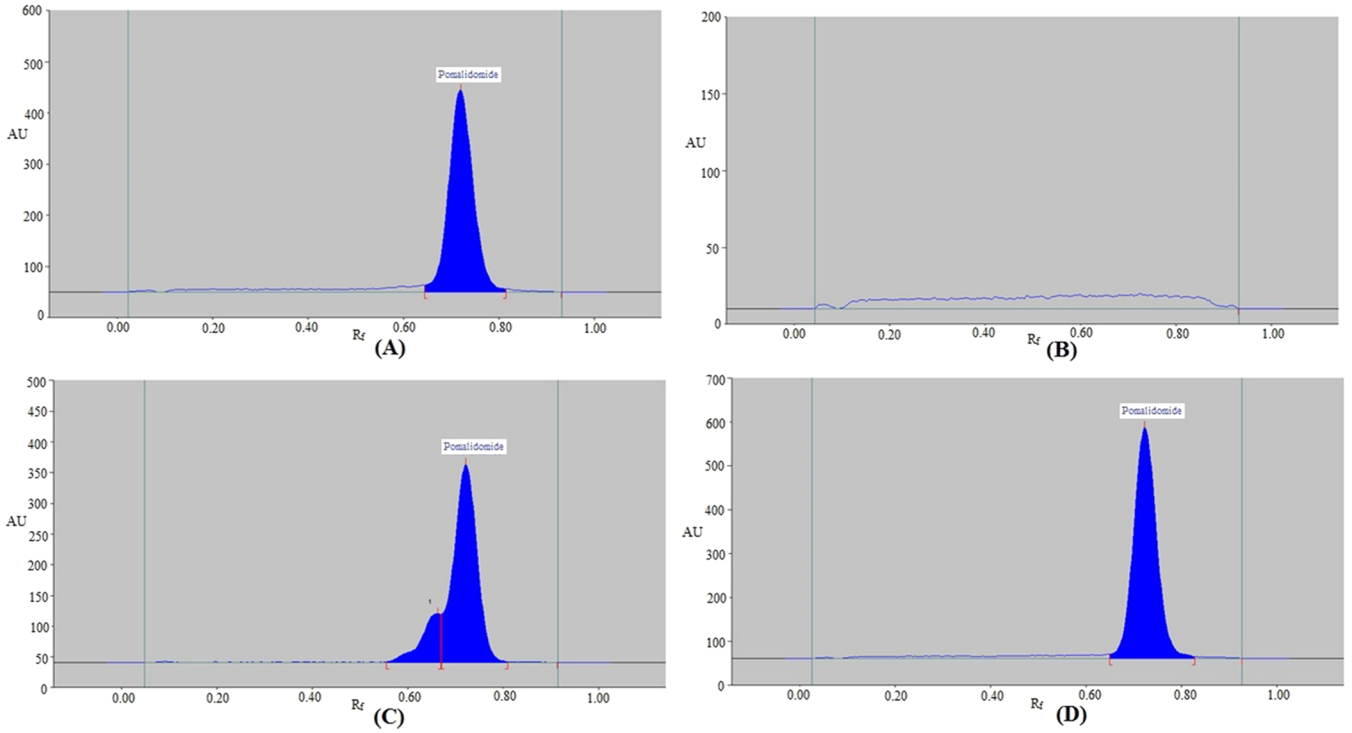

Figure 6. Spectrodensitograms of PMB recorded under (A) acid, (B) base, (C) oxidative, and (D) thermal degradations of PMD by RP-HPTLC assay.

calculated. Under acid-degradation (Figure 5A) settings, 100.0% of PMD remained intact and no breakdown of PMD was observed. As a result, PMD was highly stable under acid-degradation circumstances. The PMD R_f value during acid-degradation settings was unaltered (R_f = 0.61). During base-degradation (Figure 5B) settings, no PMD was detected and hence complete degradation of PMD was expected. Due to the complete degradation of PMD at alkaline conditions, we have also tried milder conditions such as reducing the concentration of NaOH, lowering the temperature, and reducing the treatment time. Unfortunately, we got the complete degradation of PMD under all milder conditions. As a consequence, PMD was highly unstable under base-degradation settings. Following the application of oxidative stress, 82.34% of PMD was still present, while 17.66% was degraded (Table 6 and Figure 5C). The H_2O_2-induced degradation compound (peak 1 in Figure 5C) was separated at R_f = 0.67. The PMD R_f during oxidative-degradation conditions was unaltered (R_f = 0.61). Additionally, PMD was kept at 100.0% in thermal conditions (Table 6 and Figure 5D), and there was no evidence of PMD degradation. As a result, PMD was resistant to thermal stress conditions.

Figure 6 and Table 7 contain the outcomes of the RP-HPTLC assay. PMD peak was also well separated at numerous stress settings (Figure 6). Under acid-degradation (Figure 6A) settings, 100.0% of PMD was also remained intact and no degradation of PMD was found. As a result, PMD was highly stable under acid-degradation settings. The PMD R_f value under acid-degradation settings was unaltered (R_f = 0.72). Under base-degradation (Figure 6B) settings, no PMD was detected and hence complete degradation of PMD was expected. As a consequence, PMD was highly unstable under base-degradation settings. Following the application of oxidative stress, 84.28% of PMD was still present, while

Table 7. Outcomes of Forced-Degradation Experiments of PMD for the RP-HPTLC Assay under Varied Stress Conditions (Mean ± SD; n = 3)

degradation setting	degradation products (R_f)	PMD R_f	PMD remained (ng/band)	PMD recovered (%)
1M HCl	0	0.72	600.00	100.00 ± 0.00
1M NaOH	ND	ND	0.00	00.00 ± 0.00
30% H_2O_2	1 (0.66)	0.72	505.68	84.28 ± 1.87
thermal	0	0.72	600.00	100.00 ± 0.00

15.72% was decomposed (Table 7 and Figure 6C). With an R_f value of 0.66, the H_2O_2-induced degradation peak (peak 1 in Figure 6C) was separated. Under oxidative-degradation settings, PMD's R_f value was not shifted (R_f = 0.72). In thermal stress settings, PMD was similarly kept at 100% (Table 7 and Figure 6D), and no PMD degradation was observed. Therefore, PMD was also resistant to thermal conditions. During base-degradation circumstances, the complete decomposition of PMD was recorded using both assays. These results suggested that PMD might be identified using both NP-HPTLC and RP-HPTLC assays in the presence of its degradation compounds. These results pointed to the stability-indicating features of NP-HPTLC and RP-HPTLC procedures. For PMD detection, both assays were stability-indicating.

2.4. Application of NP-HPTLC and RP-HPTLC Assays in PMD Estimation in Commercial Capsules. For the determination of PMD in commercial capsules, both assays were used. The spectrodensitogram of PMD from commercial capsules was identified by comparing its single TLC band at R_f = 0.61 ± 0.01 for PMD with standard PMD using the NP-HPTLC assay. The chromatographic peak of PMD in commercial capsules was identical to those of pure PMD by the NP-HPTLC method. The spectrodensitogram of PMD

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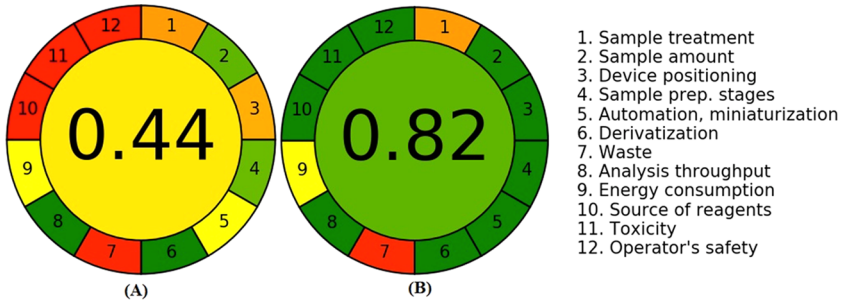

Figure 7. Representative pictograms for AGREE scores for (A) NP-HPTLC and (B) RP-HPTLC assays calculated by AGREE calculator.

Table 8. Comparison of the RP-HPTLC Method with the NP-HPTLC Method for the Determination of PMD in Terms of the Relative Hazards with respect to Chloroform (CH_{sub}/CH_{CHCl3}) Derived Using the WHN Model, in Terms of the Mass of Individual Reagents Used for One Analysis (m_{sub}), and in Terms of the ChlorTox Values Indicating the Degree of Predicted Chemical Risk

method	stage	solvent/reagent	relative hazard (CH_{sub}/CH_{CHCl3})	m_{sub} (mg)	ChlorTox (g)	total ChlorTox (g)
RP-HPTLC	sample preparation	EtOH	0.26	1500	0.39	0.78
	HPTLC analysis	EtOH	0.26	1500	0.39	
NP-HPTLC	sample preparation	$CHCl_3$	1.00	1800	1.80	3.82
		MeOH	0.56	200	0.11	
	HPTLC analysis	$CHCl_3$	1.00	1800	1.80	
		MeOH	0.56	200	0.11	

from marketed capsules was identified by comparing its single TLC band at R_f = 0.72 ± 0.01 for PMD with standard PMD using the RP-HPTLC assay. The chromatographic peak of PMD in commercial capsules was also similar to those of pure PMD by the RP-HPTLC method. Furthermore, no extra signals of capsule ingredients were detected in the procured capsules using both assays, indicating no interaction between PMD and capsule ingredients. The content of PMD in commercial capsules was derived from the PMD calibration plot for both assays. Utilizing the NP-HPTLC method, the amount of PMD in commercial capsules was derived to be 88.68 ± 1.13%. Using the RP-HPTLC assay, the amount of PMD in commercial capsules was derived to be 98.83% ± 1.28%. The % assay of PMD in commercial capsules using an HPLC method has been reported as 92.61−109.57%.[10] The % assay of PMD using the present RP-HPTLC method was superior to the reported HPLC method; however, it was inferior to the present NP-HPTLC method.[10] The solutions and instrumentations are the same for the NP-HPTLC method, but the stationary phase and mobile phase are different. The efficiency of the HPTLC method depends on the stationary phase and mobile phase. Based on the validation and pharmaceutical assay, the efficiency of the NP-HPTLC method was low compared to that of the RP-HPTLC method. As a result, the % recovery by the NP-HPTLC method was low. Based on these findings, the RP-HPTLC method was found to be superior to the NP-HPTLC method for the measurement of PMD.

2.5. Greenness Estimation. Numerous approaches are reported for the greenness measurement of analytical assays.[23−30] However, AGREE approach exclusively considers all twelve GAC principles for greenness measurement.[28,31] Hence, the greenness scores of both methods were measured by "AGREE: The Analytical Greenness Calculator (version 0.5, Gdansk University of Technology, Gdansk, Poland, 2020)". In order to confirm the AGREE results, the ChlorTox method was also used to determine the greenness of both methods.[32]

Figure 7 displays a typical diagram for the AGREE score of NP-HPTLC and RP-HPTLC assays. According to AGREE method, the AGREE score for each principle of GAC is assigned from 0 to 1. For NP-HPTLC, a score of 1 was recorded for the GAC principles 6 and 8. A score of 0.8 was recorded for the GAC principles 2 and 4. The score 0 was recorded for the GAC principles 7, 10, 11, and 12. A score of 0.5 was recorded for the GAC principles 5 and 9. A score of 0.3 was recorded for the GAC principles 1 and 3. For the NP-HPTLC method, the overall AGREE score was predicted to be 0.44 (Figure 7A). For RP-HPTLC, a score of 1 was recorded for the GAC principles 2, 3, 4, 5, 6, 8, 10, 11, and 12. A score of 0 was recorded for the GAC principle's 7. Scores 0.3 and 0.5 were recorded for the GAC principles 1 and 9, respectively. For the RP-HPTLC method, the overall AGREE score was predicted to be 0.82 (Figure 7B). These findings demonstrated the outstanding greenness features of the RP-HPTLC method compared to the NP-HPTLC method for PMD measurement.

The results of ChlorTox scores of individual solvents and total ChlorTox for both methods are included in Table 8. For NP-HPTLC, the total ChlorTox value was predicted to be 3.82, which indicated the extreme toxicity/hazardous method. However, for RP-HPTLC, the total ChlorTox value was predicted to be 0.78, which indicated that the RP-HPTLC method was relatively safe and green. Therefore, the ChlorTox results confirmed the findings of the AGREE method.

3. CONCLUSIONS

Stability-indicating HPTLC assays of PMD measurement are lacking in the published database. The published database also lacks eco-friendly analytical assays for PMD detection. As a result, this study aims to develop and validate a sensitive, and eco-friendly stability-indicating RP-HPTLC method for PMD analysis in commercial capsules, as opposed to the conventional stability-indicating NP-HPTLC assay. The RP-HPTLC method is more linear, accurate, precise, robust, and sensitive than the NP-HPTLC method for PMD measurement.

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Compared to the NP-HPTLC method, the content of PMD was greater when using the RP-HPTLC method. Both assays were discovered to be selective with stability-indicating features. The AGREE evaluation indicated the outstanding greenness score of the RP-HPTLC method to the NP-HPTLC method. Based on these findings, the RP-HPTLC method was found to be superior to the NP-HPTLC method for measuring PMD in commercial capsules. For future studies, the developed HPTLC methods can be used for the measurement of PMD in plasma samples and its pharmacokinetic assessment.

4. MATERIALS AND METHODS

4.1. Materials. Bulk PMD was procured from Beijing Mesochem Technology (Beijing, China). High-purity chromatography eluents such as EtOH, MeOH, and $CHCl_3$ were procured from Sigma-Aldrich (St. Louis, MO, USA). PMD (Imnovid) commercial capsules were obtained from a pharmacy in Riyadh, Saudi Arabia. The remaining chemicals/reagents were of analytical reagent grade.

4.2. Instrumentation and Analytical Settings. The HPTLC CAMAG TLC system (CAMAG, Muttenz, Switzerland) was used to measure PMD in procured capsule dosage forms. The solutions were applied as 6 mm bands using a "CAMAG Automatic TLC Sampler 4 (ATS4) Sample Applicator (CAMAG, Geneva, Switzerland)". The "CAMAG microliter Syringe (Hamilton, Bonaduz, Switzerland)" was loaded to the sample applicator. The application rate for PMD measurement was set to 150 nL/s. The glass-coated plates were developed in a "CAMAG automated developing chamber 2 (ADC2) (CAMAG, Muttenz, Switzerland)" at a distance of 80 mm under linear ascending mode. For 30 min at 22 °C, the development chamber was filled with the vapors from the corresponding eluent systems. PMD was measured at a wavelength of 372 nm. Scan speed and slit size were both adjusted to 20 mm/s and 4 × 0.45 mm^2, respectively. Three or six replications were employed for each measurement. "WinCAT's (version 1.4.3.6336, CAMAG, Muttenz, Switzerland)" was the software used.

The identical instrumentation and analytical procedures were used in both assays. The main variations between NP-HPTLC and RP-HPTLC assays were the eluent systems and stationary phase/TLC plates. The optimized eluent system in the NP-HPTLC method was $CHCl_3$/MeOH (90:10, v/v), whereas the optimized eluent system was EtOH/H_2O (75:25, v/v) in the RP-HPTLC assay. The stationary phase was "glass-coated plates (plate size: 10 × 20 cm) precoated with silica gel (particle size: 5 μm) 60 NP-18F254S plates" in NP-HPTLC assay. The stationary phase was "glass-coated plates (plate size: 10 × 20 cm) precoated with silica gel (particle size: 5 μm) 60 RP-18F254S plates" in RP-HPTLC assay.

4.3. Calibration Curves for PMD. The accurately measured 10 mg of PMD was dispensed into the specific volumes of $CHCl_3$/MeOH (90:10, v/v) for NP-HPTLC and EtOH/H_2O (75:25, v/v) for RP-HPTLC, producing a stock solution with a 100 μg/mL PMD concentration. For the NP-HPTLC assay, PMD concentrations in the 50−600 ng/band range were obtained by diluting the variable amount of PMD stock solution with $CHCl_3$/MeOH (90:10, v/v). However, for the RP-HPTLC assay, which involved dilution of the varying amount of PMD stock solution with the EtOH/H_2O (75:25, v/v) solvent system, PMD concentrations in the 20−1000 ng/band range were generated. For NP-HPTLC and RP-HPTLC methods, 200 μL of each PMD concentration was applied to NP and RP TLC plates, respectively. Both methods were utilized to obtain the peak response of each PMD concentration. By graphing the measured spot area vs PMD concentrations over six replications (n = 6), PMD calibration curves were created.

4.4. Sample Preparation for the Measurement of PMD in Commercial Capsules. To measure the amount of PMD in commercial capsules, twenty-five capsules containing 4 mg of PMD each were taken at random. The mean weight was then determined. The contents of the capsule were taken out and properly mixed to produce fine powder. 50 mL of the corresponding eluent system was used to disperse the fine powder, which contained 4 mg of PMD in total amounts. The resulting mixtures underwent filtration and 15 min of sonication.[34] The generated solutions were used in both HPTLC methods to assess PMD in commercial capsules.

4.5. Validation Studies. NP-HPTLC and RP-HPTLC methods to measure PMD were verified for numerous validation factors following the ICH-Q2-R1 procedures.[33] PMD linearity was evaluated by graphing recorded peak response vs PMD concentrations. The linearity for the NP-HPTLC method of PMD measurement was determined in the 50−600 ng/band range using six replications (n = 6). PMD linearity was evaluated in the 20−1000 ng/band range using six replications (n = 6) for the RP-HPTLC method.

The parameters for the system suitability for both assays of PMD measurement were assessed by measuring R_f, A_s, and N/m. Both PMD measuring assays' R_f, A_s, and N/m values were obtained by their published formula.[35]

The intra-assay and interassay accuracy for both methods of PMD measurement were calculated in terms of percent recoveries using spiking technology/standard addition approach.[33] The preanalyzed PMD solution (200 ng/band) was spiked with additional 50, 100, and 150% PMD solution for the NP-HPTLC assay to produce low QC (LQC) levels of 300 ng/band, middle QC (MQC) levels of 400 ng/band, and high QC (HQC) levels of 500 ng/band of PMD. For the RP-HPTLC assay, the preanalyzed PMD solution (300 ng/band) was spiked with additional 50, 100, and 150% PMD solution to produce LQC levels of 450 ng/band, MQC levels of 600 ng/band, and HQC levels of 750 ng/band of PMD. To assess intraday accuracy for both methods on the same day, three different PMD QC solutions underwent a reanalysis. To assess the interday accuracy for both methods over the course of three consecutive days, three distinct PMD QC solutions were reanalyzed. The percent recovery was computed using six replications (n = 6) for both methods at each QC level for both accuracies.

The NP-HPTLC and RP-HPTLC methods' intra/interassay precision for PMD was assessed. The intra-assay precision for PMD was evaluated using six replications on newly made PMD solutions at LQC, MQC, and HQC on the same day for both methods (n = 6). To assess PMD interday precision, six replications (n = 6) of freshly generated PMD solutions at the same QC samples over the course of three days for each method were employed.

By purposefully changing the content of the relevant solvent system, various intended adjustments were introduced to assess the robustness of PMD for both methods. For the NP-HPTLC experiment, the conventional eluent system of $CHCl_3$/MeOH (90:10 v/v) for PMD was modified to $CHCl_3$/MeOH (92:8 v/v) and $CHCl_3$/MeOH (88:12 v/v),

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and the uncertainties in spot area and R_f values were observed using six replications (n = 6). For the RP-HPTLC assay, the eco-friendly eluent system EtOH/H_2O (75:25, v/v) was modified to EtOH/H_2O (77:23, v/v) and EtOH/H_2O (73:27, v/v), and the uncertainties in spot area and R_f values were noted using six replications (n = 6).[33]

The sensitivity of NP-HPTLC and RP-HPTLC methods for PMD was evaluated in terms of "LOD and LOQ" using a standard deviation technique. The blank sample (without PMD) was injected in six replications for both methods, and the standard deviation of that sample was computed. Six replications (n = 6) of both methods were utilized to derive PMD "LOD and LOQ" values using their published equations.[33]

The R_f values and UV absorption spectra of PMD in commercial capsules were compared to that of pure PMD in order to assess the selectivity of NP-HPTLC and RP-HPTLC methods for PMD.[33]

4.6. Forced-Degradation Evaluation. Under acidic, alkaline, oxidative, and thermal stress circumstances, the forced-degradation investigations for NP-HPTLC and RP-HPTLC assays were performed.[35,36] For this investigation, the MQC of PMD (400 ng/band for NP-HPTLC and 600 ng/band for RP-HPTLC method) was exposed to 1M HCl (acid), 1M NaOH (alkaline), 30% v/v H_2O_2 (oxidative), and a hot air oven at 55 °C for 24 h (thermal) stress conditions. The detailed procedures as published in our recent publication were followed for these studies.[36] For NP-HPTLC and RP-HPTLC assays, PMD chromatograms were recorded and analyzed for degradation products under the above-mentioned stress conditions.

4.7. Application of NP-HPTLC and RP-HPTLC Assays in the Measurement of PMD in Commercial Capsules. For NP-HPTLC and RP-HPTLC assays, the obtained solutions of commercial capsules were applied on NP and RP TLC plates, respectively, and the peak areas for PMD were recorded in three replications (n = 3). For both assays, the content of PMD in commercial capsules was derived from the PMD calibration plot.

4.8. Greenness Estimation. The AGREE methodology[28] was applied to predict the greenness index for NP-HPTLC and RP-HPTLC assays of PMD measurement. The AGREE scores in the range from 0.0 to 1.0 for NP-HPTLC and RP-HPTLC assays, were recorded using "AGREE: The Analytical Greenness Calculator (version 0.5, Gdansk University of Technology, Gdansk, Poland, 2020)". In order to confirm the AGREE findings, the ChlorTox method was also used to determine the greenness of both methods.[32] According to ChlorTox method, the ChlorTox value is calculated using the following equation

$$\text{ChlorTox} = \frac{\text{CH}_{\text{sub}}}{\text{CH}_{\text{CHCl3}}} \times \text{m}_{\text{sub}} \quad (1)$$

where CH_{sub} is the chemical hazard of the substance of interest, CH_{CHCl3} is the chemical hazard of standard $CHCl_3$, and m_{sub} is the mass of the substance of interest required for the single analysis. The values of CH_{sub} and CH_{CHCl3} were derived using the weighted hazards number (WHN) model using the safety data sheet of Sigma-Aldrich (St. Louis, MO, USA).[32]

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Notes

The authors declare no competing financial interest.

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