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Research Article

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A stability indicating method development and validation for determination of Methylphenidate Hydrochloride and its impurities in solid pharmaceutical oral dosage form by RP-HPLC as per ICH guidelines

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ABSTRACT

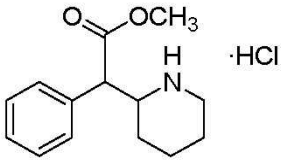
A simple, rapid, and precise, robust and rugged reverse phase high performance liquid chromatographic (RP-HPLC) method for analysis of Methylphenidate hydrochloride (MPH) and its Impurities in a tablet dosage form have been developed and validated. This method condition optimization was performed with HSSD waters symmetry C_{18} (100x 40 i.d., 3.7 µm particle column with Mobile phase Methanol: Acetonitrile: buffer (50:20:30v/v/v) and pH adjusted 4.0 with acetic acid at a flow rate of 1.5 ml/min. The eluted compounds were detected and monitored at 210 nm for Methylphenidate Hydrochloride (MPH) assay and 203 nm for related substances (RS) by PDA detector. By this method Methylphenidate Hydrochloride (MPH), Imp-A, Imp-B were eluted with retention times of 3.162, 2.349, and 2.792 min, respectively. Validation revealed the method is accurate, precise, reliable, and reproducible. Calibration curve plots were linear over the concentration ranges 0.1-2.0 µg/mL for Impurities and for MPH 500-1500 µg/. Limits of detection (LOD) for MPH, Imp-A, Imp-B were 0.03, 0.04, and 0.04µg/ml and limits of quantification (LOQ) were 0.1, 0.1, 0.1 µg/mL respectively. The statistical analysis proves the method is suitable for the analysis of MPH, Imp-A, Imp-B in bulk and tablet dosage form without any interference from the excipients. It was also proved study for degradation kinetics of the drug in tablet dosage form. The developed method separated MPH from its two known and two unknown impurities within 6.0 min. Methylphenidate Hydrochloride(MPH) was subjected to the stress conditions of oxidative, acid, base, hydrolytic, thermal and photolytic degradation. MPH was found to degrade significantly in base stress condition, degrade slightly in oxidative stress condition and remain stable in acid, hydrolytic, thermal and photolytic degradation conditions. All impurities were well resolved from each other and from the main peak, showing the stability-indicating power of the method. The developed method was validated as per International Conference on Harmonization (ICH) guidelines.

Keywords: Methylphenidate Hydrochloride (MPH), Impurities, RP-HPLC, Stability Indicating, Method Development, Validation, ICH guidelines.

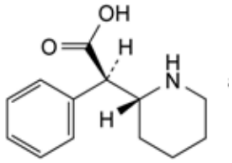
Methylphenidate Hydrochloride (MPH) is used for the treatment of attention deficit hyperactivity disorder (ADHD) and narcolepsy, the drug is a CNS stimulant and inhibits the reuptake of dopamine in the synapses [1, 2]. The use of MPH has increased considerably in recent years.

Methylphenidate hydrochloride (MPH) is used for the treatment of attention deficit hyperactivity disorder (ADHD) and it is a psycho-stimulant drug approved for the treatment of attention postural orthostatic tachycardia syndrome and narcolepsy but has also been `prescribed for the off-label treatment of other disorders such as lethargy. Methylphenidate is the most commonly prescribed psycho-stimulant and works by increasing the activity of central nervous system. [3, 4]Chemically, methylphenidate is methyl-2-phenyl-2-(piperidin-2-yl) acetate and its impurities A&B (Fig 1). It blocks dopamine uptake in central adrenergic neurons by blocking dopamine transport or carrier proteins. Methylphenidate acts at the brain stem arousal system and the cerebral cortex and causes increased

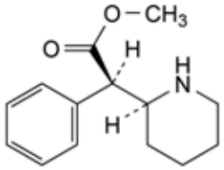
sympathomimetic activity in the central nervous system [4-6] **[**Fig1(A,B,C)] Chemical structure of Methylphenidate Hydrochloride and Its Impurities.



A)Methyl (2RS)-phenyl [(2SR)-piperidin-2-yl] acetate Hydrochloride (MPH)



B) (2RS)-phenyl [(2RS)-piperidine-2-yl] acetic acid (IMP-A)



C) Methyl (2RS)-phenyl [(2SR)-piperidin-2yl] acetate (IMP-B)

Some research studies have theorized that ADHD is caused by a dopamine imbalance in the brains of those affected. Methylphenidate is a nor epinephrine and dopamine reuptake inhibitor, which means that it increases the level of the dopamine neurotransmitter in the brain by partially blocking the dopamine transporter (DAT) that removes dopamine from the synapses. This inhibition of DAT blocks the reuptake of dopamine and nor epinephrine into the pre synaptic neuron, increasing the amount of dopamine in the synapse. [7, 8]

Literature survey revealed that only few analytical methods like spectrophotometric, RP-HPLC and LCMS methods [9-11] Gas-chromatographic and gas-chromatographic-mass spectrometric procedures recently described for quantitation of methylphenidate and ritalinic acid [12-16] require relatively large sample volumes (2-5 mL of plasma or serum) have been reported for the determination of methylphenidate hydrochloride. All these methods are expensive, time consuming, complex in nature. Consequently, there is no stability indicating method reported in the presence of known impurities and also from the unknown degradation products hence, there was still a need to develop a simple, less time-consuming and economical stability indicating method for the determination of methylphenidate hydrochloride in pharmaceutical oral dosage forms. Therefore, the attempt was made to develop a fast and reproducible RP-HPLC method for the estimation Methylphenidate in API and pharmaceutical solid oral dosage form by following ICH method validation guidelines. In the present work, we developed a simple, precise, accurate, selective and robust liquid chromatographic method for the determination of Methylphenidate hydrochloride in API and in solid Dosage forms for routine quality control.

EXPERIMENTAL SECTION

Apparatus:

The analysis was performed using waters-2695(Model alliance) High Performance liquid chromatography waters auto sampler–PDA detector 996 by using, EmpowerTM-3S-software version-3,analytical balance (Mettler Toledo) UV/Visible-Detector (Standard cell) and data handling system (Autochrome-3000), pH meter (lab India), Sonicator. The column used is HDSS waters symmetry C_{18} (100x 40 i.d., 3.7 µm) particle column.

Materials and Reagents:

Methanol (HPLC Grade), Acetonitrile (HPLC Grade), sodium acetate (AR Grade), acetic acid (AR Grade), pure drug samples of Methylphenidate Hydrochloride (MPH) and Impurity-A:(2(RS)-Phenyl(2RS)piperdine2-yl)aceticacid,Impurity-B:(methyl(2SR)-piperidine-2yl) acetate are obtained as gift samples from KDPL Pharmaceuticals Hyderabad.

Selection of wavelength by UV-Spectroscopy:
The maximum absorbance of MPH and Impurity-A, Impurity-B were found to be 210 and 203 nm respectively from the UV Visible spectrophotometer results and the detector used in eluent monitoring HPLC is PDA detector.

Preparation of sodium acetate buffer solution:
Dissolve 1.62 gms of sodium acetate in 800 ml of HPLC grade water and adjust to the pH 4.0 with acetic acid and dilute with HPLC grade water up to 1000ml.the buffer was filtered through 0.45µ nylon membrane filter and degassed.

Preparation of Mobile Phase:
The mobile phase is consisted a mixture of methanol, acetonitrile, and buffer in the ratio of 50:20:30 (v/v/v), pH is adjusted to 4.0 with acetic acid and filtered through 0.22 µm nylon membrane filter.

Diluent preparation:
Mobile phase is used as diluents

System suitability solution preparation
Suitability solution is prepared by dissolving standard substances in diluent to obtain solution containing 1000 µg/mL of MPH, 1 µg/mL of Imp-A and 1 µg/mL of Imp-B

Preparation of Placebo Solution:
Twenty tablets of placebo are crushed to fine powder. An accurately weighed portion of the powder equivalent to 25 mg of MPH is taken into 25 ml volumetric flask. About 20 ml of diluent is added to this volumetric flask and sonicated in an ultrasonic bath for 10 minutes. Dilute volume up to the mark and mixed well. It is then filtered through 0.22 µm PVDF syringe filter and the filtrate is collected after discarding first few milliliters.

Preparation of standard solution:
Standard solution are prepared by dissolving the drug MPH working standard in diluent to attain the solution containing 1000 µg/mL for assay and 1µg/mL for related substances.

Preparation of Sample Solution (Assay):
Sample solution is prepared by dissolving sample Brand name Ritalin Tablets 10 mg Novartis (twenty tablets are crushed to fine powder by mortar and pestle) in diluent to obtain the solution that containing 1000 µg/mL of MPH (for assay and related substances). It was then filtered through 0.22 µm PVDF syringe filter and the filtrate is collected after discarding first few milliliters.

Assay of Pharmaceutical Formulation:
Calculated the the quantity, in mg, of MPH in the portion of solid oral pharmaceutical formulation using the following formula:

$$\text{Assay (\%w/w)} = \frac{\text{Cstdx Rs x 10,000}}{\text{Cs x Rstd}}$$

Where,
Cstd= Concentration of standard solution in mg/ml.
Cs= Concentration of sample solution in mg/ml.
Rs= Compound peak response obtained from the sample preparation.
Rstd= Compound peak response (mean peak area) obtained from the standard preparation.

Percentage Impurity in Pharmaceutical Formulation:
For Calculating the % impurity present in the finished product formulation using the following formula:

$$\text{Impurity (\%w/w)} = \frac{\text{Cstdx Rs x 10,000x1}}{\text{Cs x R std x RRF}}$$

Where,
Cstd= Concentration of standard solution in mg/ml.
Cs= Concentration of sample solution in mg/ml.
Rs= Compound peak response obtained from the sample preparation.
Rstd= Compound peak response (mean peak area) obtained from the standard preparation.
RRF= Compound related response factor.

METHOD VALIDATION
The HPLC method was validate according to ICH Guidelines:

System suitability
System Related suitability parameters are analyzed and measured to verify the system is suitable for its performance. System precision parameter is determined on considering six replicate injections of standard solution preparation. All important characteristics features including % RSD, resolution (between MPH and Imp-B), theoretical plate number and tailing factor are measured.

Precision
The precision of the system related that is repeatability and reproducibility is determined using the sample preparation procedure as described in above for six same samples of similar solution of formulation and analysis using the same proposed method and one more is the Intermediate precision is studied by other analyst, using different columns, different HPLC, and is performed on different days.

Specificity:
Specificity is the ability to assess unequivocally the analyte in the presence of some components which may be expected to be present. Typically these might include degradants, impurities, matrix, etc. Specificity was determined by injecting a blank, placebo and also standard solution. No interference should be seen at the retention time of an analyte. The specificity has to demonstrate by induced degradation of MPH formulation and placebo samples to acid degradation, alkali degradation, water degradation, peroxide degradation, thermal degradation, and U.V. degradation.

Accuracy (Recovery)
For the confirming the recovery of the proposed method, recovery related experiments are carried out by considering the special standard addition technique for assay(MPH) and impurities(Impurity-A, Impurity-B) by addition technique for related substances. The accuracy of the assay method for MPH is evaluated in triplicate (n=3) at the five concentrations of 500, 750, 1000, 1250 and 1500 µg/mL (50, 75, 100, 125 and 150 %) of the finished dosage form product, and the recovery is calculated for each added (externally spiked) concentration. The mean of percentage recoveries (n=15) and the relative standard deviation are calculated. For all impurities, the recovery is determined in triplicate (n=3) for 0.5, 0.75, 1.0, 1.25 and 1.5 µg/mL (50, 75, 100, 125 and 150 %) of the analyte concentration (1000 µg/mL) of the drug finished product, and the recovery of the impurities is calculated. The mean of percentage recoveries (n=15) and the relative standard deviation are also calculated for related substances.

Linearity
For related impurities or substances test, the linearity is demonstrated from the parameter LOQ (0.1 µg/mL) to 2.0 µg/mL of std concentration of Impurities using a minimum of eight to nine calibration levels (LOQ, 0.2, 0.4, 0.6, 0.8, 1.0, 1.2, 1.5 and 2.0 µg/mL) for Imp-A and Imp-B. For assay test, linearity is demonstrated from 50% to 150% of standard concentration using a minimum of seven calibration levels (500,700, 800, 1000, 1200, 1400 and 1500 µg/mL) for MPH. The method of linear regression is used for data evaluation. The peak areas of the standard compounds are plotted against the respective MPH, Impurity-A and Impurity-B concentrations. Linearity is described by the linearity equation, correlation coefficient and Y-intercept bias is also determined.

Limit of detection (LOD) and limit of quantification (LOQ):
The detection limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be detected but not necessarily quantitated as an exact value. The quantitation limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be quantitatively determined with suitable precision and accuracy. Calculated the LOD & LOQ, with the calculations obtained from evaluation of the calibration curve of the linearity.LOD and LOQ values are less than the minimum linearity concentration.

Robustness
The robustness it is a measure of the capacity of a method to remain unaffected by small but deliberate changes in flow rate (± 0.05 mL/min), change in column oven temperature (± 5 °C) and change in organic solvent ratio (± 10%). These all most important characteristic including % assays, resolution (between MPH and Impurity-A, Impurity-B), tailing factor, theoretical plate's number and the retention behavior of the certain targeted compound are evaluated.

Solution stability
The stability of the sample solution is established by storage of the sample solution at ambient temperature for 24h. The sample solution is re-analyzed after 12 and 24h, and the results of the analysis are compared with the results of

the fresh sample. The stability of standard solution is established by the storage of the standard solution at ambient temperature for 24h. The standard solution is re-injected after 12, 24 and 48 hrs % RSD is calculated.

Filter compatibility

Filter compatibility is majorly performed for nylon 0.25 µm syringe filter (Whatman sciences) and PVDF 0.25 µm syringe filter (Millipore). To confirm the filter compatibility in proposed analytical method, filtration recovery experiment is carried out by sample filtration technique. Need Sample is mainly filtered via both syringe filters and percentage purity assay is determined and compared to against centrifuged sample.

RESULTS AND DISCUSSION

Method Development and Optimization

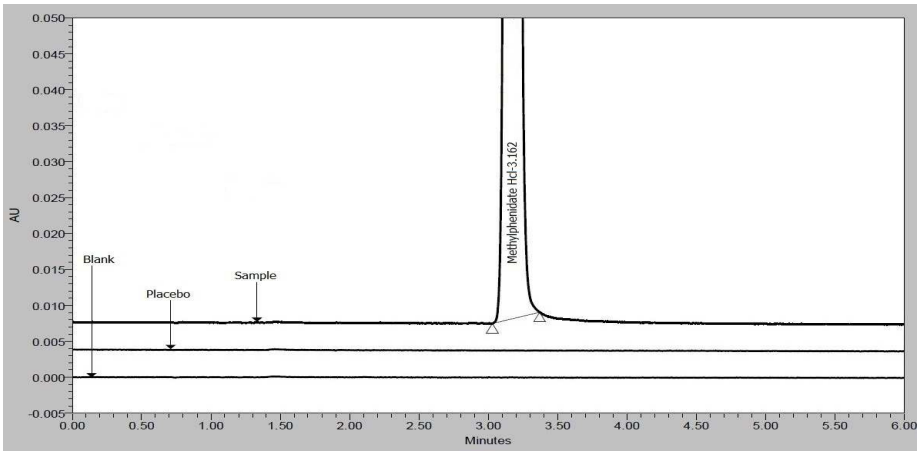
Selection of chromatographic conditions and Optimization of Mobile Phase:

The main objective of the RP-HPLC method development is to rapid assay and related substances determination of MPH in pharmaceutical formulation and the method should be able to determine assay (ASY) and related substances (RS) in single run and should be accurate, reproducible, robust, stability indicating, filter compatible, linear, free of interference from blank / placebo / impurities / degradation products and straightforward enough for routine use in quality control laboratory.

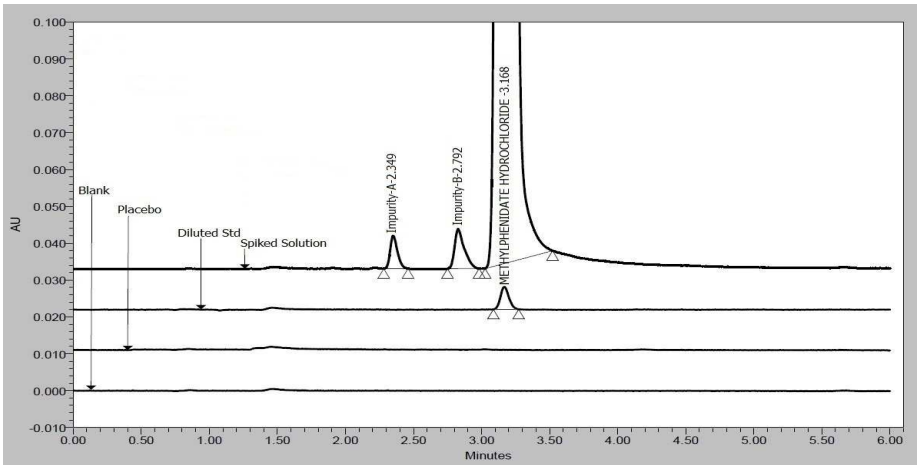
The spiked solution of MPH (1000 µg/mL), Impurity-A (1 µg/mL) and Impurity-B (1 µg/mL) is subjected to separation by RP-HPLC. Initially the separation of all compounds is studied using water as a mobile phase-A (MP-A) and acetonitrile (ACN) as a mobile phase-B (MP-B) on a BES C_{18} (100x 40 i.d., 5 µm) using a Waters (HPLC) system with the linear program. The flow rate of 1.5 mL/min is selected with regards to the backpressure and analysis time as well. Various types of MP-A and MP-B are studied to optimize the method, which are summarized with the associated observations in [Table 1].

Based on mobile phase selection experimental study, the optimized HPLC parameters are; flow rate1.5 mL/min; column oven temperature 20°C; mixture of water, ACN, MeOH and buffer in the ratio of 50:20:30: v/v/v respectively (pH adjusted 4.0 with Acetic acid). In order to achieve symmetrical peak shape of all substances and more resolution between MPH and Imp-B different stationary phases are explored. Peak merging (Imp-B and MPH) and broad peak shape of MPH is observed with an Acquity BHC C8 (100 x 2.1 mm, 4µm) column. Poor resolution (Imp-B and MPH) and broad peak shape of MPH is observed with an Acquity BHC C18 (100 x 2.1 mm, 5 µm) column.

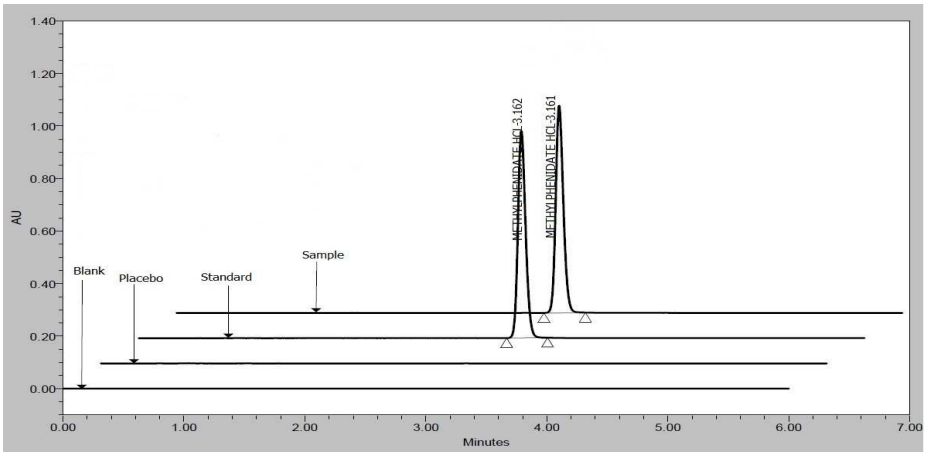
Finally the desired separation with symmetrical peaks is obtained using HSSD waters symmetry C_{18} (100x 40 i.d., 3.7 µm) column. Column oven temperature is also studied and found that 20°C is more appropriate with respect to separation and peak shape. Based on compounds UV response, 210nm (for assay MPH) and 203nm (for related substances) is found more appropriate for determination of MPH and its impurities from single run. MPH, Imp-A, and Imp-B are well resolved from each other and there is no chromatographic interference observed due to blank and placebo in a reasonable time of 6.0 minutes, optimized conditions and Overlaid specimen chromatograms of assay study are presented in [Figure 2-4] and [Table 2]



[Figure 2: Expanded overlaid specimen chromatograms of blank, placebo and sample (at 230nm)]



[Figure 3: Overlaid specimen chromatograms of blank, placebo, diluted standard and spiked impurities along with analyte (at 205nm)]



[Figure 4: Overlaid specimen chromatograms of assay study (at 230nm)]

Analytical Parameters and Validation

After satisfactory development of the method it is subjected to method validation as per ICH guideline [9]. The method is validated to demonstrate that it is suitable for its intended purpose by the standard procedure to evaluate adequate validation characteristics (system suitability, accuracy, precision, linearity, robustness, solution stability, filter compatibility and stability indicating capability).

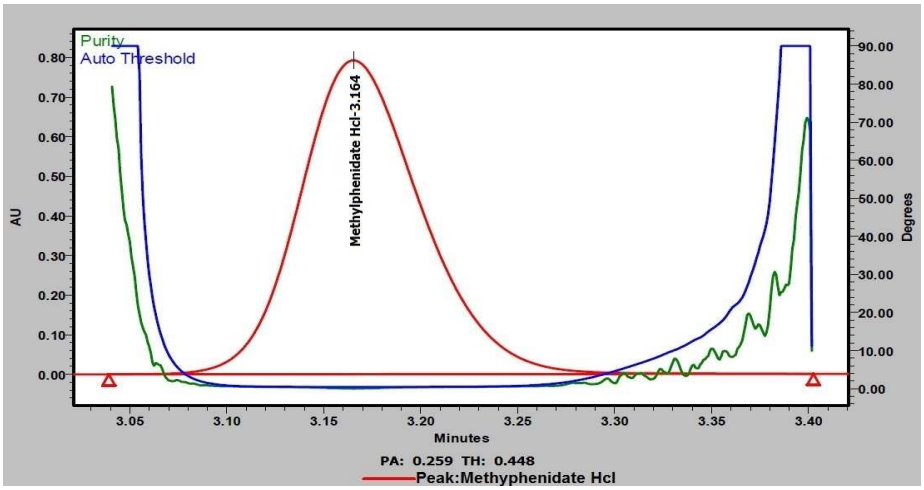
Specificity

Specificity is the ability of the method to measure the analyte response in the presence of its potential impurities. Forced degradation studies are performed to demonstrate selectivity and stability indicating capability of the proposed RP-HPLC method. [Figure 2,3] are show that there is no any interferences at the RT (retention time) of MPH due to blank, placebo and impurities. Stress studies are performed at concentration of 1000 µg/mL of MPH to provide the stability indicating property and specificity of the proposed method. Spectral purity of MPH, Imp- A and Imp-B are presented in [Figure 5-7]

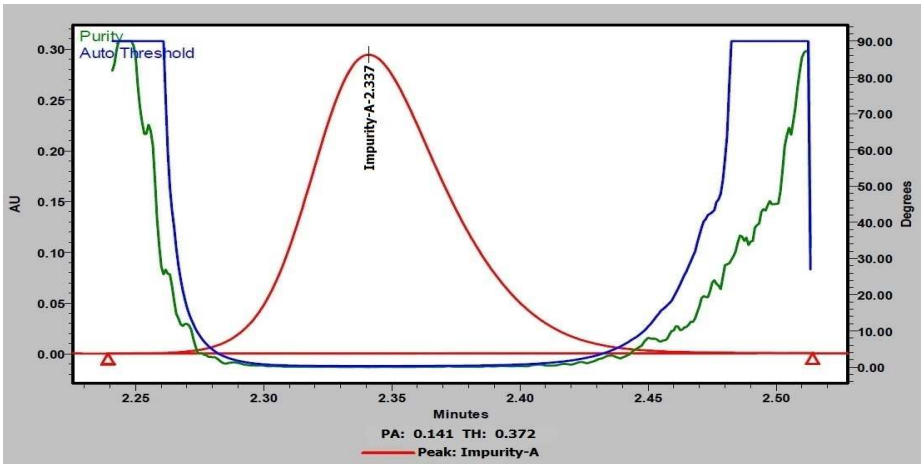
Forced degradation studies are performed by the stress conditions acid hydrolysis [1 N HCl (3 mL),60°C, 1h, Figure 8], base hydrolysis [1N NaOH (3 mL), 60°C, 30 min, Figure 9], oxidative [30% H2O2 (3 mL), 60°C, 1h, Figure 10], thermal [105°C, 6h, Figure 11], water hydrolysis [at 60 °C for 2h, Figure 12], and photolytic degradation [1.2 million Lux hours, Figure 13] to evaluate the ability of the proposed method to separate MPH from its degradation products. Minor degradation products are observed when MPH is subjected to acid, heat, photolytic and hydrolytic conditions.

Significant degradation is observed when the drug product is subjected to base hydrolysis, and slight degradation is observed when the drug product is subjected to oxidative hydrolysis. The purity of the peaks obtained from the stressed sample is verified using the PDA detector. The obtained purity angle is less than purity threshold for all the stressed samples. An assay of samples is performed by comparison with reference standards, and the mass balance [% assay + % known impurities + area % unknown impurities, at 205nm] for each of the stressed samples is

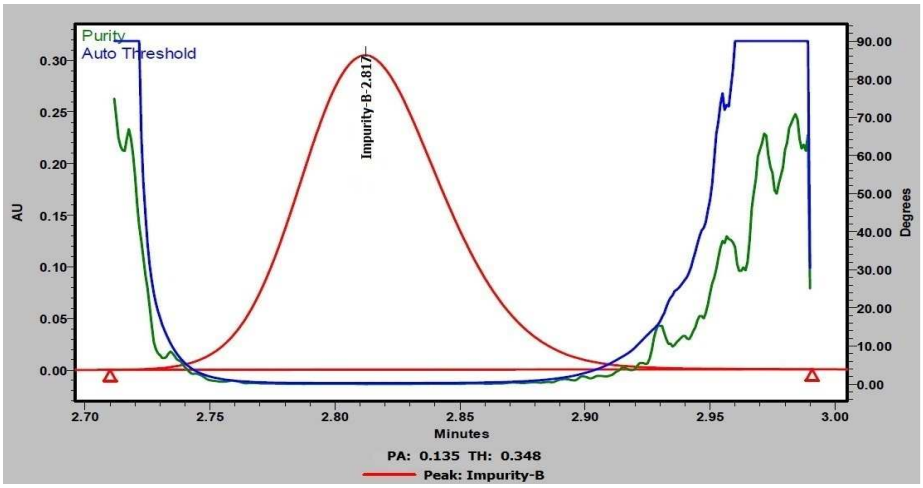
calculated. The results from forced degradation study and Peak purity plots of all stress condition samples are presented in [Figures 5, 6, 7] and [Table 3].



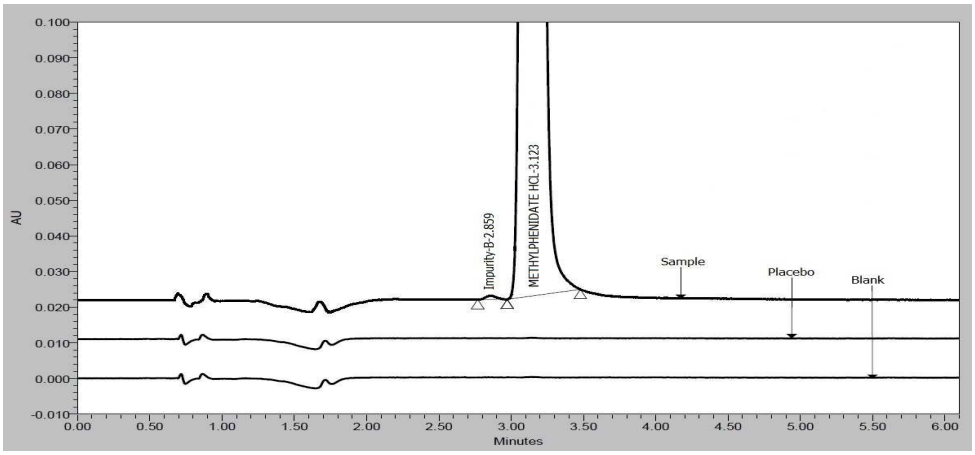
[Figure 5: Spectral purity plots of MPH]



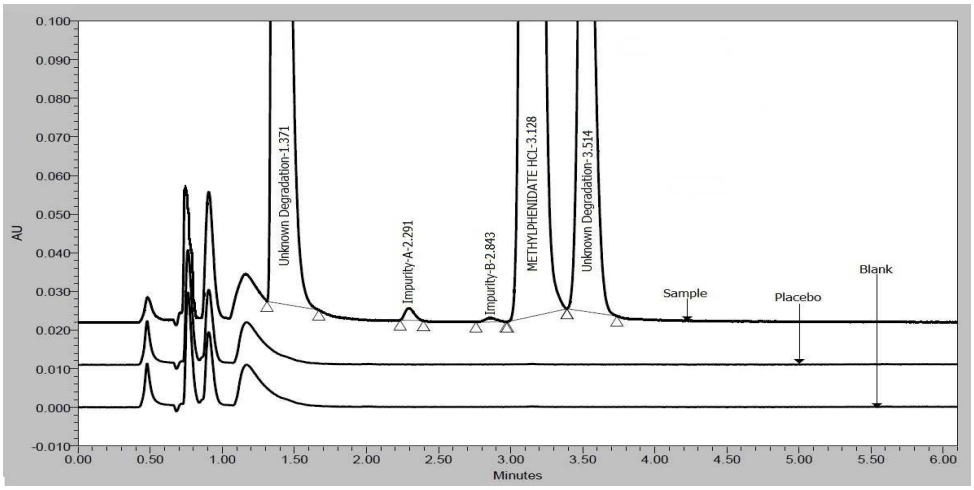
[Figure 6: Spectral purity plots of Impurity-A]



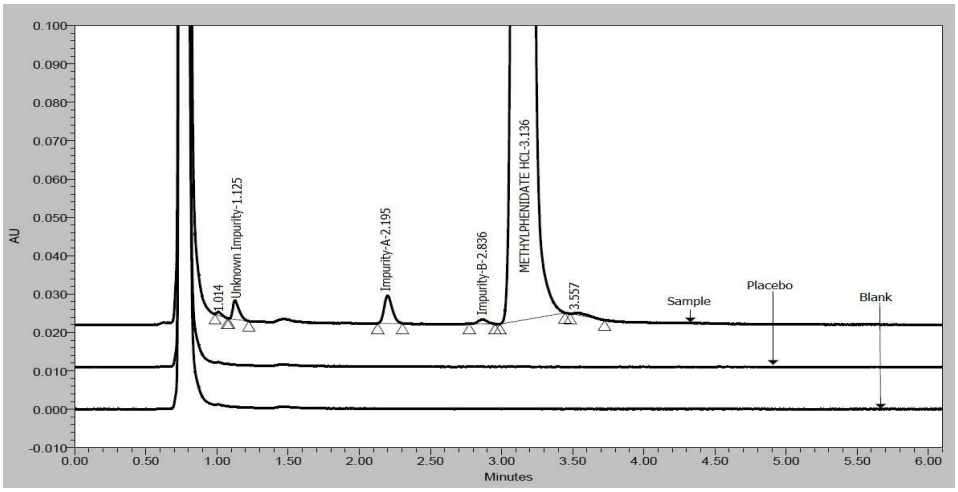
[Figure 7: Spectral purity plots of MPH, Imp-A and Imp-B]



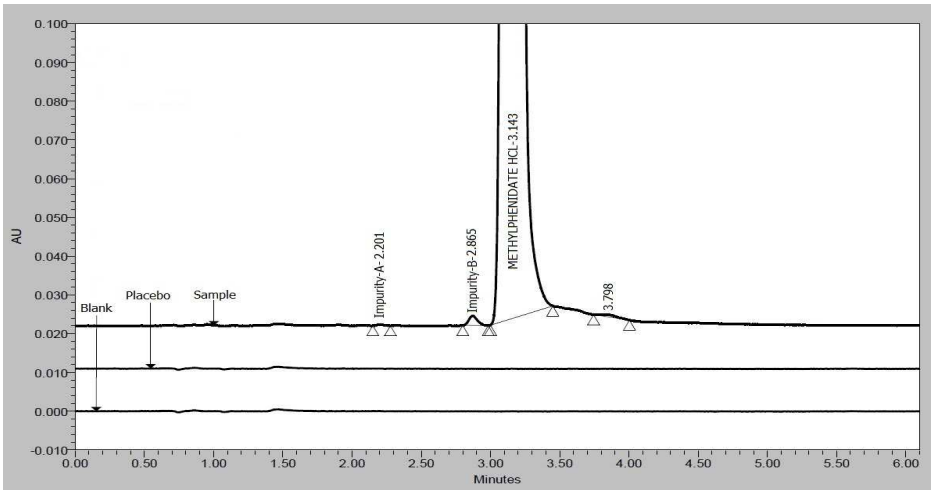
[Figure 8: Overlaid chromatograms of acid hydrolysis study]



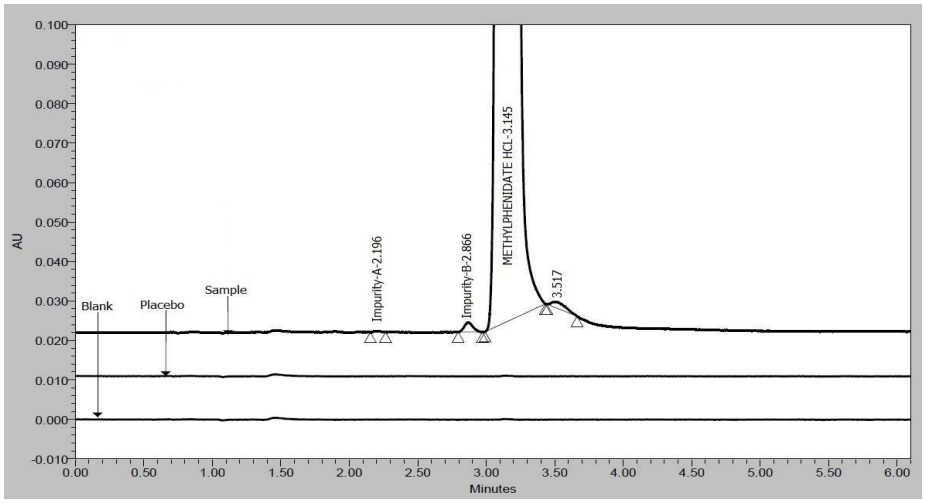
[Figure 9: Overlaid chromatograms of base hydrolysis study]



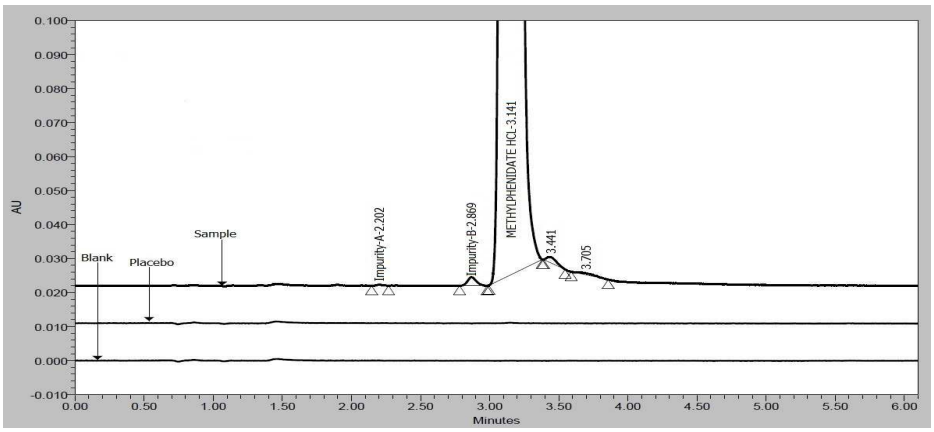
[Figure 10: Overlaid chromatograms of peroxide degradation study]



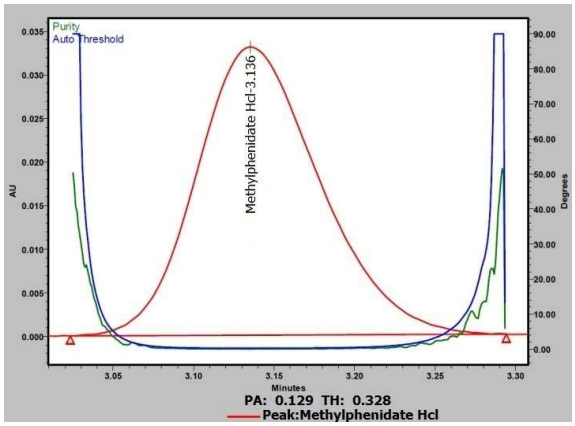
[Figure 11: Overlaid chromatograms of heat degradation study]



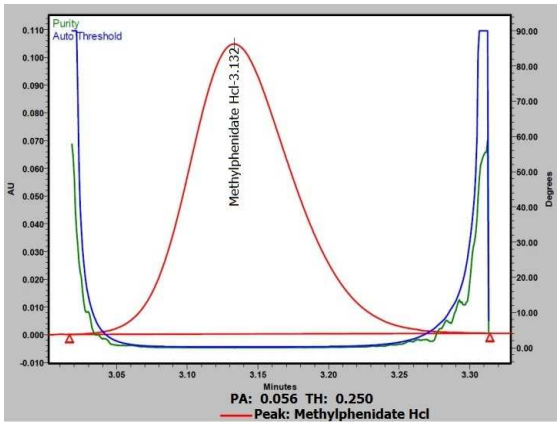
[Figure 12: Overlaid chromatograms of hydrolytic degradation study]



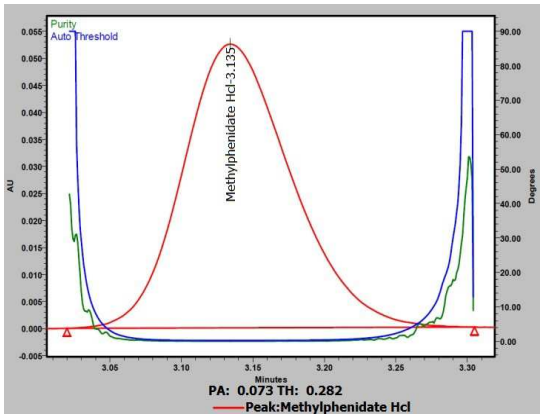
[Figure 13: Overlaid chromatograms of photolytic degradation study]



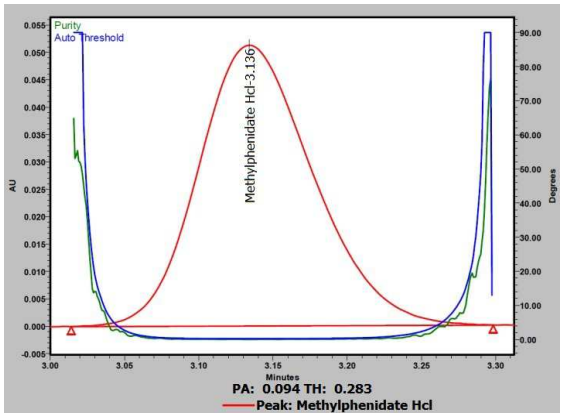
[Figure 14: Base hydrolysis]



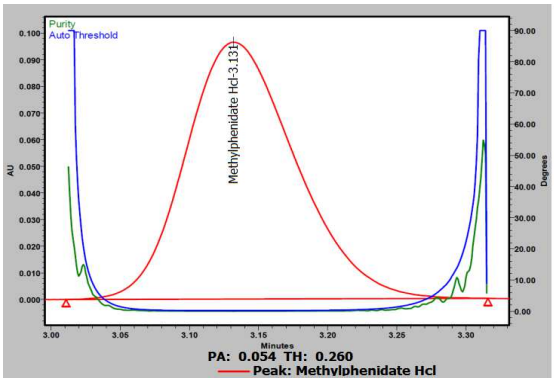
[Figure 15: Water hydrolysis]

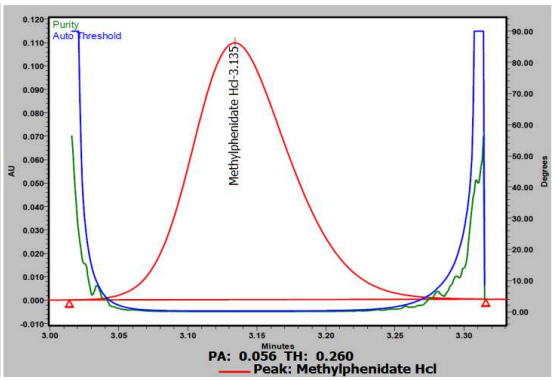


[Figure 16: Oxidative]



[Figure 17: Acid Hydrolysis]



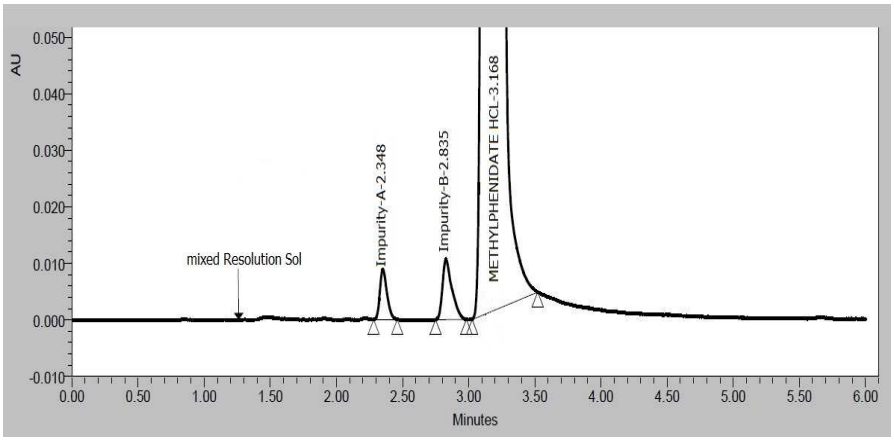


[Figure 18: Thermal] **[Figure 19: Photolytic]**

[Figure 14-19 are the Obtained spectral purity plots of MPH in forced Degradation study]

System suitability

The percentage RSD of area counts of six replicate injections is below 1.0 % .The parameters all complied with the acceptance criteria and system suitability is established. As seen from this data, the acceptable system suitability parameters would be: resolution between Imp-B and MPH is not less than 1.5, theoretical plates is not less than 5000, tailing factor for MPH is not more than 2.0. Specimen chromatogram of system suitability solution and diluted standard are presented in [Figure 20, 21] respectively and replicate in data [Table 4].

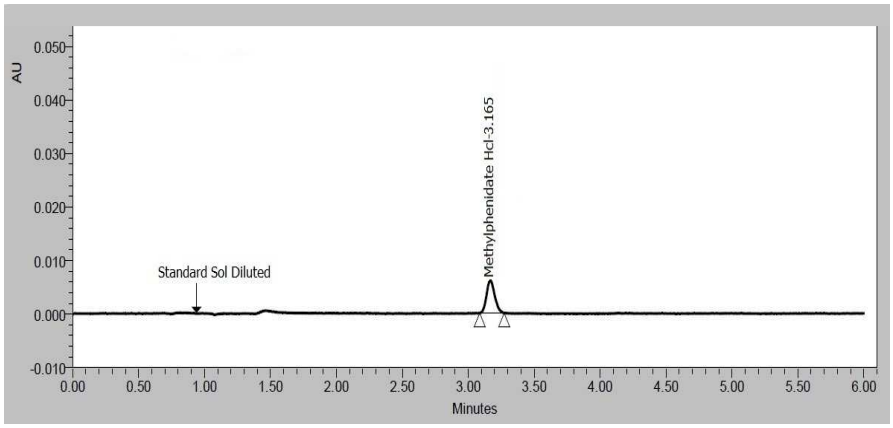


[Figure 20: Specimen chromatogram of system suitability solution]

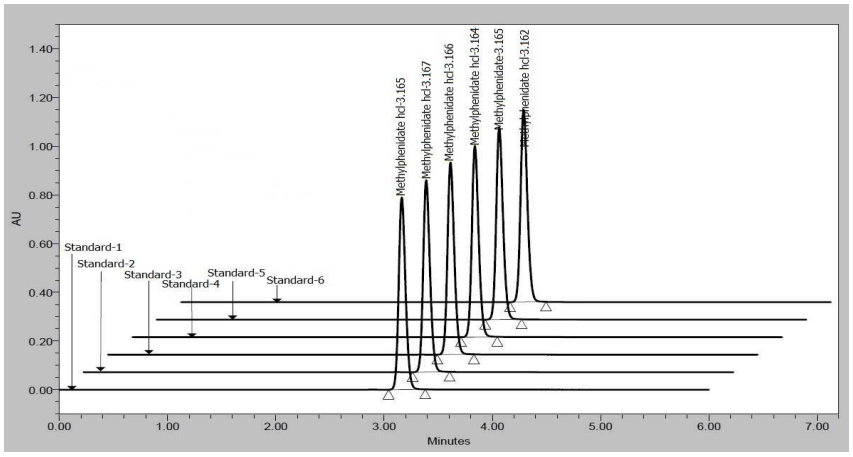
Precision

The system precision of the related substance (at 203 nm) method is verified by injecting six replicate injections of a standard solution contains MPH (1 µg/mL) and its two impurities of 1µg/mL of each. The RSD (%) of the peak areas is calculated for each compound (system precision). Method precision experiments are conducted in six individual preparations of MPH (1000 µg/mL) spiked with 1 µg/mL each of the impurities and the RSD (%) for area

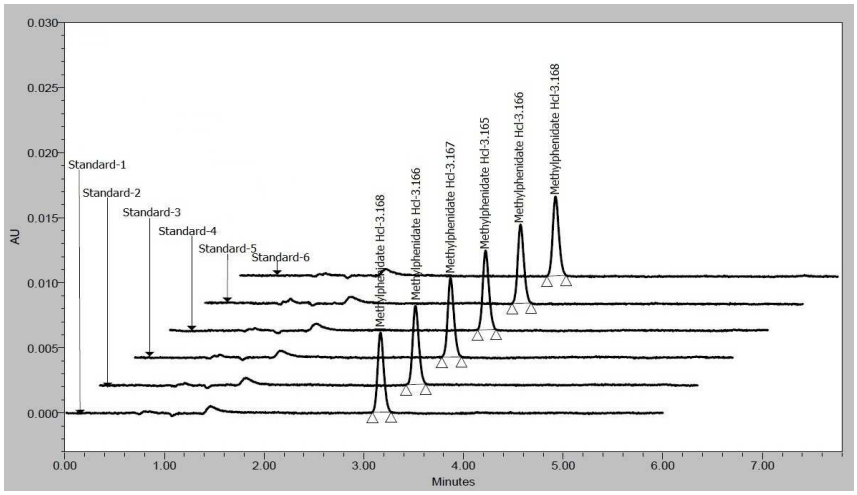
percentage of each impurities is calculated. Precision of assay (at 210 nm) method is evaluated by performing six (n=6) independent assays of MPH tablet at 1000 mg/mL level against a qualified working standard. The RSD (%) of the six results is calculated. The intermediate precision of the assay and RS method is evaluated by different analyst, instrument and day. The RSD (%) of peak area of MPH, Imp-A and Imp-B in system precision is within 1.0% .The RSD (%) results of MPH and its impurities for precision and intermediate precision are presented below table. These results confirmed the high precision of the method. Overlaid chromatogram for the assay standard preparation (at 210 nm), diluted standard preparation (at 203 nm), assay precision study and related substances precision study are presented in [Figure 22-25] and [Table 4, 5].



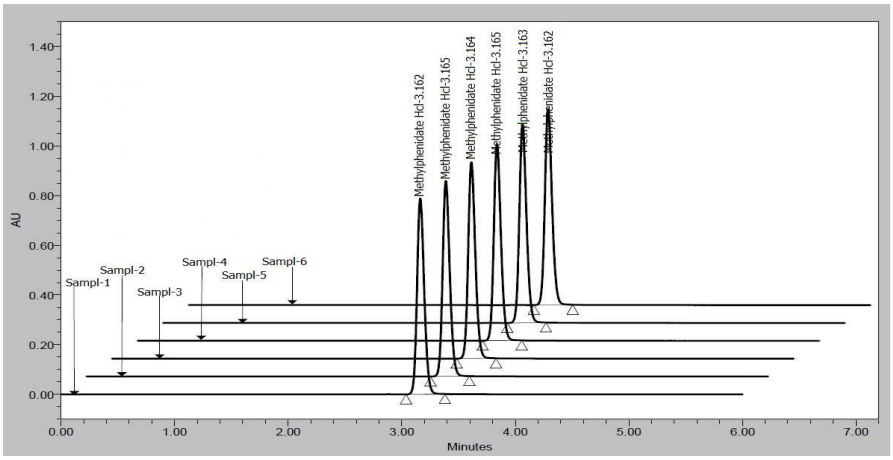
[Figure 21: Specimen chromatogram of diluted standard solution]



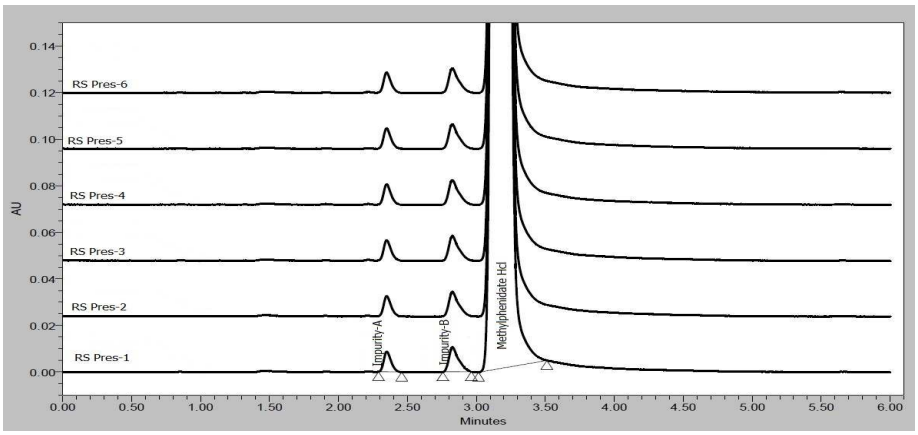
[Figure 22: Overlaid chromatograms of replicate standard injection (at 230 nm)]



[Figure 23: Overlaid chromatograms of diluted standard solution (at 205 nm)]



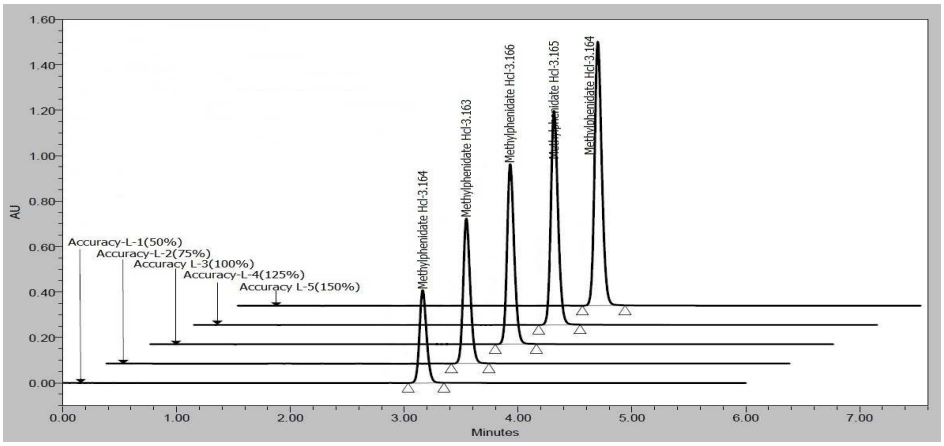
[Figure 24: Overlaid chromatograms of assay precision study (at 230 nm)]



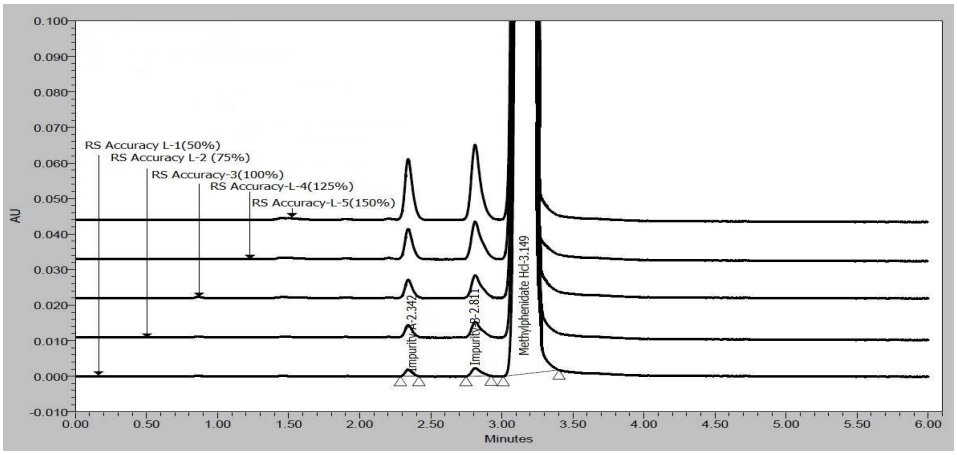
[Figure 25: Overlaid chromatograms of related substance precision study (at 205 nm)]

Accuracy

The accuracy of an analytical procedure expresses the closeness of agreement between the true value and the observed value. The accuracy of the assay method for MPH is evaluated in triplicate (n=3) at the five concentrations of 500, 750, 1000, 1250 and 1500 µg/mL (50, 75, 100, 125 and150%) of drug product, and the recovery is calculated for each added (externally spiked)concentration. For all impurities, the recovery is determined in triplicate (n=3) for 0.5, 0.75, 1.0, 1.25 and 1.5 µg/mL (50, 75, 100, 125 and 150%) of the analyte concentration (1000 µg/mL) of the drug product, and the recovery of the impurities is calculated. The amount recovered is within ± 1.5% (for assay) and ± 5.0 % (for related substances) of amount added, which indicates that there is no interference due to excipients present in pharmaceutical dosage forms. It is confirmed from results that the method is highly accurate Overlaid specimen chromatograms of assay and related substances accuracy are presented in [Figure 26, 27] and [Table 6].



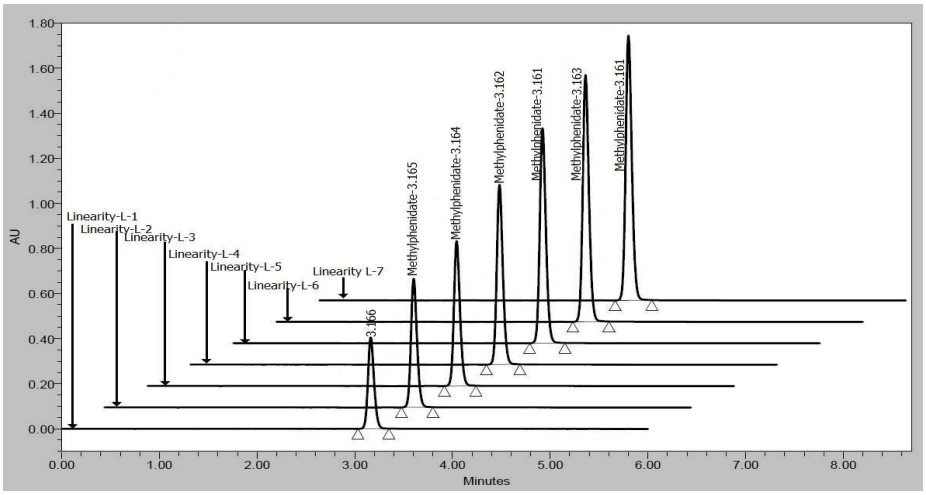
[Figure 26: Overlaid specimen chromatograms of assay accuracy study (at 230 nm)]



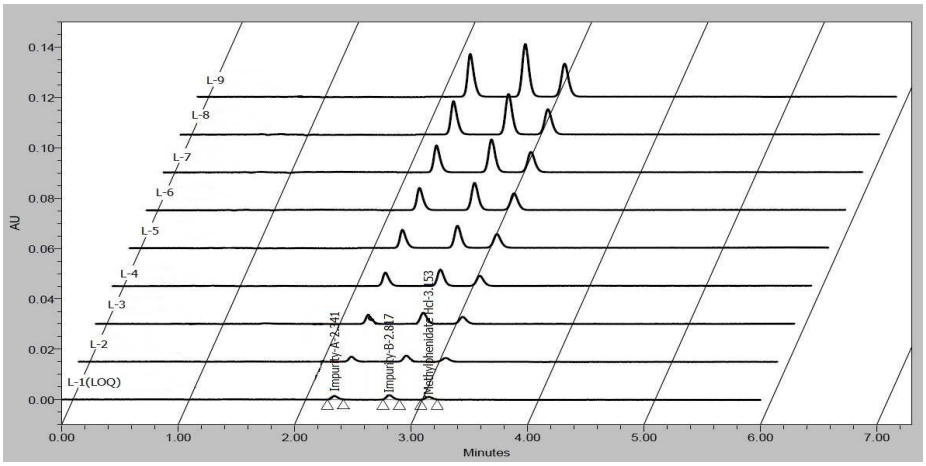
[Figure 27: Overlaid specimen chromatograms of accuracy study (related substances at 205 nm)]

Linearity:

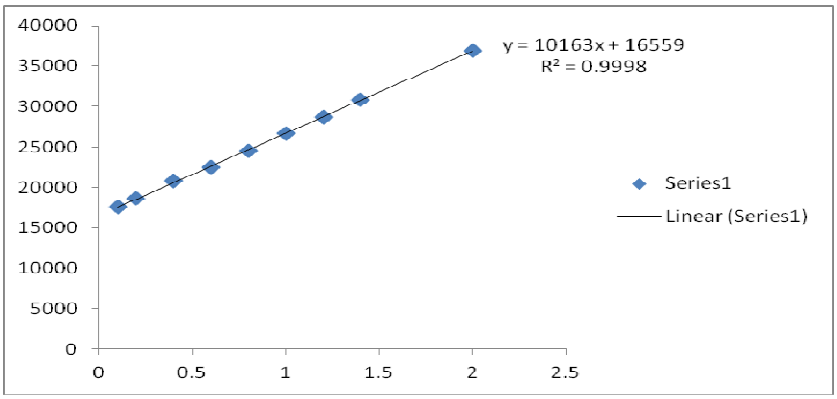
The linearity of an analytical method is its ability to elicit test results that are directly proportional, or by a well-defined mathematical transformation to the concentration of analyte in a sample within a given range. The detector response linearity for Imp-A, Imp-B and MPH are assessed by injecting nine separately prepared solutions covering the range of LOQ (0.1 µg/mL) to 2.0 µg/mL (LOQ, 0.2, 0.4, 0.6, 0.8, 1.0, 1.2, 1.5 and 2.0 µg/mL) of the normal analyte concentration (1µg/mL). For MPH assay the response function is determined by preparing standard solutions at seven different concentration levels ranging from 500 to 1500 µg/mL (500, 700, 800, 1000, 1200,1400 and 1500 µg/mL). The correlation coefficients, slopes and y-intercepts of the calibration curve are determined. The correlation coefficient obtained is greater than 0.999 in both cases. Overlaid specimen chromatograms of linearity study of assay and related substances are presented in [Figure 28, 28] respectively. Linearity graph of Imp-A, Imp-B, MPH (at 205nm) and MPH (at 230 nm) are presented in [Figure 28, 29] and [Table 7] respectively.



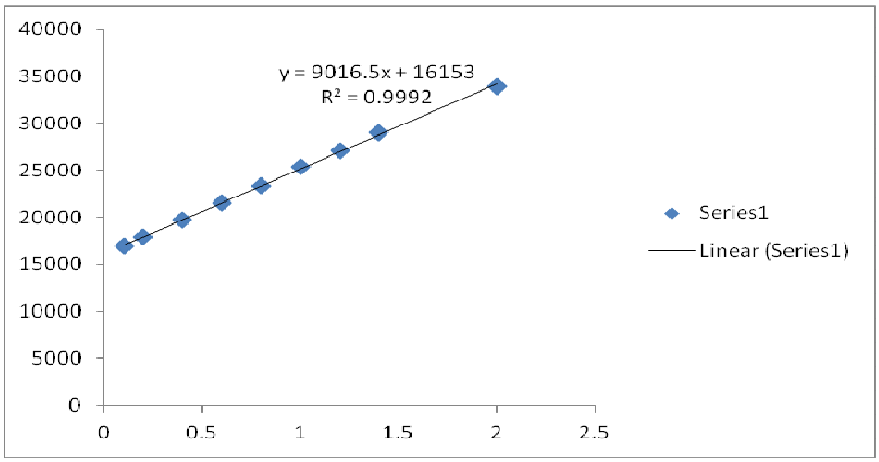
[Figure 28: Overlaid specimen chromatograms of linearity study (Assay)]



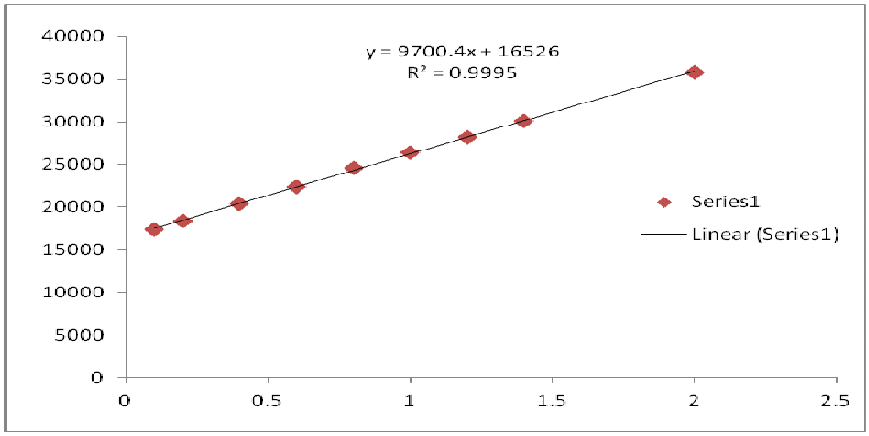
[Figure 29: Overlaid specimen chromatograms of linearity study (related substances)]



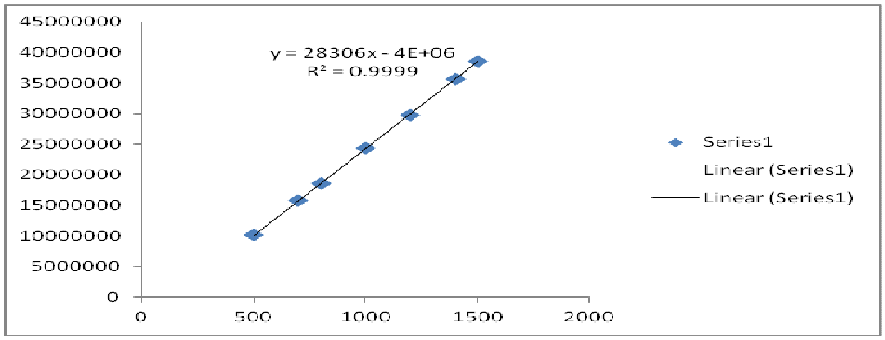
[Figure 30: Linearity curve of impurity-A]



[Figure 31: Linearity curve of impurity-B]



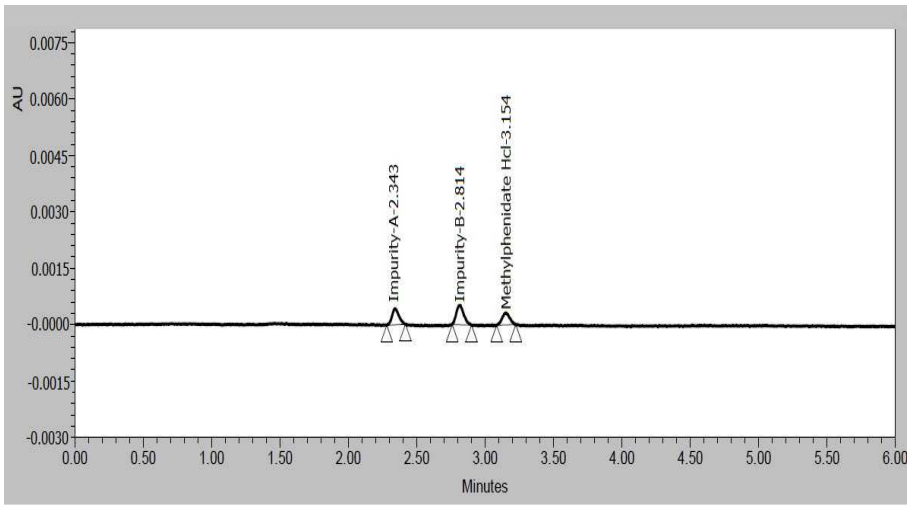
[Figure 32: Linearity curve of MPH for RS at 203 nm]



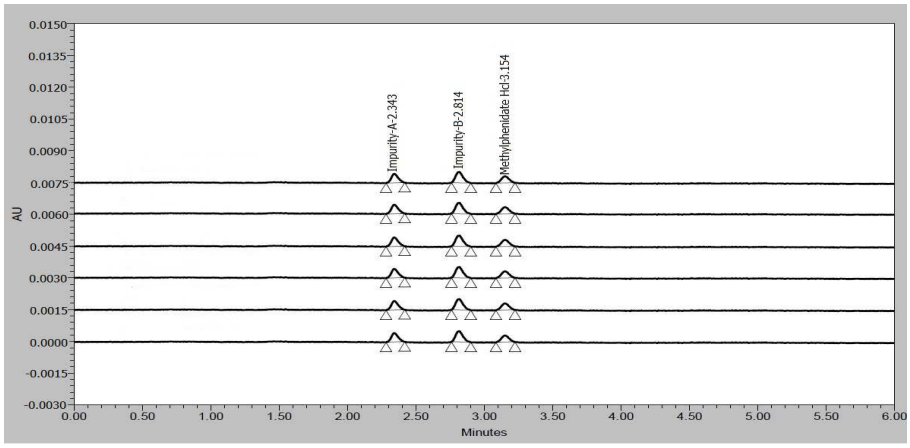
[Figure 33: Linearity curve of MPH at 210 nm]

Limit of detection (LOD) and limit of quantification (LOQ)

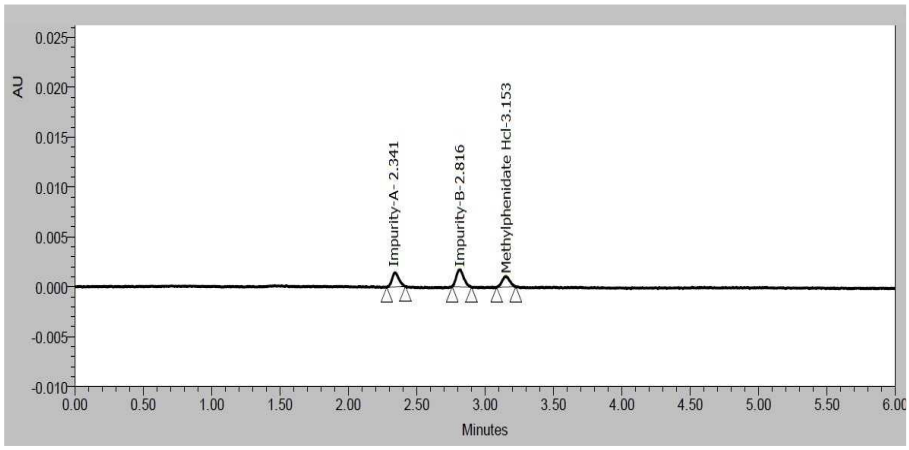
The LOD and LOQ for MPH and its impurities are determined at a signal to noise ratio of 3:1 and10:1, respectively, by injecting a series of dilute solutions with known concentrations. Precision study is also carried out at the LOQ level by injecting six (n=6) individual preparation and calculating the % RSD of the area for each impurity and for MPH. The determined limit of detection, limit of quantification and precision at LOQ levels for MPH, Imp-A and Imp-B are presented .Specimen chromatogram of LOD [Figure 34] And LOQ study is presented in [Figure 36] and Overlaid chromatograms of LOD and LOQ precision study is presented in [Figure 37] and [Table 8].



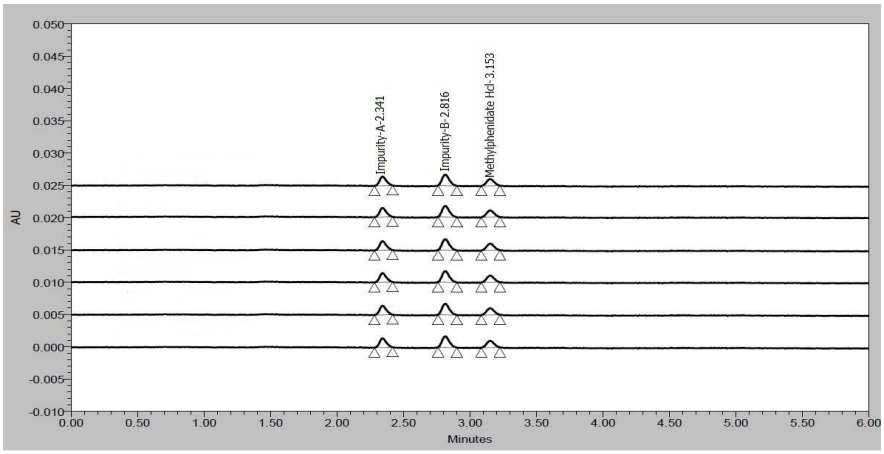
[Figure 34: Spiked Specimen chromatogram of LOD for IMP-A, IMP-B, and MPH]



[Figure 35: Overlaid spiked chromatograms of LOD precision study for IMP-A, IMP-B, and MPH]



[Figure 36: Spiked Specimen chromatogram of LOQ for IMP-A, IMP-B, and MPH]



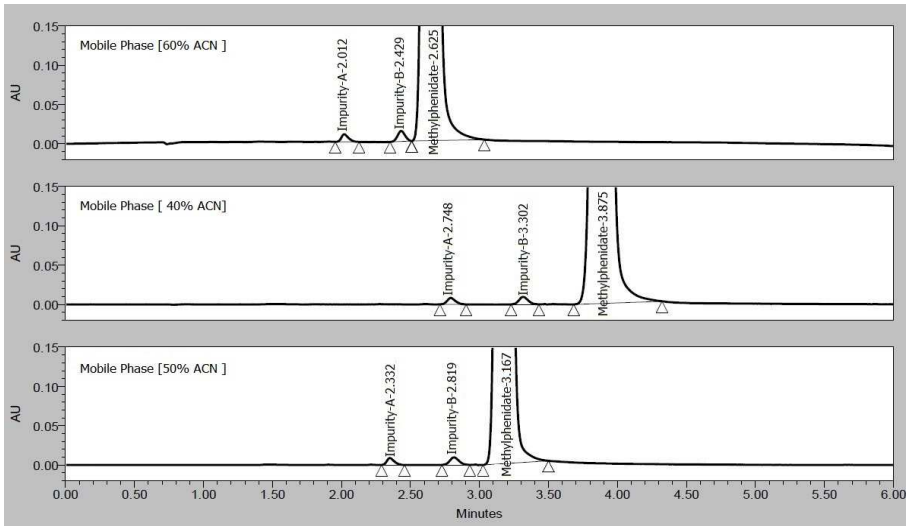
[Figure 37: Spiked Overlaid chromatograms of LOQ precision study for IMP-A, IMP-B, and MPH]

Robustness

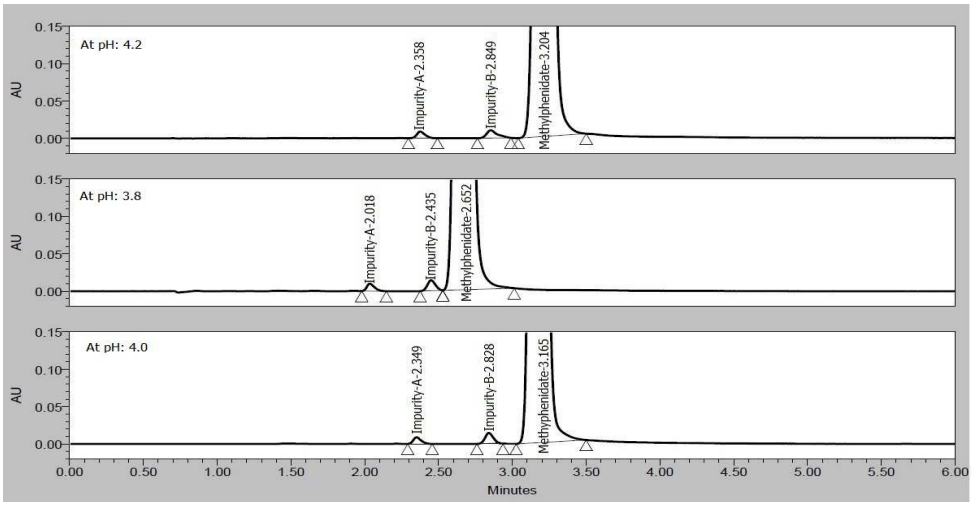
To determine the robustness of the method, the experimental conditions are deliberately changed. The resolution of MPH and imp-B is evaluated. The effect of change in flow rate ± 0.05mL/min (1.45 and 1.55 mL/min), column oven temperature ± 5°C (15 and 25°C), mobile phase pH ± 0.2 units (3.8 and 4.2 pH) and mobile phase composition ± 10% (for ACN) are studied. During study other chromatographic conditions are kept same as per the experimental section.

In all the deliberately varied chromatographic conditions, all of the analytes are adequately resolved, and the order of elution remained unchanged. Robustness study obtained results are presented in Specimen chromatograms

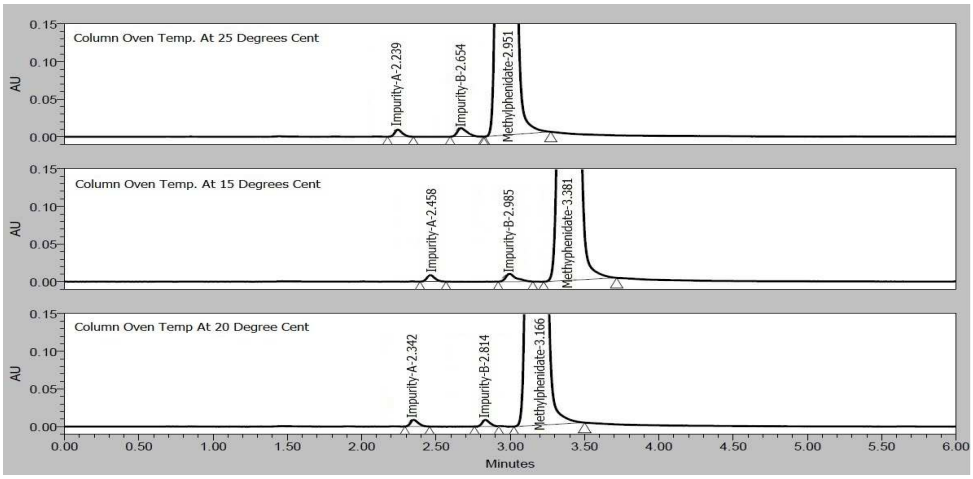
of robustness study are presented in [Figure 38 - 41]. [Figure 38] indicate that USP resolution 1.56 is also adequate for the peak separation in developed method data was represented in [Table 9].



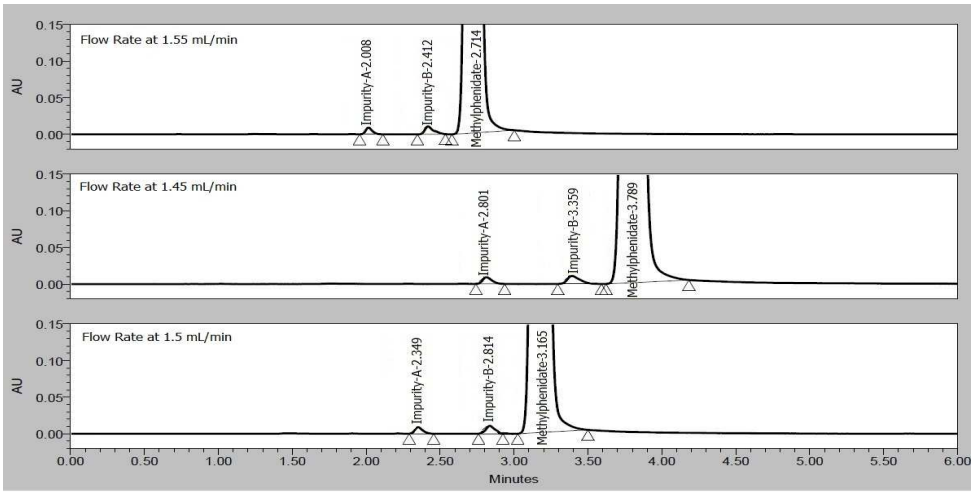
[Figure 38: Specimen chromatograms of IMP-A, IMP-B, MPH robustness study (change in organic solvent ratio, ± 10%)]



[Figure 39: Specimen chromatograms of IMP-A, IMP-B, MPH robustness study (change in mobile phase pH)]



[Figure 40: Specimen chromatograms of IMP-A, IMP-B, MPH robustness study (column oven temperature)]

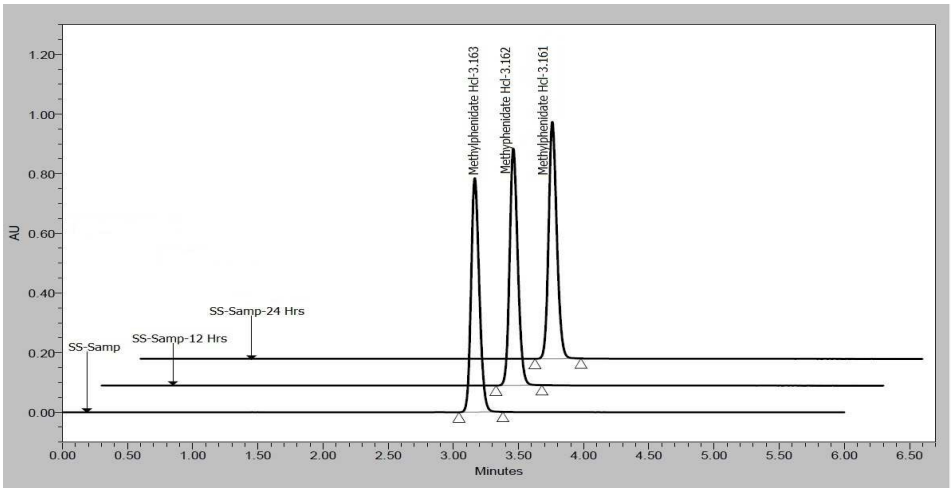


[Figure 41: Specimen chromatograms of IMP-A, IMP-B, MPH robustness study (flow rate variation)]

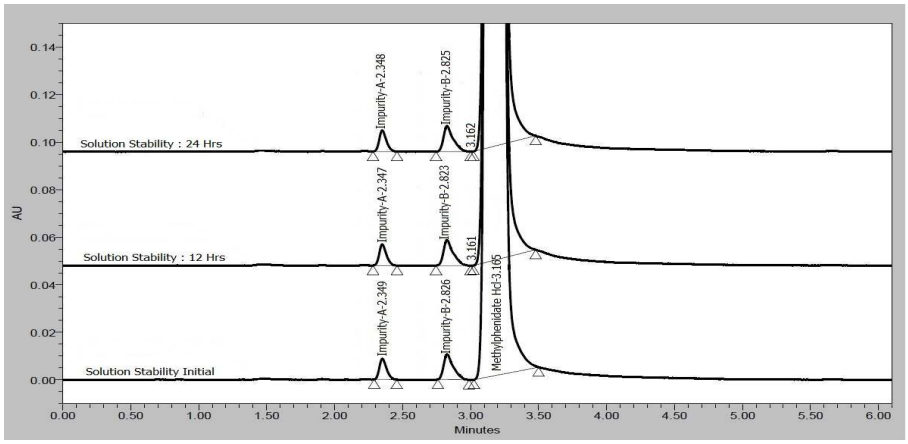
Ruggedness: The ruggedness can be evaluated by using different analysts but in the same chromatographic conditions in different laboratories and different instruments. The result of ruggedness of developed method is stated in table and the results are shown during by different analysts but in the same chromatographic condition of the test solution wasn't affected & in the accordance with the actual. The suitability parameters are also been found to be good hence this method was concluded as rugged [Table 10].

Solution stability

Drug stability in pharmaceutical formulations is a function of storage conditions and chemical properties of the drug and its impurities. Conditions used in stability experiments should reflect situations likely to be encountered during actual sample handling and analysis. Stability data is required to show that the concentration and purity of the analyte in the sample at the time of analysis corresponds to the concentration and purity of the analyte at the time of sampling. MPH (1000 µg/mL) spiked solution (with 1 µg/mL of each impurity) is prepared in the diluent by leaving the test solutions at room temperature. The spiked solution is re-analyzed at 12h and 24h time intervals, assay and related substances are determinate for the compounds and compared against fresh sample. The sample solution does not show any appreciable change in assay and related substances value when stored at ambient temperature up to 24hrs data are presented and the results from solution stability experiments confirmed that sample solution is stable for up to 24h during assay and related substances determination. Standard solution is re-injected after 12 and 24h time intervals and % RSD of all injected standard injections are calculated. Standard solution does not show any appreciable change in % RSD (RSD for MPH is less than 1.0%) value when stored at ambient temperature up to 24h. Overlaid specimen chromatograms for sample solution stability of assay and related substances are presented in [Figure 42-43] and [Table 11] respectively.



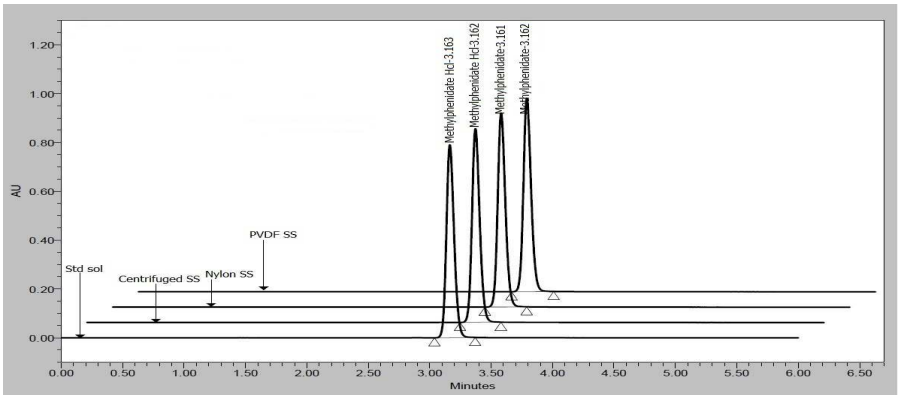
[Figure 42: Specimen chromatograms of MPH assay sample solution stability]



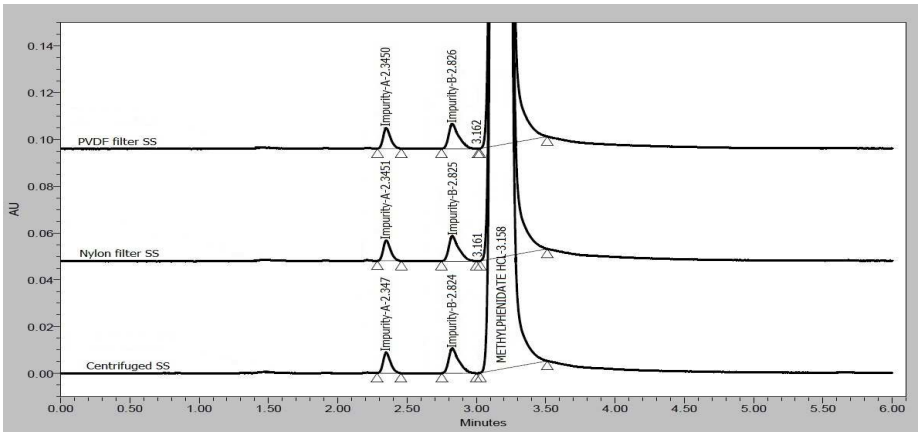
[Figure 43: Overlaid specimen chromatograms of related substance sample solution stability]

Filter compatibility

Filter compatibility is performed for PVDF 0.22 µm syringe filter (Millipore) and nylon 0.22 µm syringe filter (Analytical Tech Labs). To confirm the filter compatibility in proposed analytical method, filtration recovery experiment is carried out by sample filtration technique. Sample is filtered through both syringe filters. Assay and related substances is determined (in µg/mL) and compared against centrifuged sample. The sample solution does not show any significant changes in assay and related substances with respect to centrifuged sample. Filter compatibility results are presented in which indicates that both syringe filters are compatible with sample solution. Overlaid specimen chromatograms of assay and related substance test (filter compatibility) are presented in [Figure 48, 49] & [Table 12], respectively.



[Figure 44: Overlaid specimen chromatograms of MPH assay filter compatibility study]



[Figure 45: Overlaid specimen chromatograms of related substance filter compatibility study]

Table 1: Summary of mobile phase optimization Trails and its observations

MP-A	MP-B	Observation
Water	ACN	Co-eluting peak of Imp-B and MPH
50% SAB	ACN	Co-eluting peak of Imp-B and MPH
20% SAB	ACN:MeOH (30:50 v/v)	Poor resolution (Imp-B and MPH), USP tailing more than 2.0 for MPH peak
Mixture of water, ACN, MeOH and THF [50:30:30 v/v/v]	N.A	Poor resolution (Imp-B and MPH), USP tailing more than 3.0 for MPH peak
Mixture of water, ACN, MeOH and TEA [40:30:30:0.1 v/v/v/v], pH adjusted 6.2with OPA	NA	Poor resolution (Imp-B and MPH), and broad peak shape of MPH
Mixture of ACN, MeOH and Buffer [50:20:30 v/v/v], pH adjusted 4.0 with Acetic acid	NA	Good Resolution (Imp-A,Imp-B, MPH) and good peak symmetrical shape was achieved.
USP = United state pharmacopoeia; ACN=Acetonitrile,MeOH=Methanol;AA=Acetic Acid OPA= Orthophosphoric acid; THF=Tetrahydrofuran ,SAB=Sodium Acetate Buffer		

Table 2: Optimized Chromatographic Conditions

Parameters	Description
Stationary Phase(Column)	HSSD waters symmetry C_{18} (100x 40 i.d., 3.7 µm)
Mobile phase	Methanol:Acetonitrile:buffer (50:20:30 v/v/v) and pH adjusted 4.0 with acetic acid
pH	4
Flow rate	1.5 ml min^{-1}
Run time (minutes)	6
Column temperature	20 ± 1^0C
volume of Injection loop (µl)	20 µl
Detection wavelength	210 nm
Impurities & Drug RT (min)	2.349,2.792 & 3.168

Table 3: Summary of forced degradation results

Degradation condition	Mass balance	Purity		Observation
		Angle	Threshold	
Control sample	99.8	0.056	0.26	N.A.
Acidic hydrolysis	99.2	0.094	0.283	No degradation observed
Alkaline hydrolysis	98.5	0.129	0.328	Significant degradation observed
Oxidation	98.4	0.073	0.282	No significant degradation
Observed Water hydrolysis	99.5	0.056	0.25	No significant degradation
observed Thermal (solid)	100.1	0.054	0.26	No significant degradation
Photolytic	100.2	0.056	0.26	No significant degradation observed

Table 4: System suitability results (system precision, method precision and intermediate precision)

Test	Parameters	Imp-A (1µg/mL)	Imp-B (1µg/mL)	MPH (1000µg/mL)	MPH (1 µg/mL)
System Precision	Area % RSD	0.8	0.9	0.9	0.3
Presicison(n=6)	USP Resolution	NA	NA	NA	NA
	USP tailing	1.34	1.52	1.12	1.19
	USP plate count	8831	7219	11975	11994
Intermediate precision (n=6)	USP resolution	NA	NA	NA	NA
	USP tailing	1.34	1.52	1.12	1.19
	USP plate count	8831	7219	11975	11994
NA= Not applicable					

Table 5: Precision (n=6) and Intermediate precision (n=6) results

Drug Substance	Precisional Values		Intermediate precision	
	Mean %	% RSD	Mean %	% RSD
MPH (1000µg/mL)	99.3	0.9	99.5	1.1
Imp-A (1µg/mL)	0.098	2.4	0.101	2.3
Imp-B (1µg/mL)	0.103	2.7	0.097	2.6

Table 6: Accuracy results

Substance	At 50% (n=3)		At 50% (n=3)		At 50% (n=3)		At 50% (n=3)		At 50% (n=3)	
	%REC*	%RSD	%REC*	%RSD	%REC*	%RSD	%REC*	%RSD	%REC*	%RSD
MPH (1000µg/mL)	100.6	0.8	100.1	0.4	99.4	1.1	99.7	0.7	99.1	1.3
MPH (1µg/mL)	95.5	3	97.2	2.1	97.8	3.5	96.8	2.3	98.7	3.3
Imp-A (1µg/mL)	95.3	4.6	98.3	2.8	103.1	3.5	98.7	3.1	99.5	4.2
Imp-B (1µg/mL)	102.5	3.5	101.2	3.5	104.5	4.4	101.3	2.7	97.5	3.8
*= Recovery										

Table 7: Regression statistics

Compound	Linearity range (µg/mL)	Correlation coefficient (r^2)	Linearity (Equation)	Linearity (Equation)
MPH	500 to 1500	0.9999	y=28306(x)-4E+06	1.452
MPH	0.1 to 2.0	0.9995	y =9700.4(x) + 16526	0.366
Imp-A	0.1 to 2.0	0.9998	y =33041(x)-208.53	-0.635
Imp-B	0.1 to 2.0	0.9992	y =9016.5(x) +16153	1.886

Table 8: Results of LOD, LOQ and LOQ precision (n=6)

	MPH	Impurity-A	Impurity-B
LOD (µg/mL)	0.03	0.04	0.04
LOQ (µg/mL)	0.1	0.1	0.1
LOQ precision (% RSD)	4.5	4.9	5.7

Table 9: Evaluation data results of Robustness study of IMP-A, IMP-B, MPH

Condition	Parameters	Imp-A	Imp-B	MPH
	USP resolution	--	--	2.35
	Retention time in min	2.349	2.792	3.162
	USP tailing	1.28	1.49	1.17
	USP plate count	8829	7215	11968
Normal methodology	Assay % w/w	0.097	0.101	99.4
	USP resolution	--	--	2.29
	Retention time in min	2.801	3.391	3.789
	USP tailing	1.29	1.46	1.17
	USP plate count	9281	7871	7990
At flow rate 1.45 mL/min	Assay % w/w	0.096	0.102	99.3
	USP resolution	--	--	2.48
	Retention time in min	2.008	2.412	2.714
	USP tailing	1.32	1.49	1.17
	USP plate count	9083	9298	7786
At flow rate 1.55 mL/min	Assay % w/w	0.096	0.103	99.3
	USP resolution	--	--	2.75
	Retention time in min	2.458	2.985	3.381
	USP tailing	1.34	1.39	1.16
	USP plate count	9820	9889	8380
At 15°C column oven temp	Assay % w/w	0.098	0.102	99.5
	USP resolution	--	--	2.16
	Retention time in min	2.239	2.654	2.951
	USP tailing	1.26	1.37	1.26
	USP plate count	9081	7545	7126
At 25°C column oven temp	Assay % w/w	0.097	0.103	98.8
	USP resolution	--	--	1.69
	Retention time in min	2.018	2.435	2.652
	USP tailing	1.29	1.39	1.12
	USP plate count	7379	8964	7536
At mobile phase pH 3.8	Assay % w/w	0.098	0.101	99.4
	USP resolution	--	--	2.38
	Retention time in min	2.358	2.849	3.204
	USP tailing	1.29	1.36	1.18
	USP plate count	8649	8138	7848
At mobile phase pH 4.2	Assay % w/w	0.097	0.103	99.4
	USP resolution	--	--	2.74
	Retention time in min	2.788	3.302	3.875
	USP tailing	1.14	1.43	1.3
	USP plate count	9969	10774	8252

Mobile phase [-10% ACN]	Assay% w/w	0.098	0.102	99.5
Mobile phase [+10% ACN]	USP Resolution			1.53
	Retention time in min	2.012	2.429	2.625
	USP tailing	1.4	1.53	1.38
	USP plate count	7247	8758	7436
	Assay % w/w	0.099	0.103	99.7

[Table 10: Evaluation data of Ruggedness study of IMP-A, IMP-B, and MPH]

ID Precisions	No. of Injections	IMP-A		IMP-B		MPH	
		Peak Area	RT	Peak Area	RT	Peak Area	RT
Analyst ID Precision - 1	1	897912	2.347	188537	2.79	311221	3.167
	2	902826	2.349	188577	2.792	311666	3.165
	3	903754	2.346	190256	2.794	316841	3.167
Analyst ID Precision - 2	1	901972	2.345	189433	2.791	316709	3.165
	2	899897	2.347	189558	2.792	317747	3.167
	3	900583	2.346	189746	2.792	318175	3.167
MEAN		901157.3	2.346	189351.17	2.792	315393	3.164
STDEV		2127.971	0.002	676.209	0.003	3111.45	0.005
% RSD		0.236138	0.123	0.357	0.121	0.986	0.121

Table 11 : Solution stability results of IMP-A, IMP-B, and MPH

Compound	0hrs	12hrs	24hrs	48 hrs
MPH (1000µg/mL)	1005	1004	1003	1002
IMP-A (1µg/mL)	1.003	1.007	1.008	1.006
IMP-B (1µg/mL)	0.975	0.967	0.978	0.969

Table 12 : Filter compatibility results of IMP-A, IMP-B, and MPH

Compound	Ultra Centrifugation	PVDF filter membrane 0.22µm	Nylon membrane filter 0.22µm
MPH (1000µg/mL)	1005	1004	1003
IMP-A (1µg/mL)	0.951	0.961	0.964
IMP-B (1µg/mL)	0.985	0.972	0.981

CONCLUSION

The rapid isocratic RP-HPLC method is developed for quantitative and related substances analysis of MPH in pharmaceutical formulation. Satisfactory results are obtained from validation of the method. The run time (6 min) enabled rapid determination of MPH. This method exhibited an excellent performance in terms of sensitivity and speed. This stability-indicating method can be applied for the routine analysis of production samples and to check the stability of MPH in bulk drug and formulation. Moreover, it can be applied for determination of assay, blend uniformity, content uniformity and in vitro dissolutions of pharmaceutical products, where sample load is higher and high throughput is essential for faster delivery of results

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