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A VALIDATED RAPID RP-UHPLC METHOD FOR DETERMINATION OF ASSAY AND RELATED SUBSTANCES IN TTBB

L. Vaikunta Rao[1]*, K. Tirumala Rao[2] and KVV. Krishna Mohan[3]

[1]Department of Chemistry, GITAM Institute of Science,
GITAM (Deemed to be) University,
Visakhapatnam-530 045, Andhra Pradesh, India,
[2]Analytical Research, Product and Technology,
Development, ecoLogic Technologies Limited,
Hyderabad-500 072, Telangana, India.
[3]Process Research, Product and Technology,
Development, ecoLogic Technologies Limited,
Hyderabad-500 072, Telangana, India.

ABSTRACT

A simple eco-friendly linear gradient liquid chromatographic method (RP-UHPLC) is recommended for the simultaneous determination of assay and its related compounds in methyl 5-(4'-(bromomethyl)-[1,1'-biphenyl]-2-yl)-1-trityl-1H-tetrazole (TTBB) in commercial bulk samples. The chromatographic separation is achieved on Zorbax Eclipse Plus C18 RRLC column (100×4.6 mm; 3.5 µm) and eluent A used as 0.05% v/v ortho phosphoric acid and eluent B used as acetonitrile using Agilent UHPLC system. The mobile phase flow rate was set at 1.0 mL/min and the eluted peaks were monitored at 220 nm for related substance and assay method. The adequate separation was achieved between TTBB and its related impurities and those were eluted within 16 min of the chromatographic run time. The enactment of the method is validated according to the present ICH guidelines for specificity, limit of detection, limit of quantification, linearity, accuracy, precision, and robustness. Repeatability, intra, and inter-day precision results were well within the tolerable limits. Limits of detection were found to be 0.084, 0.084 and 0.085 ppm and limits of quantitation as 0.251, 0.253 and 0.256 ppm for TTBB, TTMB and Dibromo TTBB respectively. The correlation coefficient of linearity for TTBB and its impurities were found to be ≥0.999. The related substances method recoveries were found between 80 and 120 % and also assay method recovery was found between 98.0 to 102.0%. Peak homogeneity data for TTBB and its related compounds is indicates that specificity of method.

Keywords: UHPLC, TTBB; Related substances; Assay and Method Development.

INTRODUCTION

TTBB is chemically, methyl 5-(4'-(bromomethyl)-[1,1'-biphenyl]-2-yl)-1-trityl-1H-tetrazole (TTBB) and structure of molecule given in Figure 1, Molecular weight 557.50 and CAS number of compound is 124750-51-2[1]. Commercially synthesis of this compound is mentioned from 5-[2-(4-methylphenyl) phenyl]-1-trityltetrazole (TTMB) (CAS No. 124750-53-4) and the route of synthesis is mentioned in Figure 2. Elevated blood pressure is one of the most important causes of death and disability worldwide, accounting for 7.6 million premature deaths and 92 million disability-adjusted life years annually[2]. Controlling blood pressure and prevention of its complications such as coronary heart disease, stroke, renal failure and eye damage are the main objectives for the treatment of hypertension[3,4]. This compound is used for preparation of few AT 1 receptor antagonists of Active Pharma Ingredient (API) sartans and these API are

commonly used for angiotensin II receptor antagonist, acting on the AT1 subtype & used for treatment of high blood pressure, of congestive heart failure (CHF), and post-myocardial infarction (MI). By blocking the action of angiotensin, sartan dilates blood vessels and reduces blood pressure, the drug works by inhibiting effects of angiotensin II, a potent vasoconstrictor and one of the key contributors to cardiovascular and renal disease[5-10]. Sartan contains a biphenyl moiety substituted with an imidazol-1-ylmethyl and a tetrazol-5-yl group at C-4 and C-2′, respectively. Aside from OM, four other sartans used in clinical practice, *i.e.*, losartan, valsartan, irbesartan and candesartan, are 5-(biphenyl-2-yl)tetrazole derivatives. [2′-(*N*-Triphenylmethyltetrazol-5-yl)biphenyl-4-yl]methyl bromide is a common intermediate used in the synthesis of these sartan drugs i.e., Irbesartan, Losartan, Valsartan and Olmesartan (Structure of API were shown in Figure 3). Despite its wide synthetic application, the available literature data and chemical databases are not in agreement on its chemical structure, giving formulas of the two bromides that differ substitution with trityl group at tetrazole *N*-1 (CAS No. 124750-51-2) and *N*-2 (CAS No. 133051-88-4). The protection and deprotection of the tetrazole *N*-atom are essential steps during the synthesis of the sartan drugs containing 5-(biphenyl-2-yl)tetrazole moiety, and are often accompanied by side reactions and process-related impurities formation, influencing the high quality of the final active pharmaceutical ingredient (API). The available literature data indicate that tetrazolic acids of sartans, formed as a result of undesired tetrazole *N*-atom deprotection from trityl group, may exist as mixtures of 1*H*- and 2*H*-tautomeric forms that subsequently undergo adverse alkylation reactions to afford mixtures of *N*-1 and *N*-2-alkyl region isomeric impurities[11, 12].

As this kind ofintermediates in active pharmaceutical ingredient (API) synthesis often afford numerous impurities affecting the quality of the final drug product, their structure explanation is essential for impurities identification, characterization and quantification, not only for an API, but also of its key synthetic intermediates, has recently become a requirement of both the U.S. FDA and the European Medicine Agency (EMA)[13-18] and these regulatory bodies are keenly reviewing integrity of the manufacturing process and very cautious about the chemistry and manufacturing controls on corresponding intermediate, raw materials to show the process capability and also method capability. Therefore it was very necessary to have a suitable method for the impurity identification and quantification and also potency of the compound of such key intermediates used in the process.

During literature review majorly methods were reported HPLC assay, LC-MS, UV & CE on respective API compounds[19-26] and unfortunately very few intermediate methods were HPLC methods were reported for the public interest, but research scientists are spending more time for method development on very key intermediate/starting material compounds. Therefore it is essential to have a suitable method for the intermediates/starting materials quality which are used in drugs preparation. Hence, the research work is carried out on one of key intermediate compound method development and validation as per ICH[27] for the determination of assay and its related compounds.

EXPERIMENTAL

MATERIALS

Working in-house reference standard of TTBB and related impurities (shown in Figure 4) were synthesized and further confirmation was performed by using spectral techniques (NMR, MASS, IR & UV) and samples are obtained from ecoLogic Technologies Limited (Hyderabad, India) for this entire study. Gradient grade acetonitrile R1, Orthophosphoric acid HPLC grade (Merck, India) was used and HPLC grade water was obtained by Millipore water purification system.

Equipment

The ultra-high performance liquid chromatographic method (UHPLC) system was used model of Agilent 1260 Infinity series with a diode array detector (quaternary pump: G1311B, column thermostat: G1316B, Auto sampler with cooler: G1329B & G1330B and detector: G4212B) for method development, validation experiments and the chromatographic data was recorded, peak purity of the TTBB was tested by using Chemstation DAD software (Agilent Technologies, Clara, US). A column Zorbax Eclipse Plus C18 100×4.6 mm; 3.5 µm RRLC column manufactured by Agilent (Agilent Technologies, Clara, US) was procured from LCGC India.

METHODS

Optimized Chromatographic conditions

The chromatographic separations were achieved on Zorbax Eclipse Plus C18 UHPLC/RRLC column (100×4.6 mm; 3.5 µm) using mobile phase A as 0.05% *v/v* ortho phosphoric acid and mobile phase B as acetonitrile with a linear gradient programme :

time (min)/% B is 0/40, 5/90, 11/90, 11.1/40 and 16/40 at flow rate of 1.0 mL/min with UV detection of 220 nm at column temperature was maintained at 25 °C using 5.0 µL injection volume.

Preparation of Solutions

Preparation of standard solution

TTBB Standard solution is prepared about 500 µg/mL solution for the assay and the same stock solutions are used for related compounds estimation by diluting known concentration.

Preparation of impurities stock solutions

TTBB related compound stock solutions were prepared about 100 µg/mL solution for the related compounds estimation.

Preparation of sample solutions

Sample solutions were prepared about 500 µg/mL solution for the assay & related compounds estimation.

Preparation of spike sample solutions

Spike sample solutions were prepared by spiking impurities about 2.5µg/mL for related compounds for recovery and specificity experiments.

Validation parameters

Specificity/Selectivity

Specificity of the method was evaluated by injecting the blank, individual related compounds and sample solution prepared by spiking related compounds of TTBB at 0.5% level of test concentration and injected into UHPLC system to check the co elution between peaks and the system suitability test evaluation.

Precision

For related compound method six solutions containing TTBB (500 µg/mL) were spiked with related compounds solutions 2.5 µg/mL (0.50% of TTBB concentration). Chromatography was performed and value of %RSD was calculated considering peak area for TTBB and each related compound. Similarly, intermediate precision of the method was also evaluated by another analyst, on a different day in the same laboratory. For assay method six individual sample solutions were prepared TTBB (500 µg/mL) and calculated assay of the compound against standard solution and also checked %RSD for assay values for six determinations. Similarly, intermediate precision of the method was also evaluated by another analyst, on a different day in the same laboratory.

Limit of detection and Limit of quantitation

The limit of detection (LOD) and limit of quantitation (LOQ) for TTBB and related compounds were determined. Precision study was also carried out at the LOQ level by injecting six (n=6) individual preparations and calculating the %RSD considering peak area for TTBB and each related compound.

Linearity

For the related compound determination method linearity was checked for related compound and TTBB at lower concentration levels 0.05% to 1.0% (i.e., 0.25 µg/mL to 5 µg/mL). The responses were measured as peak areas and plotted against concentration.

The similar experiment was performed for assay method linearity by preparing the standard concentrations 80% to 120% at assay concentration level (i.e., 400 µg/mL to 600 µg/mL). The calibration curve was drawn by plotting the each impurity peak area versus its corresponding concentration. The correlation co-efficient, slope and Y-intercept for each impurity was determined.

Accuracy

The accuracy of the assay method was evaluated in triplicate (n=3) at the concentration levels of TTBB 400, 500 and 600 µg/mL (80%, 100% and 120%) and the % recovery was calculated at each level. Similarly accuracy of the related substances method evaluated in triplicate (n=3) at the concentration levels of each related compound 0.05%, 0.1%, 0.5% and 1% level and the % recovery was calculated for each related compound.

Stability of the solution

A sample solution of assay method and related substance method was checked at different time intervals up to 48 h by keeping solution at room temperature and checked cumulative %RSD for the peak area of TTBB and its related compounds.

RESULTS AND DISCUSSION

Method development

The TTBB compound UV spectrum was recorded using PDA detector and the maximum absorption at 220 nm, at the same wavelength monitored related impurities and product assay determination (UV spectra shown in Figure 5).

The main objective of the method is to develop a common chromatographic method for the estimation of assay and related compounds from TTBB samples. As TTBB is containing trityl protection and tetrazole ring and the compound would be in neutral nature therefore the development was tried in acidic condition

were attempted with Trifluoroacetic acid and orthophosphoric acid (OPA) on three C18 columns (Table 1). At these chromatographic conditions, resolution between TTMB and TTBB is more than 2.0 condition. But the trifluoro acetic acid condition base line noise is more and it can't be attained sensitivity for the related compounds. Therefore orthophosphoric acid mobile phase is chosen for the method optimization.

Specificity/Selectivity

There was no interference from the blank also from the related compounds at the retention time of TTBB and related compound peaks. The peak purity data shows the peaks are pure and there was no co eluting peak at the retention time of TTBB peak and related compound peaks. The system suitability test results are given in Table 2 & 3 and typical blank, system suitability and specificity chromatograms are given in Figure 6, 7 & 8.

Precision

The precision of the method for assay determination, the % RSD is showing below 0.45 and it was well within the acceptance value. For related compound determination method precision of the method, the %RSD is found to be less than 1.6%. The %RSD of TTBB assay results obtained in the intermediate precision was with in 1.0 and related substance method was found be less than 2 %.The experiments precision results are given in Table 4 & 5.

Limit of detection and Limit of quantitation

The LOD and LOQ for TTBB and related compounds were found be 0.08 and 0.25 µg/mL, precision of LOQ is shown below 5.1 and recovery at LOQ level is above 93.6%. The results are given in Table 5.

Linearity

Calibration curve obtained by least square regression analysis between peak areas versus concentration showed linear relationship with correlation coefficient of 0.9997 for TTBB and ≥ 0.9992 for related compounds respectively over the calibration ranges tested. The results are demonstrated that an excellent correlation between the peak area and concentration. The linearity results Table 5 & 6 and the obtained calibration curve was given in Figure 9 -12.

Accuracy

The accuracy of the assay method was determined in percentage recovery of TTBB from bulk drug samples ranged from 99.3% to 99.8%. The percentage recovery of the five impurities from bulk drug samples ranged from 91.5% to 96.5% and the results are shown in Table 7.

Solution stability

The RSD percentage of TTBB and its related compound peak areas were found be less than 1% and 5% respectively. The stability of TTBB sample solution by assay method and also related substances method was stable up to 24 h at room temperature.

CONCLUSION

The developed linear gradient RP-UHPLC method had excellent selectivity between impurities along with TTBB and the objective of method development is achieved on short RRLC column Zorbax Eclipse Plus C18 100 x 4.6 mm, 3.5 µm to reduce the run time of the method to achieve quick turnaround time (TAT) for the routine samples analysis, which was more economic and environment friendly to minimize the HPLC effluent waste. The developed method is showing all known related compounds and TTBB were eluted within 16 minutes. The present method was simple, linear gradient method and it was found to be specific, precise, linear and accurate based on method validation experimental data. This method can be successfully used for the quality determination of TTBB in commercial manufacturing batches and also it can be used for stability monitoring (Accelerated, Long term stability studies) in quality control laboratories.

ACKNOWLEDGEMENTS

The authors wish to thank the management of ecoLogic Technologies Limited for supporting this work. We also thank to colleagues of Analytical Research of ecoLogic Technologies Limited for their support.

Table 1: Method development trials

S.No.	Condition	Observation
1	Column: Lichrospher RP18 250 x 4.6 mm, 5 µm Mobile phase: 0.1% TFA in water and Acetonitrile (50:50 *v/v*) Flow rate: 1.5 mL/min, column temperature: 25 °C	Co-eluted with TTBB peak
2	Column: Lichrospher RP18 250 x 4.6 mm, 5 µm Mobile phase: 0.1% TFA in water and Acetonitrile (20:80 *v/v*) Flow rate: 1.5 mL/min, column temperature: 25 °C	Co-eluted with TTBB peak
3	Column: Zorbax Eclipse Plus C18,(100×4.6 mm; 3.5 µm) Mobile phase-A: 0.1% TFA in water Mobile phase-B: 100% Acetonitrile Flow rate: 1.0 mL/min, column temperature: 25°C Gradient programme (Time in min./%B) 0/40, 5/90, 11/90, 11.1/40 and 16/40	All impurities were eluted but the base line drift and noise due to this method sensitivity is not attained.
4.	Column: Zorbax Eclipse Plus C18,(100×4.6 mm; 3.5 µm) Mobile phase-A: 0.05%*v/v* OPA in water Mobile phase-B: 100% Acetonitrile Flow rate: 1.0 mL/min, column temperature: 25°C Gradient programme (Time in min./%B) 0/40, 5/90, 11/90, 11.1/40 and 16/40	Separation is very good and eluted within 16 min run time.

Table 2: System suitability test (SST) results

Name	Retention time (tR) in min	USP Resolution (Rs)	USP Theoretical plates (N)	USP Tailing factor (T)
TTBB	8.570	---	54000	1.12
TTMB	8.903	2.4	---	---
Dibromo TTBB	9.243	2.8	---	---

Table 3: Specificity results

Name	Retention time (tR) in min	USP Resolution (Rs)	USP Theoretical plates (N)	USP Tailing factor (T)
TTBB	8.570	---	54000	1.12
TTMB	8.903	2.4	---	---
Dibromo TTBB	9.243	2.8	---	---

Table 4: Precision results

Analyte/impurity	TTBB	TTMB	Dibromo TTB
Method precision #	0.18%	1.21%	1.48%
Intermediate precision #	0.45%	1.98%	2.01%
# Six determinations 0.5% level impurities with respect to analyte concentration (500 µg/mL) and 500 µg/mL for assay of TTBB.			

Table 5: LOD, LOQ and linear regression for related substances method

Parameter	TTBB	TTMB	Dibromo TTBB
LOD (µg/mL)	0.084	0.084	0.0853
LOQ (µg/mL)	0.251	0.253	0.256
Regression equation			
Slope (m)	356711	390955	342449
Intercept (c)	7568.9	-1532.1	12620
Correlation coefficient	0.9997	0.9996	0.9992
Y-intercept at 100% level	1.30%	1.40%	1.40%

Table 6: Linear regression

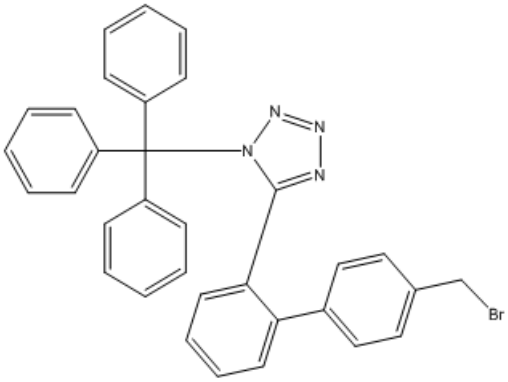
for assay method

Parameter	TTBB
Regression equation	Y=354068x-105833
Slope (m)	354068
Intercept (c)	-105833
Correlation coefficient	0.9997
Y-intercept at 100% level	0.06%

Table 7: Accuracy results

Name	% Level	Amount added	Amount found	% Recovery
TTMB [a]	LOQ	0.251	0.235	93.63
	0.10	0.502	0.478	95.22
	0.50	2.51	2.298	91.55
	1.00	5.02	4.658	92.79
Average % recovery	93.30			
Std dev.	1.54			
%RSD	**1.65**			
Name	**% Level**	**Amount added**	**Amount found**	**% Recovery**
Dibromo TTBB[a]	LOQ	0.256	0.247	96.48
	0.10	0.512	0.489	95.51
	0.50	2.56	2.397	93.63
	1.00	5.12	4.894	95.59
Average % recovery	95.30			
Std dev	1.20			
%RSD	**1.26**			
Name	**% Level**	**Amount added**	**Amount found**	**% Recovery**
TTBB[b]	80	398.5	397.8	99.82
	100	499.5	496.8	99.46
	120	598.5	594.5	99.33
Average % recovery	99.54			
Std dev.	0.26			
%RSD	**0.26**			

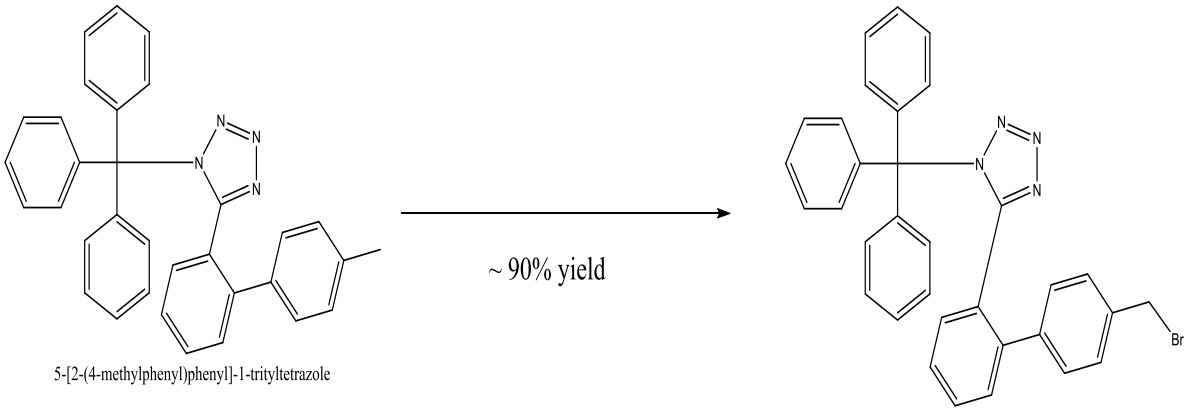
a – Impurity level,
b -Assay conc. level i.e., 500µg/mL



5-(4'-(bromomethyl)-[1,1'-biphenyl]-2-yl)-1-trityl-1*H*-tetrazole
Chemical Formula: $C_{33}H_{25}BrN_4$
Exact Mass: 556.13
Molecular Weight: 557.50

TTBB

Fig. 1: Chemical structures of TTBB



5-[2-(4-methylphenyl)phenyl]-1-trityltetrazole

5-(4'-(bromomethyl)-[1,1'-biphenyl]-2-yl)-1-trityl-1H-tetrazole

Reference:
WO2011/73703 A2, ; Page/Page column 6-7 ;

Fig. 2: Route of synthesis (ROS) for TTBB

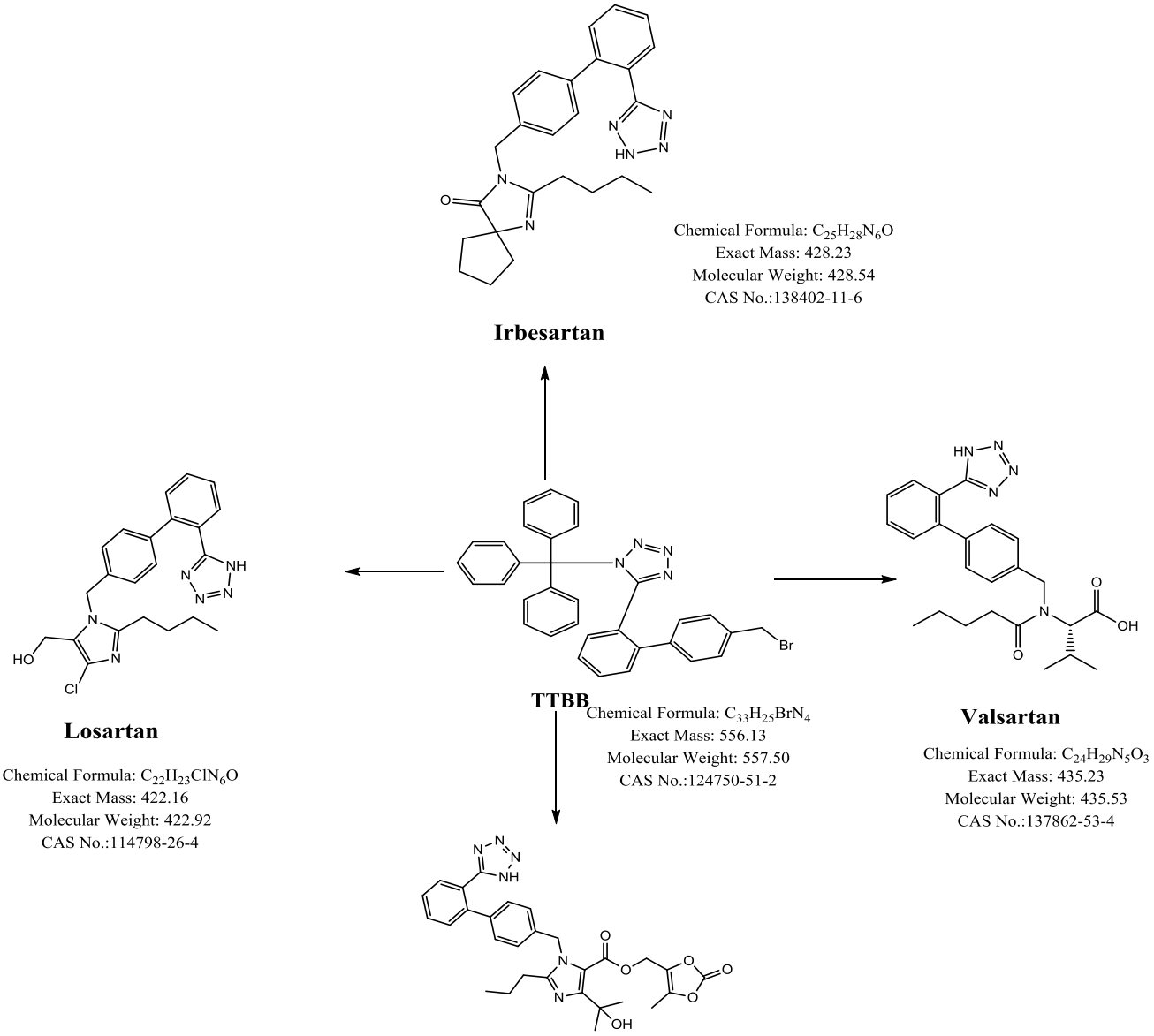
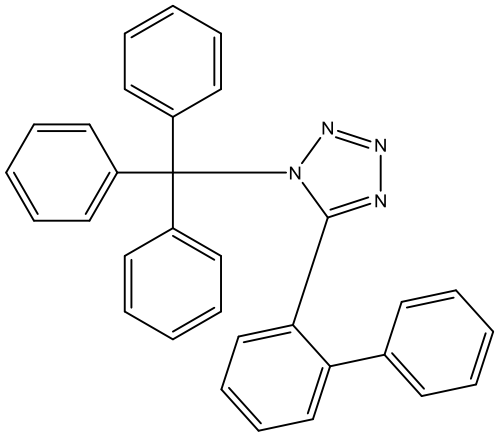
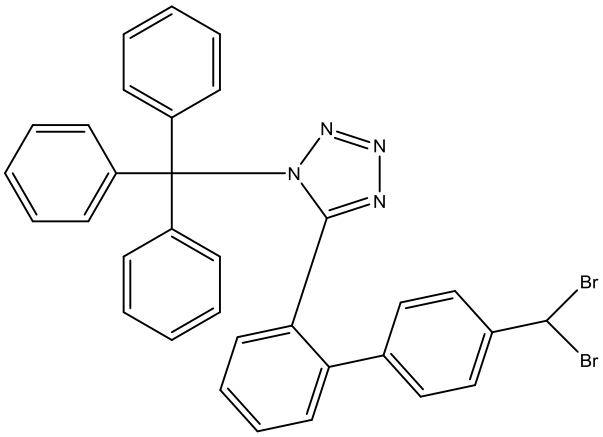


Fig. 3: TTBB used for API's



5-(4'-methyl-[1,1'-biphenyl]-2-yl)-1-trityl-1*H*-tetrazole
Chemical Formula: $C_{33}H_{26}N_4$
Exact Mass: 478.22
Molecular Weight: 478.60

TTMB



5-(4'-(dibromomethyl)-[1,1'-biphenyl]-2-yl)-1-trityl-1*H*-tetrazole
Chemical Formula: $C_{33}H_{24}Br_2N_4$
Exact Mass: 634.04
Molecular Weight: 636.39

Dibromo TTBB

Fig. 4: Chemical structures of related compounds of TTBB

Fig. 5: UV Spectrum of TTBB

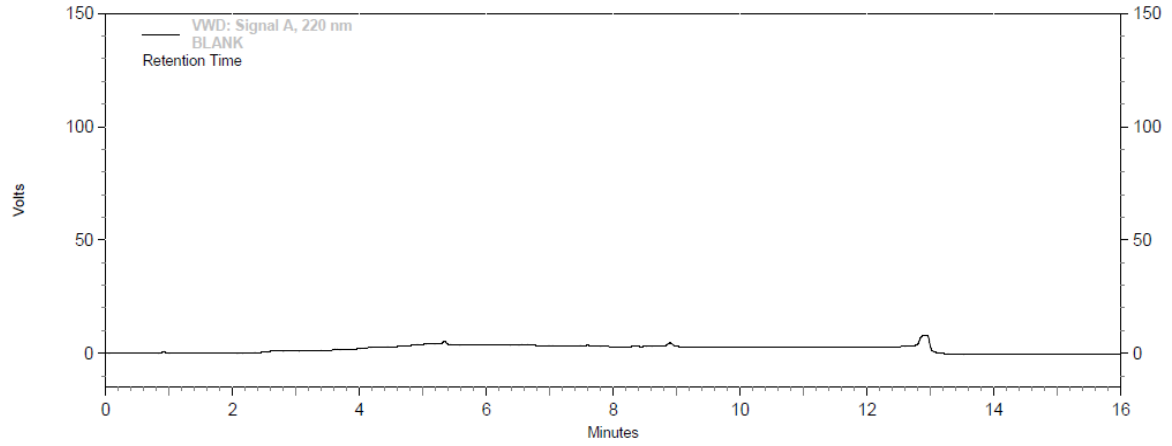


Fig. 6: Typical blank chromatogram

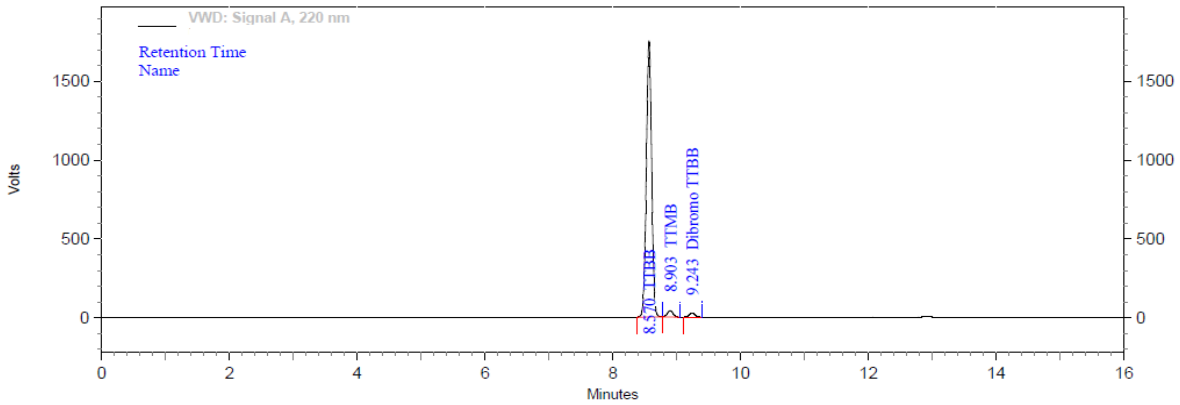


Fig. 7: System suitability chromatogram

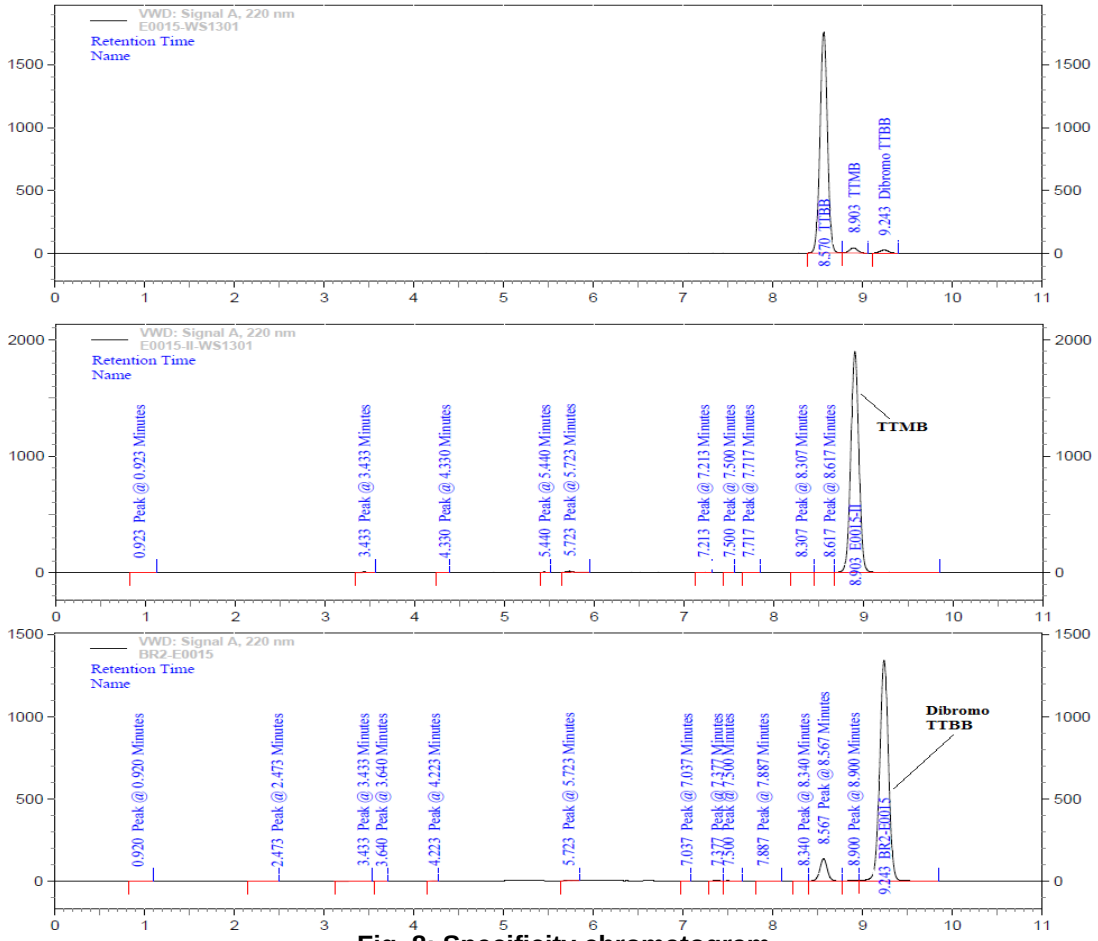


Fig. 8: Specificity chromatogram

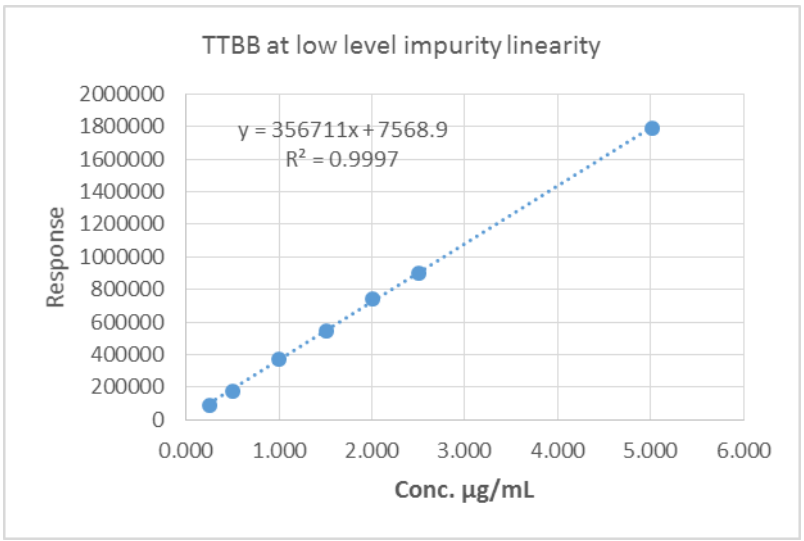


Fig. 9: Linearity plot of TTBB at low level

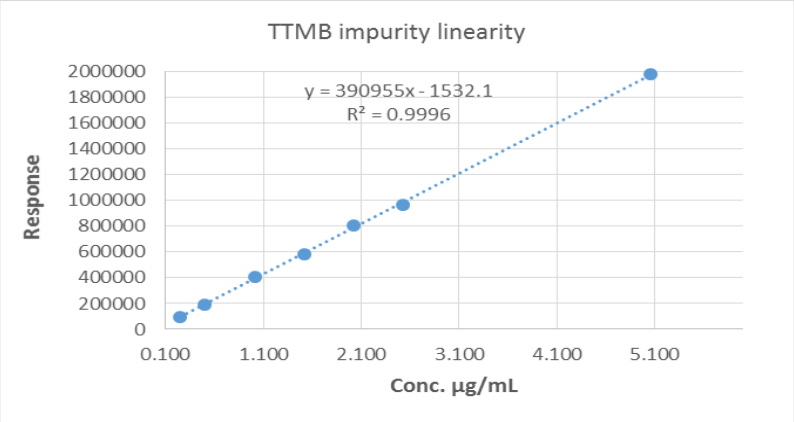


Fig. 10: Linearity plot of TTMB impurity

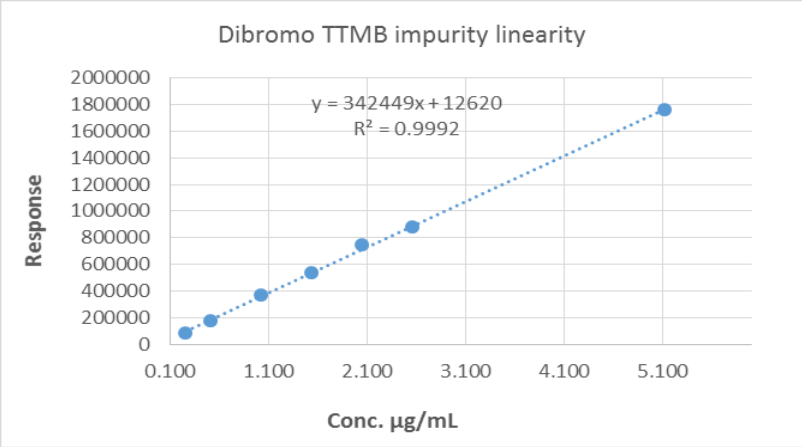


Fig. 11: Linearity plot of Dibromo TTBB impurity

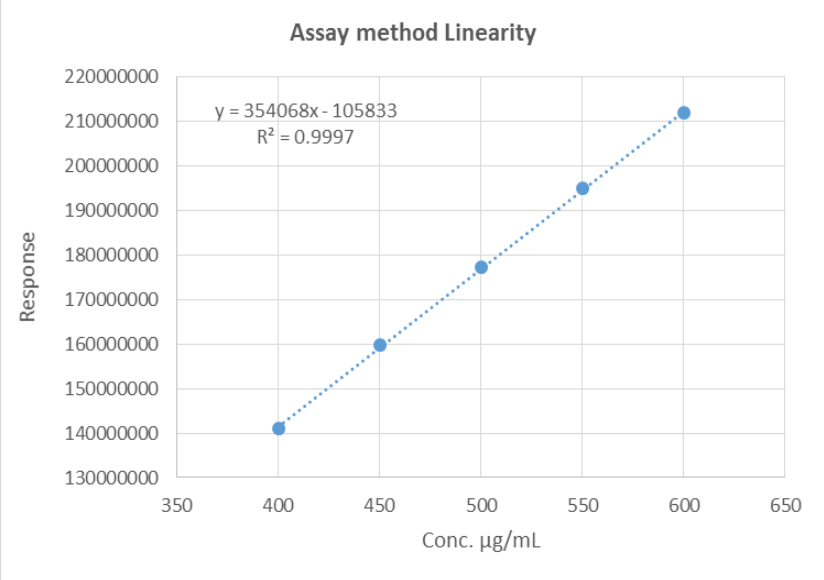


Fig. 12: Linearity plot of TTBB at assay conc. level

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