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Validation of stability-indicating reverse phase HPLC method for the determination of related substances in dabigatran etexilate mesylate drug substance

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ABSTRACT

A gradient reversed phase high performance liquid chromatography (RP-HPLC) method has been developed and validated for the determination for eight related substances of Dabigatran Etexilate Mesylate. The successful chromatographic separation of Dabigatran Etexilate from its related substances was achieved on octadecyl silane chemically bonded to porous silica particles stationary phase i.e Inertsil ODS-4, 250mm x 4.6mm, i.d., 5µ column maintained at temperature at 25°C by using phosphate buffer pH 3.0 and acetonitrile as mobile phases A & B respectively. Wavelength for UV detection: 220nm, flow rate: 1.0ml/min and Injection volume: 10µl. The performance of the method was validated according to the ICH guidelines for specificity, linearity, accuracy, precision, limit of quantification, limit of detection robustness and ruggedness and also DEM was subjected to stress conditions of thermal, hydrolysis, humidity, peroxide and photolytic to observe the degradation products. Limit of detection of impurities was in the range of 0.007%–0.008% indicating the high sensitivity of the developed method. The experiment results are given in detailed in this paper.

Keywords: Dabigatran Etexilate Mesylate, HPLC, Related substances, Validation

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INTRODUCTION

Dabigatran Etexilate (DE) is a new oral thrombin inhibitor [1] to reduce the risk of clotting in patients with atrial fibrillation and is a low-molecular-weight prodrug that exhibits no pharmacological activity. After oral administration, dabigatran etexilate is rapidly absorbed and quickly and completely hydrolyzed to its active moiety, dabigatran, by nonspecific ubiquitous esterases in the gut, plasma, and liver [2]. DE is a mesylate salt of a base which also contains two ester functional groups (ethyl ester and etexilate ester). The di-ester is essentially a prodrug for the corresponding zwitter ion and its brand name is Pradaxa, the nomenclature and strength is based on the relevant di-ester, intrinsic neutral form [3]. It is available as 75mg and 150 mg capsules for twice daily oral administration. DEM is a reversible thrombin inhibitor licensed for the use of stroke prevention in atrial fibrillation (AF) granted on the basis of data from the RE-LY (Randomized Evaluation of Long term anti-coagulation Therapy) study and is an alternative to anticoagulation with warfarin [4]. The empirical formula of DEM is $C_{34}H_{41}N_7O_5.CH_4O_3S$ and the molecular weight is 723.86 (mesylate salt), 627.75 (free base) and DEM is chemically

known as *β*-alanine, *N*-[[2-[[[4- [[(hexyloxy)carbonyl]imino]amino methyl]phenyl]amino] methyl]-1-methyl-1*H*-benzimidazol-5-YL] carbonyl]-*N*-2-pyridinyl, ethyl ester, methanesulfonate.

There are several process and degradation impurities of DEM, which are originated through synthesis process and as well as degradation during stability storage. The chemical structures of DEM and its eight impurities [Impurity –I to VIII] are represented in Figure 1. There is no HPLC method was specified for determination of DEM and its related substances in official Pharmacopoeias (i.e. USP , European Pharmacopoeia). However, a few of methods have been reported in literature for the determination of DE in formulated products and plasma. A HPLC-UV method for determination of DE in rat plasma was established in year 2013 by Sun Min etal by using Kromasil C_{18} column [5], assay for routine quantification of dabigatran in human plasma by UPLC MS/MS technique by D. Xavier etal, published in 2012 [6] and a stability indicating HPLC method for the determination of DE in capsules has been published by Bernardi etal in 2013 [7] have been reported. Recently in 2015, Dare, Jain and Pandey reported the determination of dabigatran etexilate related substances by HPLC [8]. In reported method, ion-pair reagent was used in the preparation of mobile phase. In ion-pair mobile phases, stabilization of column is quite difficult and time taking procedure. Hence, stability indicating RP-HPLC method has been developed for the quantification of impurities related to DEM with simple buffer. The limit of each impurity is considered 0.15% level accordance with ICH guideline based on daily intake 300mg of DE [8]. The developed chromatographic method can resolve all these eight impurities with passable resolution to achieve good chromatography and the optimized methodology have been validated to accomplish ICH guidelines on validations [9].

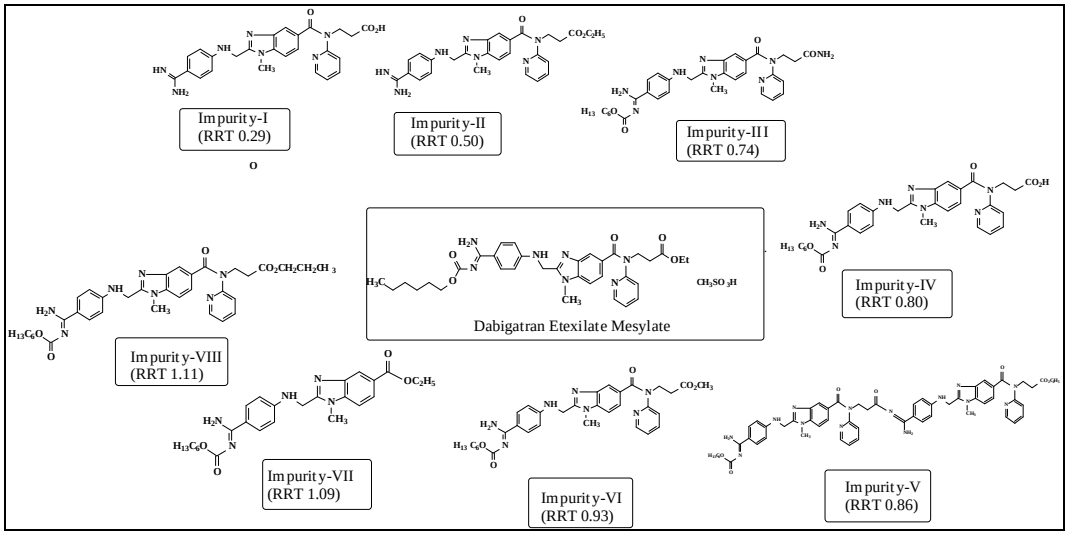


Fig. 1: Chemical structures of DEM and its impurities

MATERIALS AND METHODS

Chemicals, reagents, standards and samples

The investigated samples of DEM drug substance, its related impurities and DEM for system suitability (DEM enriched with Impurity-VII) were gifted from APL Research Centre-II Laboratories (A division of Aurobindo Pharma Ltd., Hyderabad). AR grade of Potassium dihydrogen orthophosphate were procured from Spectrochem, India. Acetonitrile and Orthophosporic acid (~88%) were procured from Merck, India and pure milli-Q water was used with the help of millipore purification system (Millipore®, Milford, MA, USA).

Instrumentation and methodology

The HPLC system used for method development, method validations as well as forced degradation studies were Waters Alliance 2695 separation module equipped with 2996 photo diode array detector with Empower data handling system i.e Empower 2 software, Build No: 2154 [Waters Corporation, MILFORD, MA 01757, USA] was used.

HPLC column: Inertsil ODS-4, 5µ (250mm × 4.6mm) (Make: GL Sciences)],column oven temperature: 25°C. Mobile phase A: Dissolve 2.72 g of Potassium dihydrogen orthophosphate in 1000 ml of water, adjust pH to 3.0±0.05 with orthophosphoric acid and filter this solution through 0.45 µ or finer porosity membrane filter. Mobile phase B: Acetonitrile. Diluent: water and acetonitrile in the ratio of 70:30%v/v. Flow rate: 1.0 ml/min, injection volume: 10µl, data acquisition time: 40 min and UV detection: 220 nm. Retention time of dabigatran etexilate: about 24 minutes. The pump is in gradient mode and the program is as follows: Time (min)/ A (v/v): B (v/v); T0.01/85:15, T15/60:40, T40/30:70,T42/85:15,T50/85:15.

Preparation of solutions

System suitability solution

0.6 mg/ml concentration of DEM for system suitability (DEM enriched with impurity-VII) in diluent.

System suitability evaluation: The USP resolution between Dabigatran etexilate and Despyridyl dabigatran etexilate is not less than 3.0.

Standard solution

0.0009mg/ml concentration of solution using DEM standard in diluent.

Sample solution

0.6mg/ml concentration of solution using DEM sample in diluent.

RESULTS AND DISCUSSION

Method Validation

Specificity

Specificity is the ability of assess unequivocally of analytic in the presence of components which may be expected to be present. For determination of specificity, injection of blank, all individual eight impurities solutions were prepared and injected to confirm the individual retention times. The solutions of DEM drug substance (Control Sample) and DEM spiked with known related substances at specification level (Spiked Sample) were prepared and injected into HPLC. Peak purity was established by using Empower Software. The specificity results are tabulated in Table 1. A typical representative HPLC chromatogram of DEM drug substance spiked with all impurities is shown in Fig. 2.

Tab. 1 Specificity experiment from spiked sample

Spiked sample			
Name	RRT	Peak Purity	
		Purity Angle	Purity Threshold
Impurity-I	0.29	0.697	1.289
Impurity-II	0.51	0.668	1.155
Impurity-III	0.74	0.594	1.067
Impurity-IV	0.80	0.525	0.886
Impurity-V	0.86	0.707	1.272
Impurity-VI	0.94	0.486	0.943
Impurity-VII	1.09	0.511	0.891
Impurity-VIII	1.11	0.643	1.157
DEpeak-control sample/diluted		0.048	0.254
DEpeak-spiked sample/diluted		0.047	0.254

Forced degradation

The degradation behavior of DEM has been studied by performing forced degradation studies. DEM was subjected to different stress conditions [10] i.e acid/base hydrolysis [1M HCl/85°C/45 min & 5M NaOH/Initial/RT], peroxide degradation under oxidative stress [5%w/v hydrogen peroxide solution, 85°C/45min], thermal degradation [105°C/120Hours], humidity degradation study (90% RH/25°C) and photolytic degradation [white Fluorescent light, 1.2million Lux hours and UV light, 200 watt hours / m2] w.r.t ICH option 2 of Q1B [11]. Peak purity of DE peak was established by using PDA detector in these stress samples. The forced degradation results are tabulated in Table 2. The typical representative HPLC chromatograms of forced degradation experiment are shown in Fig. 3.

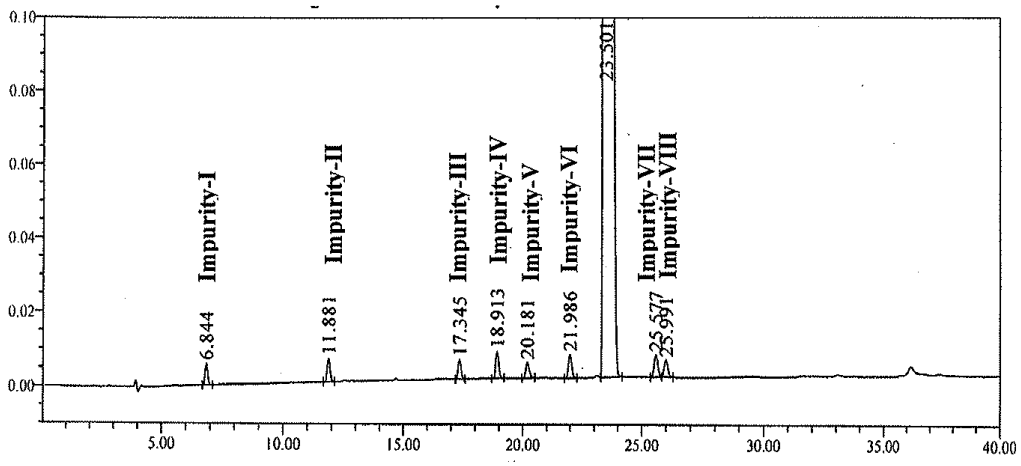
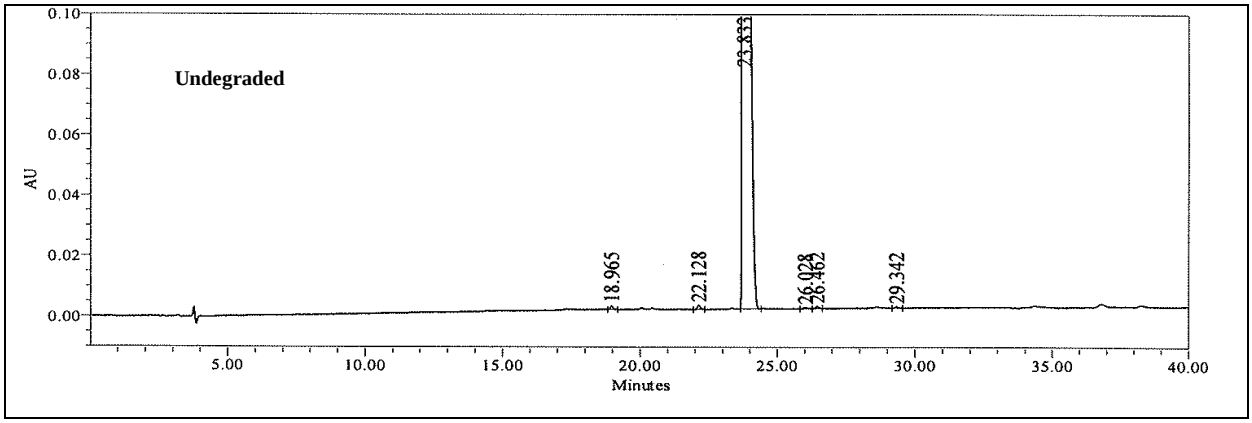


Fig. 2. A typical representative HPLC chromatogram of DEM drug substance spiked with all impurities

In acid degradation (1M HCl / 85°C / 45min), impurity-IV was degraded up to 15% in base degradation (5M NaOH / RT / Initial), impurity-IV was degraded up to 21%. In peroxide degradation (5% H2O2 / 85°C / 45 min), impurity-II was degraded up to 15%. In thermal degradation, impurity-II was degraded up to 1% was degraded up to 0.2%.In photolytic & humidity degradation conditions, there was no degradation observed with respect to undegraded sample. The above results of various stress conditions employed to degrade DEM indicate that DEM is susceptible to degrade under acidic, basic hydrolysis and oxidative conditions and moderately sensitive to heat whereas, it is found to be stable to photolytic and humidity stress conditions. Based on the forced degradation data generated, it can be concluded that impurity-II and impurity-IV are potential degradants.

Tab. 2. Specificity experiment –forced degradation studies

Degradation mechanism	Degradation condition	Degradation (%)	Peak purity of DE	
			Purity angle	Purity threshold
-	Undegraded Sample	-	0.043	0.256
Acid	1M HCl / 85°C / 45min	17.1	0.046	0.251
Base	5M NaOH / RT / Initial	19.9	0.011	0.264
Peroxide	5% H_2O_2 / 85°C / 45min	23.7	0.069	0.327
Thermal	105°C / 120 hours	3.4	0.051	0.260
Photolytic	1.2million Lux hours and UV light, 200 watt hours / m^2	Nil	0.031	0.260
Humidity	90% RH / 25°C / 120 hours	Nil	0.033	0.255



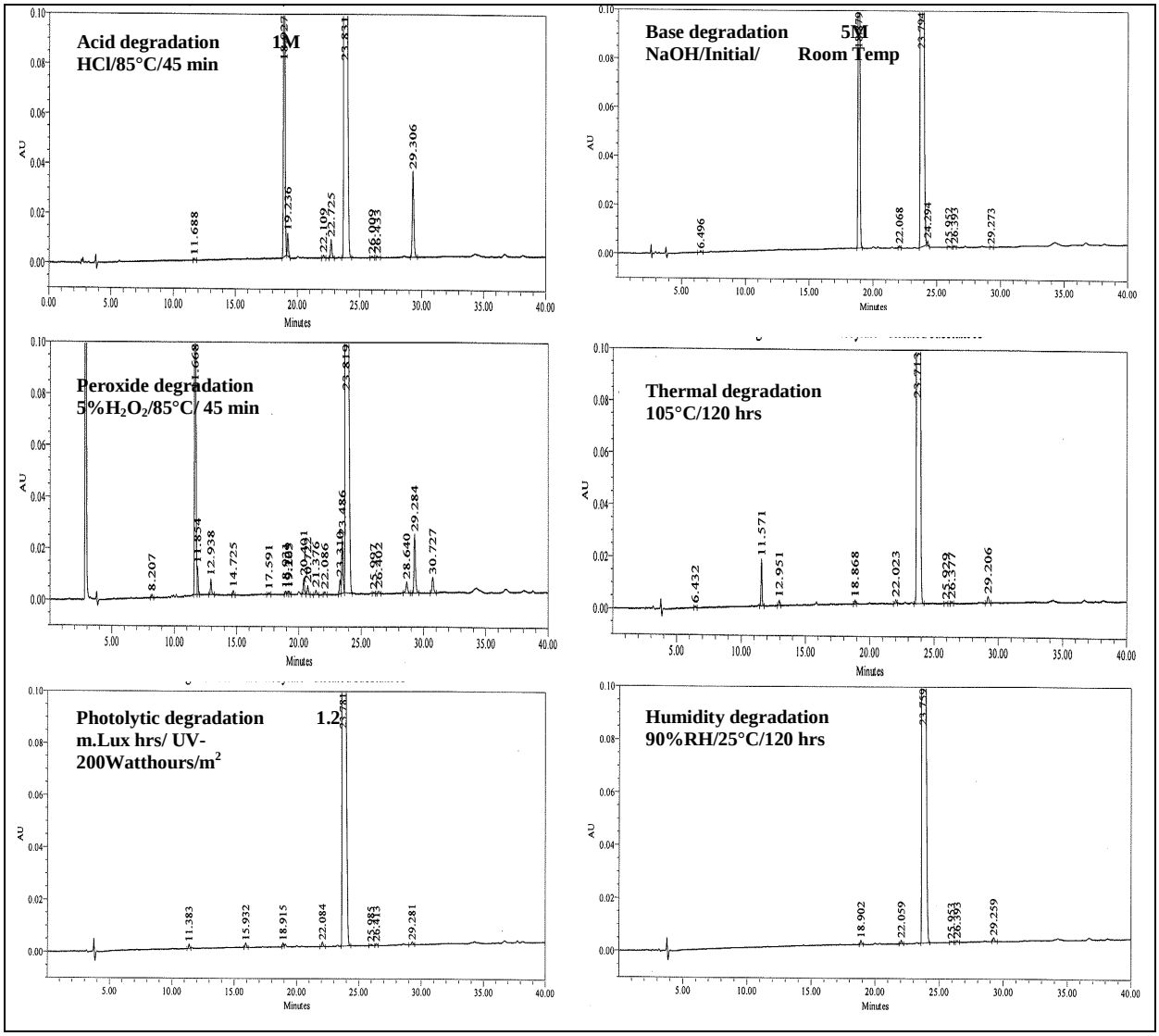


Fig. 3. The typical representative HPLC chromatograms of forced degradation Experiments

Limit of Detection (LOD)/ Limit of Quantification (LOQ)

LOD and LOQ were calculated on the basis of response and slope of the regression equation. These are calculated from the formula 3.3δ/S and 10 δ /S respectively where 'δ is standard deviation of the y-intercept of the regression line and 'S' is slope of the calibration curve which were predicted from linearity experiment. The precision study was carried out at about predicted LOD and LOQ levels by injecting six replicates and calculating the % RSD of the area of each impurity.

Linearity

A series of solutions were prepared using DEM and its impurities at concentration levels from LOQ to 150% of specification level and each solution was injected and calculated the statistical values like slope, intercept, STEYX and correlation coefficient from linearity plot drawn for concentration versus area. The statistical values are presented in Table 3.

Tab. 3. Statistical evaluation of linearity and LOD/LOQ experiments

	Concentration range(µg/mL)	Slope	Intercept	STEYX	RF	Correlation Coefficient	LOD		LOQ	
							(%w/w)	%RSD	(%w/w)	%RSD
Impurity-I	0.107 - 1.439	48968	-265	264	0.80	0.9999	0.007	1.4	0.021	0.6
Impurity-II	0.109 - 1.425	48821	-459	413	0.80	0.9998	0.007	2.5	0.022	0.9
Impurity-III	0.120 - 1.342	44315	-116	338	0.98	0.9998	0.008	3.2	0.023	0.6
Impurity-IV	0.132 - 1.359	42296	-1097	518	0.92	0.9997	0.008	0.9	0.025	0.3
Impurity-V	0.139 - 1.365	40904	-1620	1186	1.06	0.9983	0.009	4.3	0.027	2.6
Impurity-VI	0.131 - 1.369	39730	-1068	481	0.98	0.9997	0.009	0.8	0.026	0.7
Impurity-VII	0.108 - 1.277	53582	-1656	390	0.73	0.9998	0.007	8.1	0.020	0.8
Impurity-VIII	0.126 - 1.361	40432	360	354	1.07	0.9998	0.008	3.2	0.024	0.7

Precision

The precision (system precision) was evaluated by injecting six injections of DEM standard solution and calculating the % relative standard deviation. The method precision was checked by injecting six individual preparations of DEM spiked with each impurity with 0.15% with respect to sample concentration. % RSD of content of each impurity was calculated. The intermediate precision of the method was also evaluated using different analyst, different instrument, different lot of column on different day. The inter day variations were calculated. The precision experiments results are given in Table 4.

Tab.4. Precision experiment results

System Precision										
	Inj-1	Inj-2	Inj-3	Inj-4	Inj-5	Inj-6	Mean	SD	% RSD	95% Confidence Interval (±)
DE Peak area	41621	40453	39099	38570	39407	37486	39439	1447	3.7	1519

Method Precision & Ruggedness								
Name	Mean (%w/w)[*n=6*]		SD		% RSD		95% Confidence Interval (±)	
	MP	RUG	MP	RUG	MP	RUG	MP	RUG
Impurity-I	0.167	0.162	0.001	0.001	0.6	0.6	0.001	0.001
Impurity-II	0.159	0.163	0.001	0.002	0.6	1.2	0.001	0.002
Impurity-III	0.171	0.176	0.001	0.001	0.6	0.6	0.001	0.001
Impurity-IV	0.240	0.242	0.001	0.003	0.4	1.2	0.001	0.003
Impurity-V	0.191	0.170	0.006	0.005	3.1	2.9	0.001	0.005
Impurity-VI	0.247	0.258	0.001	0.003	0.4	1.2	0.006	0.003
Impurity-VII	0.172	0.194	0.001	0.002	0.6	1.0	0.001	0.002
Impurity-VIII	0.203	0.227	0.001	0.003	0.5	1.3	0.001	0.003

MP: Method Precision RUG: Ruggedness

Tab. 5. Accuracy experiment results

Recovery details (average 3 replicates)		Impurity-I	Impurity-II	Impurity-III	Impurity-IV	Impurity-V	Impurity-VI	Impurity-VII	Impurity-VIII
--	% Level								
Added (%w/w)	LOQ	0.0210	0.0209	0.0215	0.0247	0.0266	0.0251	0.0210	0.0238
	50	0.075	0.076	0.078	0.077	0.081	0.080	0.084	0.079
	100	0.149	0.152	0.156	0.154	0.162	0.159	0.168	0.156
	150	0.224	0.228	0.233	0.232	0.243	0.239	0.252	0.234
Recovered (%w/w)	LOQ	0.0213	0.0208	0.0214	0.0246	0.0271	0.0248	0.0207	0.0288
	50	0.074	0.077	0.080	0.076	0.0783	0.076	0.084	0.079
	100	0.149	0.150	0.154	0.157	0.168	0.162	0.171	0.152
	150	0.223	0.227	0.232	0.238	0.250	0.245	0.264	0.231
Recovery (%)	LOQ	101.3	99.7	99.1	101.6	102.2	98.8	98.7	96.1
	50	98.7	100.4	101.7	99.6	96.7	95.0	100.4	100.0
	100	100.0	98.7	98.9	101.5	103.5	102.1	102.0	97.0
	150	99.9	99.4	99.6	102.8	103.0	102.4	104.9	98.7

Accuracy

The accuracy of the method was determined by analyzing DEM (n=3) samples spiked with impurities at different levels (LOQ, 50, 100 and 150% of specification, i.e 0.15%). The percentage recovery values for all the impurities are calculated and tabulated in Table.5.

Robustness

To determine the robustness of the method, experimental conditions were deliberately changed and to evaluate system suitability requirement as per methodology. For this evaluation, system suitability solution and sample solution spiked with impurities at specification level were prepared as per test method and injected into HPLC. To study the effect of flow rate, 10% variation (±0.1 units) of flow rate was changed. The effect of column temperature was studied by keeping 20°C and 30°C instead of 25°C. The effect of pH was studied by varying ±0.2 units of methodology value. In the same manner, detection wavelength (±3 nm) and organic in mobile phase (±2% absolute in Gradient Composition) have been verified and the results obtained from these experiments are summarized in Table 6.

Tab. 6. Robustness experiment results

Condition	Variation	System Suitability			Spiked Sample (RRT)							
		USP Resolution	USP Plate count	USP Tailing	Imp-I	Imp-II	Imp-III	Imp-IV	Imp-V	Imp-VI	Imp-VII	Imp-VIII
STP	**-**	5.6	188940	1.1	0.28	0.50	0.74	0.81	0.86	0.94	1.09	1.11
Flow	**-10%**	6.3	186028	1.1	0.30	0.52	0.74	0.81	0.85	0.94	1.09	1.11
	+10%	6.6	160157	1.1	0.27	0.49	0.73	0.80	0.86	0.93	1.09	1.11
Wavelength	**-3 nm**	5.6	183954	1.0	0.28	0.50	0.74	0.81	0.86	0.94	1.09	1.11
	+3 nm	5.7	183027	1.1	0.28	0.50	0.74	0.81	0.86	0.94	1.09	1.11
% of Organic in MP	**-2% absolute**	6.3	189427	1.1	0.31	0.52	0.74	0.81	0.86	0.94	1.09	1.10
	+2% absolute	6.3	158790	1.1	0.26	0.48	0.73	0.80	0.85	0.93	1.09	1.11
pH of Buffer	**-0.2 units**	6.5	163006	1.1	0.27	0.49	0.73	0.80	0.85	0.93	1.09	1.11
	+0.2 units	6.3	172449	1.1	0.30	0.52	0.74	0.81	0.86	0.94	1.09	1.10
Column Oven Temperature	**-5°C**	6.4	161440	1.1	0.29	0.50	0.74	0.81	0.85	0.94	1.10	1.11
	+5°C	6.2	188384	1.1	0.28	0.50	0.73	0.80	0.85	0.93	1.08	1.11

Stability of solutions

Standard solution and sample solution spiked with impurities were prepared and analyzed initially and at different time intervals by keeping the solutions at room temperature (~ 25°C) and refrigerator condition (~6°C). Test results reveal that standard solution is stable for at least 24 hours at room temperature (~25°C), sample solution is not stable at room temperature (~25°C) and stable for at least 6 hours at refrigerator condition (~6°C).

CONCLUSION

A reverse phase stability indicating HPLC method was developed and validated for the quantitative determination of the process and degradation impurities of DEM. The results obtained from validation experiments proved that the chromatographic method is well separated all eight impurities from drug substance. The present study will help the manufacturers and suppliers of DEM to quantify and quality the purity based on degradation data. Thus, it can be used for routine analysis, quality control and for determining quality during the stability studies of pharmaceutical analysis.

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