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STABILITY INDICATING RP-UPLC METHOD DEVELOPMENT FOR RELATED SUBSTANCES OF ANTI-EMETIC TRIMETHOBENZAMIDE HYDROCHLORIDE, ITS VALIDATION AND MASS BALANCE STUDY

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ABSTRACT

A simple and rapid reverse phase ultra-performance liquid chromatographic (RP-UPLC) method for determination of related substances of an antiemetic drug Trimethobenzamide Hydrochloride (TMB·HCl) is reported. The RP-UPLC method is developed and validated as per the International Council for Harmonisation (ICH) guidelines Q2(R1). The effective chromatographic separations were achieved on Acquity CSH Phenyl-Hexyl, 2.1 x 100 mm 1.7µm column and reverse phase with linear gradient elution. The mobile phase-A is 0.1% Nonaflurobutane -1 sulfonic acid in water, while mixture of 35:65 ratio of 0.1 % Nonaflurobutane-1-sulphonic acid in purified water to acetonitrile is mobile phase-B. The flow rate of 0.4 mL min^{-1} and PDA/UV detector are used. The method is linear in the range of limit of quantitation (LOQ) to 150 % level with respect to specification concentration limit of impurities. The correlation coefficient of all impurities and TMB is greater than 0.999. The LOQ of all known impurities and TMB is in between 10 to 30% of its specification limits. The relative response factor are calculated to all eigth known impurites. The unknown peaks are well separate, observed results are comparable to initial values. This RP-UPLC method is accurate, precise and robust. The forced degrdiation study of the TMB·HCl has been carried out and the mass balance is proven.

KEY WORDS

Anti-emetic, forced degradation, mass balance, RP-UPLC, Related substances, Stability Indicating, TMB·HCl

INTRODUCTION:

Trimethobenzamide (TMB), 4- (2-dimethyl Amino ethoxy)-N-(3, 4, 5-trimethoxy benzoyl) benzylamine (see **Table 1**), is a specific anti-emetic drug and marketed as its hydrochloride salt (TMB·HCl). The double-blind studies have shown that, the TMB·HCl is significantly better in treating nausea and vomiting without any side effect [1]. The combination of TMB and diphenhydramine is an inexpensive formulation, which is moderately effective in a majority of acute migraines. However, it has less efficiency compared to the sumatriptan [2,3]. Many antiemetic agents have also been used as antihistamines, reserpine, and phenothiazine derivatives. The TMB incorporates parts of their chemical structure.

The action of Trimethobenzamide hydrochloride (TMB·HCl) is speculated due to its probably involvement in the chemoreceptor trigger zone. The TMB·HCl pre-treatment inhibits the emetic response to apomorphine in animal and show in significant protection against emesis by intragastric $CuSO_4$. In earlier days TMB·HCl formulation was approved injection, later administration as an oral capsule was also approved. The pharmacokinetics TMB·HCl studied in healthy adults have shown that the oral capsule takes ~50% more time to reach the maximum plasma concentration compared to the intramuscular injection, which requires

about half an hour. The elimination half-life of TMB is 7 to 9 h. Moreover, the metabolic disposition of Trimethobenzamide is still not known in humans [4]. The reproduction experiments in rats and rabbits suggested that no teratogenicity and the only observed effects are in the increased percentage of embryonic resorptions or stillborn pups of one or two candidates [5].

Table 1: Chemical structure and name of TMB·HCl as well as its related impurities

Name	Chemical Structure and Name	Category	Name	Chemical Structure and Name	Category
TMB·HCl	N{[4-(2-dimethylaminoethoxy) phenyl] methyl}-3,4,5-trimethoxy-benzamide hydrochloride	Active Pharmaceutical ingredient	Impurity E	N- [4-(2 methylamino) ethoxy] benzyl]-3,4,5-trimethoxy-benzamide	Degradant Impurity
Impurity-A	4-Hydroxy benzaldehyde	Starting material	Impurity F	N-[4-(2 dimethylamino)ethoxy] benzyl]-3,4,5-trimethoxy-benzamide N-Oxide	Degradant Impurity
Impurity-B	3,4,5-Trimethoxybenzoic acid	Starting material	Impurity G	3,4,5-Trimethoxybenzoic acid methyl ester	Degradant Impurity
Impurity-C	4-(2-Dimethylaminoethoxy) benzaldehyde	Process Impurity	Impurity H	N-[4-(2-dimethylchloromethylamin) ethoxy]-benzyl]-3,4,5-trimethoxybenzamide	Degradant Impurity
Impurity-D	4-(2-Dimethylaminoethoxy) benzylamine	Process Impurity			

TMB has been approved in anti-emetic prescriptions, use as a complete therapeutic substitute for an IV anti-emetic at the time of chemotherapy treatment [6]. Naviasky have performed the first quantitative assay of TMB·HCl in capsule as well as injection formulation, using the ion-pair column chromatography [7]. The UPLC for separation of the TMB·HCl and its one impurity was performed; however, the details of the analytical method having 21 min runtime were not reported [8]. However, there may presented eight known impurities in TMB, which requires detailed separation and quantification of these impurities. Recently we have presented an efficient, fast RP-UPLC method for quantitative analysis of TMB. The use of Nonaflurobutane-1-sulfonic acid (NFSA) for mobile phase preparation was shows the significant effect for the separation of impurities. Furthermore, the recent FDA norms have also necessited the mass balance

studies for the drug and its related impurities. To attempt this issue we are herewith developing and validation a new RP-UPLC method for determination of related substances of TMB·HCI.

EXPERIMENITAL:

Materials and Methods:

TMB·HCl and its eight related impurities (A to H) were synthesized and characterized in Emcure Pharmaceutical Limited. Analytical grade acetonitrile, methanol, NFSA and purified water as milli-Q were used for preparation of mobile phase and diluent. The analytical balance (Metler Toledo) and the Acquity H-class UPLC (Waters) system with Empower 2 software for data acquisition and PDA/UV detector are used. All the instruments were calibrated during method development and validation.

Method Development:

The TMB and its related impurities are polar molecules, therefore the method for the related substances is developed using reversed phase (RP) chromatography. The stationary phase in reversed phase chromatography is non-polar like C4, C8, C18, while the mobile phase is polar such as water, acetonitrile, methanol and/or buffer solution. The very few literatures is available for estimation of related substances in TMB·HCI [8]. Thus, the UPLC technique is utilized for determination of related substances in TMB·HCI.

During UPLC method development [9] along with stationary and mobile phase other parameters such as column temperature, diluents, wavelength, and pH are also playing a crucial role. Stationary phase of UPLC method screened from particular Acquity CSH phenyl hexyl and Acquity BEH columns both with the C18 having 2.1 mm internal diameter and 1.7µm particle size. These Acquity CSH phenyl hexyl and Acquity BEH columns having 50 mm and 100 mm length. When the Acquity CSH phenyl hexyl, C18 (2.1X 100 mm) 1.7µm is used in the method, the better separation of impurities, peak sharpness and appropriate system suitability parameters i.e. tailing factor and theoretical plates are met. The pKa value of target molecules is mainly directes the selection of buffer for mobile phase. Here, an ion pair buffer of NSFA is selected for the mobile phase preparation. NFSA has a capacity to elute close peaks with suitable resolution and it also obeys other system suitability parameters. Thus 0.1% NFSA in water used as mobile phase A,where as a mixture of 35:65 ratio of NFSA in purified water and acetonitrile is a mobile phase B. The appropriate gradient program, flow rate, column oven temperature is selected by performing different trial runs of standard preparation. Method development in chromatographic conditions for TMB·HCI is listed in **Table 2**.

Table 2: Chromatographic conditions for the related substances RP-UPLC method of TMB·HCI

Instrument	UPLC equipped with an injector, pump UV/PDA detector and recorder
Column	Acquity CSH Phenyl-Hexyl, (2.1 x 100 mm), 1.7µm
Wavelength	220 nm
Flow rate	0.4 mL/min
Injection volume	1.0 µL.
Column oven temperature	40°C
Sampling rate	5 points/s
Run time	21 min

Furthermore, the gradient program has been used to perform the UPLC analysis, initially the composition of mobile phase A and B in 75:25 ratio has been used, which is then gradually changed to 65:35 up to 10 min. It is further changed to 25:75 ratio up to 17 min. It is constant up to 18.9 min. The composition is then abruptly brought to initial values of 75:25 in 0.1 min and maintain throughout the run i.e. up to 21 min.

RESULTS AND DESCUSION:

The analytical method validation is carried out as per ICH guideline Q2(R1) [9-12]. All parameters in the method validation are discussed below.

Specificity:

Specificity of method measures the analyte response in presence of related impurities. In impurity profiling method should present discrimination of such impurities. Selectivity data are given in **Table 3** and the corresponding chromatograms are shown in **Figure 1**. Specificity can be done by spiking pure substances with

appropriate levels of impurities (up to 0.15 % of concentration) and demonstrating the results are unaffected by the presence of these spiked impurities. Blank run does not show any interfering peak with TMB peak and its related impurities. The TMB peak is well resolved from known impurities (see **Figure 1**). Peak purity angle of TMB peak and the peaks of its related impurities are below the threshold value for respective standards and un-spiked as well as spiked test solutions (see **Table 3**). This indicates that these peaks are pure. The peak at retention of about 0.6 min is the maleic acid, which comes from Impurites C. The % relative standard deviation (%RSD) of six replicates of standard preparation with a mixture of impurities A to H and TMB·HCl are 0.39, 0.40, 0.28, 0.56, 0.55, 0.40, 0.35, 0.28 and 0.80%, respectively. The resolution between close eluting impurity-E and TMB·HCl is 4.09, which indicates well sepration between these peaks.

Table 3: The selectivity data in the TMB·HCl over the spiked impurities up to 0.15 % of concentration in test solution

Impurities*	Retention Time	Area	RT Ratio	Resolution	Purity Angle	Purity Threshold	Peak Purity
A	1.647	19711	0.15	--	0.373	0.758	Pass
B	3.160	20097	0.28	18.34	1.790	3.078	Pass
C	3.715	13338	0.33	5.38	0.821	1.153	Pass
D	4.582	11928	0.44	9.61	6.121	9.709	Pass
G	7.402	20169	0.67	16.46	3.610	5.543	Pass
E	10.358	29840	0.93	16.29	2.394	3.758	Pass
TMB·HCl	11.111	10895438	1.00	4.09	2.546	4.473	Pass
F	12.285	16149	1.11	6.82	2.546	4.473	Pass
H	13.098	14524	1.18	7.12	3.119	4.586	Pass

* Impurities are represented by alphabets A to H.

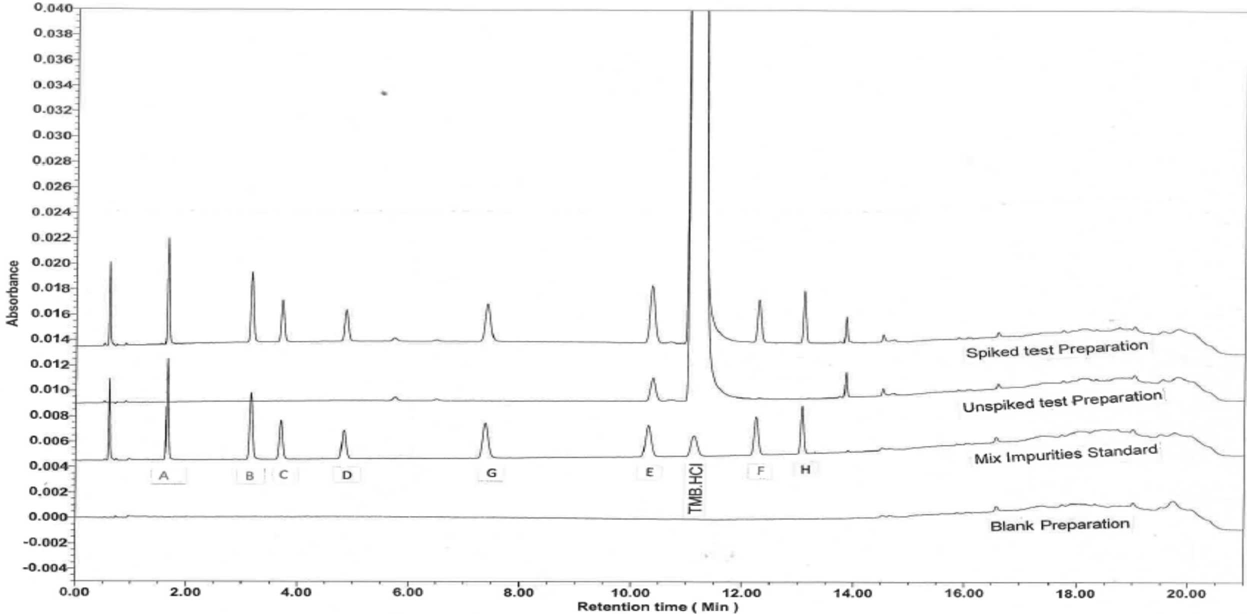

Figure 1: Typical chromatogram for selectivity in the TMB·HCl over the spiked impurities

Determination of limit of detection and limit of quantitation:

The LOD is the point at which a measured value is larger than the uncertainty associated with it. It is the lowest concentration of analyte in a sample that can be detected. In chromatography, LOD is the injected amount that results in a peak with a height at least two or three times as high as the baseline noise level. The LOQ of an individual analytical procedure is the lowest

amount of analyte in a sample that can be quantitatively determined with suitable precision and accuracy.

Furthermore, determination of LOD and LOQ, three methods are describing in ICH guideline based on, visual inspection, signal-to-noise ratio and standard deviation of the response and slope. The visual inspection method is used for non-instrumental methods. While in standard deviation based on response and slope method, LOD and LOQ concentration can be calculated by using the observed slope value. In signal-to-noise ratio method, s/n ratio is performed by measured signals from the sample. In TMB·HCl signal-to-noise ratio method applied for determination of LOD and LOQ concentration of TMB·HCl and its impurities (A to H). To establish the predicted LOD and LOQ concentration, injecting the various concentration levels (between 10 to 120%) of standard solutions both of TMB·HCl (0.1%) and impurities A to H (0.15%) of its limit level concentrations. The observed LOQ values for are TMB·HCl and its impurities (A to H) found at lower concentrations

(between 10 to 20% of limit concentration). The LOD concentration evaluate by multiplying factor 0.33 to predicated LOQ concentration. The predicated LOD and LOQ data shown in **Table 4**.

Table 4: The predicted LOD, LOQ concentration and s/n ratio value of LOQ level of TMB·HCl and its related impurities A to H.

Impurities	LOQ in % w.r.t test	S/N of LOQ level	LOD in % w.r.t test
A	0.015	26	0.005
B	0.015	18	0.005
C	0.030	21	0.010
D	0.030	11	0.010
E	0.030	17	0.010
F	0.015	11	0.005
G	0.030	19	0.010
H	0.015	14	0.005
TMB·HCl	0.030	17	0.010

Linearity and Range:

The linearity of an analytical procedure is its ability to obtain test results which are directly proportional to the concentration of analyte in the sample. It may be directly demonstrated on the analyte, or on spiked samples using at least five concentrations over the whole working range. The linearity of the method was determined by using standard solutions of impurities with LOQ Level to 150 % (encompassing 50, 80, 100, and 120 %) of specification limit concentration of TMB·HCl. The LOD and LOQ concentrations, correlation coefficient, slope and intercept of the linearity data are reported in **Table 5** and respetvie linearity graphs are shown in **Figure 2**. The peak area verses concentration data was treated by least squares linear regression analysis and the correlation coefficienst obtained for all the impurities are greater than 0.999. The % Y intercept of calibration curve not more than 5 %. The linearity graph shows that, impurities A to H present in the test sample of TMB·HCl has difference response. The impurity D has lower response while impurity B has higher response over all known impurities present in TMB·HCl.

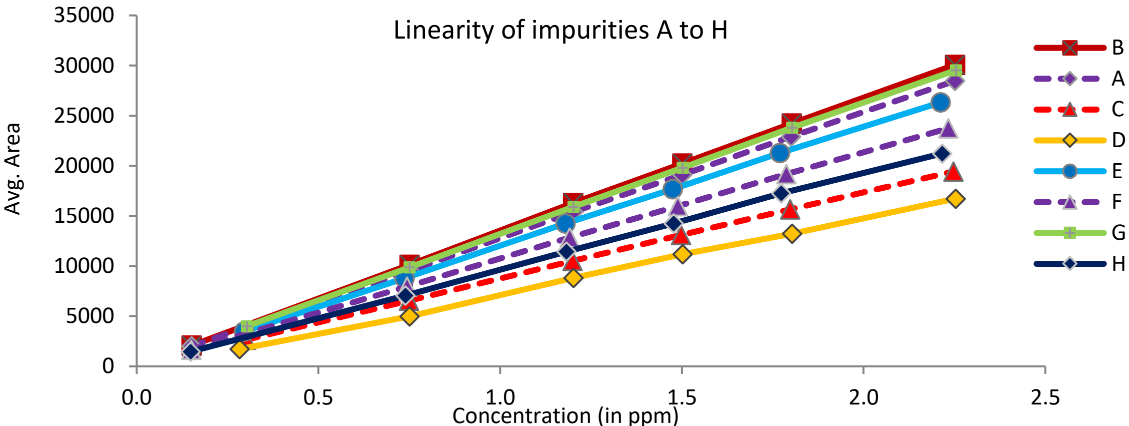

Figure 2: Linearity graph of impurities A to H from LOQ – 150 % concentration levels.

Table 5: The concentration of LOQ, LOD, Area for the correlation coefficient, slope, intercept as well as RRF of Impurites A to H and TMB·HCl.

Impurities	LOQ in % w.r.t test	LOD in % w.r.t test	Area for 0.15 % conc.	Correlation Coefficient	Slope	Intercept	% Y intercept	RRF
A	0.015	0.005	19112	0.99996	12627.32	128.77	0.67	0.92
B	0.015	0.005	20232	0.99996	13355.05	128.83	0.66	0.87
C	0.030	0.010	13115	0.99995	8638.35	99.50	0.76	1.35
D	0.030	0.010	11184	0.99958	7677.97	526.19	4.70	1.52
E	0.030	0.010	17679	0.99993	11951.68	23.76	0.13	0.98
F	0.015	0.005	15973	0.99996	10632.63	101.30	0.63	1.10
G	0.030	0.010	19816	0.99997	13119.39	70.16	0.35	0.89
H	0.015	0.005	14237	0.99989	9616.59	37.66	0.19	1.21
TMB·HCl	0.030	0.010	11690*	0.99989	11660.13	23.79	0.20	1.00

* The concentration of TMBHCl is 0.10 %.

The linearity for TMB·HCl is also estabilished from its LOQ Level to 150 % (0.1% w.rt. test concentration). The observed correlation coefficient, slope and intercept of the linearity data are reported in **Table 5** and respetvie linearity graph is shown in **Figure 3**.

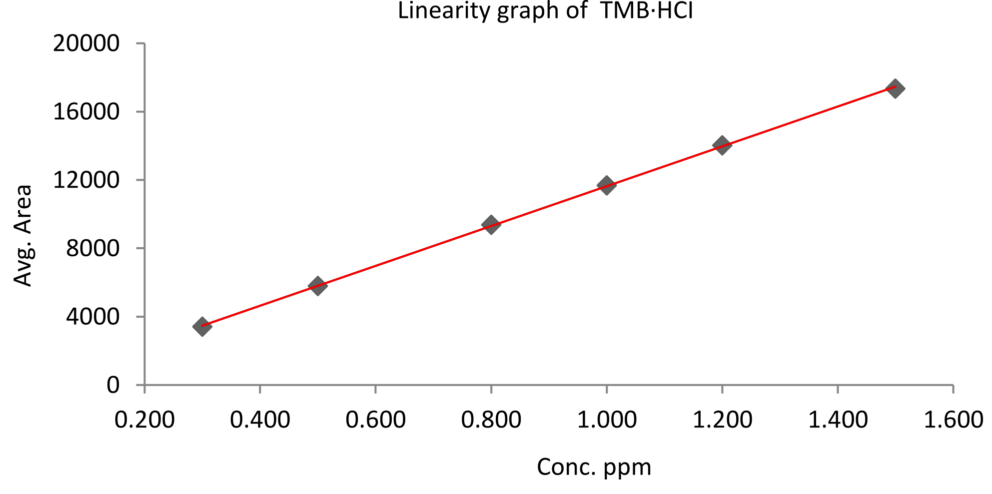

Figure 3: Linearity graph of TMB·HCl from LOQ – 150 % concentration levels.

Relative response factor:

The relative response factor (RRF) is an alternative method for determination of the quantity of impurities present in pharmaceutical products. The RRF is the ratio of the response of impurity to active pharmaceutical ingredient (API) under the identical chromatographic conditions. The RRF are generally different for every component of the sample and should be determined individually. The RRF values determined on one particular type of detector cannot be used for the determination of concentration respective components using other type of detectors. The RRF depends on the response of TMB·HCl and its impurities. If higher the response of impurities against TMB·HCl, RRF will be lower side and vice versa. The observed RRF values for known impurities A to H is between 0.87 to 1.52 and considered RRF of TMB·HCl (1.00) for calculation of unknown impurities.

Precision:

Precision expresses closeness between individual results when the same test procedure is applied to multiple samplings of same homogeneous sample under given condition. The precision categorized into repeatably, intermediate precision and reproducibility. These precision studies are performed when the entire analytical method procedure is finalized.

In reputability study, system precision is studied by carrying out six replicates of TMB·HCl with the mixed impurities standard. The repeatability of related substances has been performed by injecting six individual test sample preparation with spiking related impurities of its limit level concentration. The % impurities content and % RSD of the spiked test sample are calculated. The intermediate precision is also evaluated using different instruments and different columns by different analysts on different days in different laboratory. % RSD of results of impurities was calculated in repeatability (n=6) and intermediate precision (n=12). The %RSD of six spiked test preparation in repeatability for impurities A to H and total impurities are 0.52, 0.66, 0.32, 0.38, 0.52, 0.67, 0.33, 0.66 and 0.40, respectively, and overall %RSD of these impurities for twelve samples (i.e. six from repeatability and six from intermediate precision) are 0.52, 1.12, 0.78, 4.00, 0.87, 0.53, 1.13 and 0.49, respectively. The reprodubility shows collaboration study commonly applied for the standardization of methodology. The comparative data for % related impurities of repeatability and intermediate precision are shown in **Table 6**.

Table 6: Comparative data of repeatability (REP) and intermediate precision (IP) in spiked test (0.15% spiked of respective impurities) (continue…)

Test	Impurity concentrations in %							
	% of A		% of B		% of C		% of D	
	REP	IP	REP	IP	REP	IP	REP	IP
Spiked Test- 1	0.153	0.152	0.150	0.153	0.154	0.151	0.156	0.147
Spiked Test- 2	0.152	0.153	0.149	0.155	0.154	0.154	0.156	0.145
Spiked Test- 3	0.153	0.152	0.151	0.152	0.154	0.156	0.156	0.144
Spiked Test-4	0.152	0.153	0.150	0.153	0.153	0.153	0.155	0.143
Spiked Test- 5	0.154	0.151	0.152	0.152	0.153	0.154	0.157	0.147
Spiked Test- 6	0.153	0.153	0.151	0.153	0.154	0.153	0.156	0.143
Mean (n=6)	**0.153**	**0.152**	**0.151**	**0.153**	**0.154**	**0.154**	**0.156**	**0.145**
SD (n=6)	0.001	0.001	0.001	0.011	0.001	0.002	0.001	0.002
% RSD	**0.52**	**0.53**	**0.66**	**0.72**	**0.32**	**1.04**	**0.38**	**1.24**

(n=6)				
Mean (n=12)	**0.153**	**0.152**	**0.154**	**0.150**
SD (n=12)	0.002	0.002	0.001	0.006
%RSD (n=12)	**0.52**	**1.12**	**0.78**	**4.00**

Table 6: Comparative data of repeatability (REP) and intermediate precision (IP) in spiked test (0.15% spiked of respective impurities) (continued)

Test	Impurity concentrations in %							
	% of E		% of F		% of G		% of H	
	REP	IP	REP	IP	REP	IP	REP	IP
Spiked Test- 1	0.149	0.148	0.149	0.151	0.155	0.157	0.151	0.149
Spiked Test- 2	0.148	0.149	0.149	0.151	0.153	0.156	0.149	0.148
Spiked Test- 3	0.150	0.150	0.150	0.152	0.154	0.158	0.151	0.148
Spiked Test-4	0.149	0.152	0.149	0.153	0.154	0.159	0.150	0.154
Spiked Test- 5	0.151	0.151	0.150	0.153	0.154	0.157	0.152	0.151
Spiked Test- 6	0.149	0.151	0.150	0.156	0.155	0.161	0.151	0.150
Mean (n=6)	**0.149**	**0.150**	**0.150**	**0.153**	**0.154**	**0.158**	**0.151**	**0.150**
SD (n=6)	0.001	0.002	0.001	0.002	0.001	0.002	0.001	0.002
% RSD (n=6)	**0.67**	**1.00**	**0.33**	**1.24**	**0.52**	**1.14**	**0.66**	**1.53**
Mean (n=12)	**0.150**		**0.151**		**0.156**		**0.150**	
SD (n=12)	0.001		0.001		0.002		0.002	
%RSD (n=12)	**0.87**		**0.53**		**1.54**		**1.13**	

Accuracy:

The accuracy of an analytical method is the extent to which test results generated by the method and the true value agree. Accuracy can also be described as the closeness of agreement between the value that is true and value found. For the quantitative approaches, at least nine determinations across the specified range should be obtained (three replicates at three concentration levels each). Accuracy study of impurities was carried out triplicate at its LOQ level, 50 %, 100 %, and 150 % of the specification level in the test preparation. The % accuracy for known impurities well within acceptance criteria. The average % accuracy and its standard deviation of known impurities from its LOQ level, 50 %, 100 %, and 150 % of the specification level given in **Table 7**.

Table 7: %Accuracy data of related impurities of TMB·HCl

Impurities	LOQ Level (n=3)	50 % Level (n=3)	100 % Level (n=3)	150 % Level (n=3)
A	106.67±0.00	101.33±0.00	101.78±0.37	101.93±0.25
B	102.22±3.85	100.00±1.33	100.00±0.67	100.00±0.44
C	104.44±1.92	102.22±0.77	102.90±0.40	103.13±0.45
D	100.00±0.00	104.96±0.79	105.17±0.41	105.98±0.26
E	104.60±1.99	101.80±0.78	102.04±0.68	102.11±0.26
F	102.22±3.85	101.35±0.00	101.59±0.39	102.11±0.26
G	102.22±1.92	102.67±0.00	102.21±0.38	101.92±0.51
H	111.11±3.84	100.44±0.77	100.89±0.77	101.19±0.68

Solution Stability:

The stability of test prepared solution was performed at the room temperature on the day basis up to 3 days. The % recovery of known impurities was calculated for the study period of test preparation. Cumulative % RSD values of all known impurities are within acceptance criteria. This indicates that the test preparations are stable up to 3 days, when stored at room temperature.

Robustness:

In liquid chromarographic analysis, the method parameters such as, flow rate of mobile, column oven temperature, different serial number column etc plays vital role for system suitability parameter (tailing factor, theoretical plates, resolution etc). In future, avoid such types of reflections, it needs to performed the robustness study. In the robustness study, purposely altering experimental condition such as flow rate of mobile phase, change in the column oven temperature and also using the different lot of column. Considering all extreme possible variation in flow rate as well as column oven temperature. it is decided that, robustness study performed by changed by flow rate ±10 % of its actual flow rate given in method. The actual flow rate of mobile phase is 0.40 mL/min, it is altered as 0.36 mL/min and 0.44 mL/min. The column oven temperature is changed with ±5°C from 40°C in the original method it altered as 45°C and 35°C. The intermediate precision data performed on different lot number of column and shows no variation in the results. In all above cases, the retention times are varied by ±0.2 mins compared to actual retention times. In all deliberate varied chromatographic conditions (flow rate, column oven temperature and different lot number of column), significant change are not observed for the system suitability criteria like tailing factor, theoretical plates and % RSD. The values of these criteria are well within acceptable limits. The overall mean and %RSD of n= 8 test preparation (6 from repeatability and 2 from robustness) are given in **Table 8.**

Table 8: The overall mean ($\bar{x}$) and % RSD of repeatability and robustness study in spiked test preparations (n=8)*

Impurities	Change in flow rate				Change in column oven temperature			
	0.44 mL/min		0.36 mL/min		45 °C		35 °C	
	$\bar{x}$	% RSD	$\bar{x}$	% RSD	$\bar{x}$	% RSD	$\bar{x}$	% RSD
A	0.152	1.25	0.152	1.38	0.152	0.79	0.152	0.86
B	0.150	1.20	0.149	1.81	0.149	1.54	0.150	1.40
C	0.153	1.31	0.152	1.91	0.153	1.31	0.153	1.05
D	0.155	1.10	0.154	2.01	0.155	1.55	0.154	2.01
E	0.149	0.81	0.148	1.62	0.148	1.35	0.149	0.67
F	0.149	0.47	0.148	1.76	0.150	0.33	0.149	0.47
G	0.153	1.37	0.153	1.96	0.153	1.76	0.153	1.31
H	0.150	1.10	0.149	2.08	0.150	1.13	0.150	1.27
Total Impurities	1.254	1.05	1.249	1.80	1.253	1.28	1.254	1.12

***** 6 spiked tests from repeatability study and 2 from robusrness study taken for comparison

Forced degradation Studies:
Forced degradation [13-15] study is helpful to selection of stability-indicating analytical method. The degradation studies carried out for thermal, photolytic, humidity, aqueous, acidic, basic and oxidative stress conditions. The TMB. HCl sample is subjected to the thermal, photolytic and humidity stress conditions, while for acid, alkali and oxidation degradation studies 50 mg of TMB. HCl is dissolved in 5.0 mL of diluent is exposed to respective stress conditions. The degradation data under these conditions are shown in **Table 9.**
In photolytic degradation the test samples were exposed to near UV light of 200 W·h·m^{-2} intensity till the energy of 1.2 x 10^6 lux h. The humidity degradation carried out with 75% relative humidity at 40 °C for 24 h, while in the thermal degradation test sample was heated to 105 °C for
24 h. All above tests are prepared by using similar concentration (1000 ppm) of the present analytical method of TMB·HCl. During heat, humidity and photolytic stress condition study, no any physical as well as chemicals changes was observed. The observed peak area of the TMB·HCl remained constant without any degradient peak, which indicates that this molecule is stable to heat, humidity and photolytic stress.

Table 9: The stress conditions with % assay and impurities degradation of TMB·HCl

Stress Condition	Exposure period	% Assay*	% Impurities degradation	Major degradants	Mass balance
Untreated Test Preparation	-	99.52	---	---	Pass
Humidity degradation	40 °C, 75% RH for 24 h	99.53	---	---	Pass
Thermal degradation	105 °C for 24 h	99.45	---	---	Pass
Photolytic degradation	Light energy of 1.2 million lux hours and near UV 200-watt hrs./m^2	100.28	---	---	Pass
Aqueous degradation	10.0 ml 24 h at room temperature.	99.48	---	---	Pass
Acid Degradation	4.0 ml 5.0 N HCl kept the solution for 24 h at 85 °C in Oil bath.	84.19	17.06	Impurity B (8.03 %), H (5.41 %) and D (2.13 %)	101.25
Alkali Degradation	5.0 ml 1.0 N NaOH kept the solution for 24 h at 85°C in Oil bath.	94.50	7.46	Impuity-B (3.01 %)	101.95
Oxidation degradation	10.0 ml 50 % H_2O_2 kept the solution for 1 h at 85°C in Oil bath.	86.87	11.84	Impurity-F (4.20 %)	98.71

* The values of TMB.HCl assay are taken from reference 16.

The chromatograms of the acid, alkali and oxidation degradations are depicted in the **Figure 4 and Figure 5.** All known, as well as degradients impurities are well separated from the TMB·HCl peak and shows the peak purity criteria. The oxidation degradation carried out by addition 10.0 mL of 50 % H_2O_2 to the test solution and heating it in an oil bath at 85 °C for 1 h. The TMB is degraded by 11.14 % majorly to impurity F (4.20 %) (which is the N-oxide analogue of the TMB. The alkali degradation was performed by adding 5.0 mL of 1.0 N NaOH to test sample to 85 °C in oil bath for 24 h. after completion of time, solution removed from oil bath, cooled, as well as neutralized with HCl and diluted to the mark. The total 7.46 % degradation of TMB is observed. The major degradient are the impurities B (3.01 %) and D (0.99 %) (see **Figure 4**). The impurities G, E, and F are minor degradients. Apart from it the 2/3 unknown degradient are observed in alkali degradiation at 5.59, 6.25 and 11.84 min. In acid degradation, test sample was exposed to 4.0 mL of 5.0 N HCl at 85°C in oil bath

for 24 h. After 24 h solution was removed from oil bath, cooled and neutralized with NaOH. The maximum degradation (17.06 %) of TMB is observed in acid degradation compared to all other stress conditions. The major degradants in this case are impurity B (8.03%), H (5.41 %) and D (2.13 %) (see **Figure 5**), due to the acid hydrolysis of the amide C-N bond in the TMB.HCl.

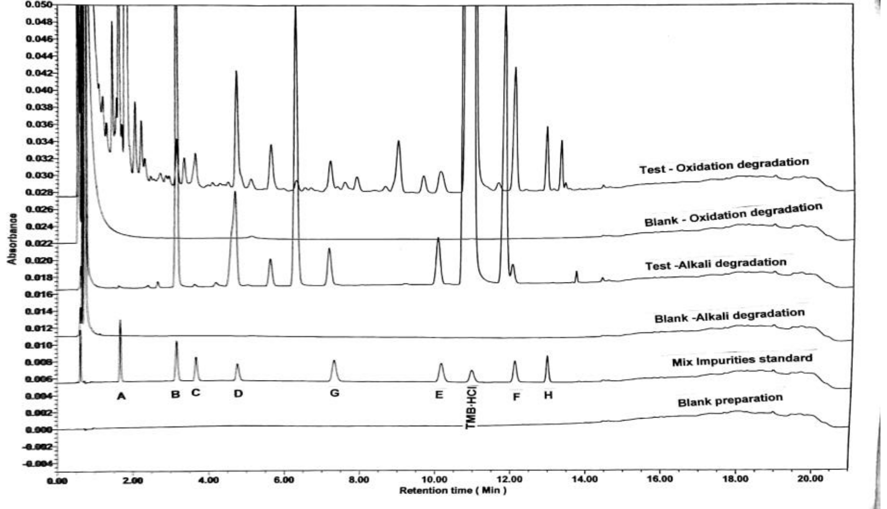

Figure 4: Chromatogram for the alkali and oxidation degradation studies of TMB·HCl

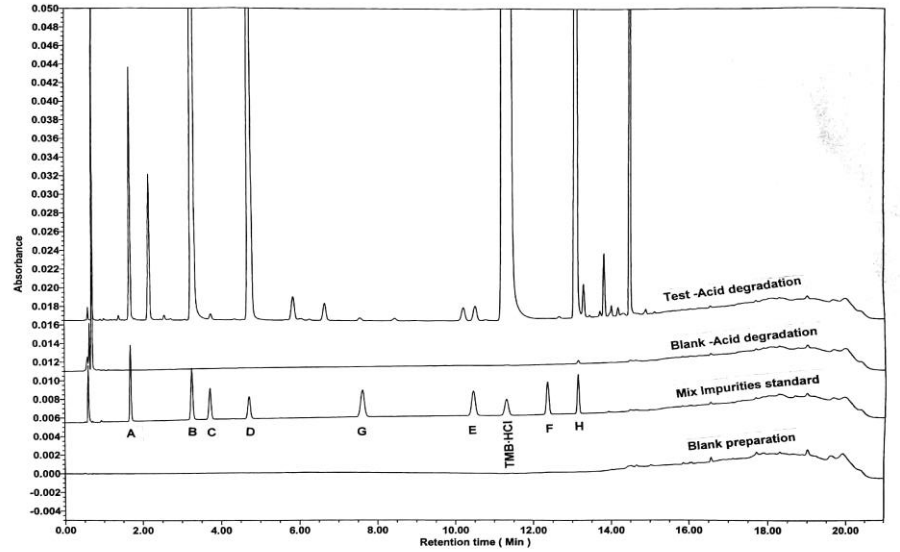

Figure 5: Chromatogram for the acid degradation studies of TMB·HCl

Maas balance study:

For the determination of mass balance of impurities in the assay and related substances the data of forced degradation study are used. The % impurity contents were determined in related substances method as well as determined the % assay by quantitative method. All the degraded samples the sum of % total impurities and % assay was found to be between 90 to 110 %. The evaluation of the forced degradation data indicates that the increase in known and unknown impurities observed in related substances method is verified by a corresponding reduction in drug content in quantitative method. The observed results are shown in **Table 9.** The % Assay values adapted from our reported method for quantitative analysis of TMB·HCl [16]. Thus, mass balance was achieved in all of the stress conditions studied.

CONCLUSION:

A highly accurate, linear and stability indicating RP-UPLC method for the related substances analysis of antiemetic TMB·HCl an API is developed and successfully validated as per the ICH guidelines Q2(R1). The specificity shows that, TMB peak is well resolved

from known as well as unknown impurities. The method is linear with correlation coefficient of all known impurities being greater than 0.999. Robustness studies do not show any significant change in the system suitability criteria like tailing factor, theoretical plates and % RSD. The values of these criteria are well within acceptable limits. The heat, humidity and photolytic stress condition have not shown any change in the physical appearance of the sample and the peak area of the TMB·HCI, indicating its stability for these stress conditions. In case of acid, alkali and oxidation degradations all known as well as impurities are well separated from the TMB·HCI peak and the peak purity criteria are also passed. Alkali degradation resulted in impurities B and D as major degradants, while in oxidation degradation only impurity F is formed. Similarly, acid degradation resulted in impurities B, H and D. The mass balance values for stress condition are within the criteria. The method was completely validated shown satisfactory data for all the tested method parameters. The present method is specific, linear, precise, selective, robust, as well as stable and can be used for the routine analysis in quality control.

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