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A SIMPLE AND SENSITIVE RP-HPLC METHOD FOR TRACE-LEVEL QUANTIFICATION OF METHYL VINYL KETONE, A POTENTIAL GENOTOXIC IMPURITY, IN DRUG SUBSTANCES

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Methyl Vinyl Ketone, Potential Genotoxic Impurity, Threshold of Toxicological Concern

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ABSTRACT: An HPLC method was developed with the aim of quantifying the levels of Methyl Vinyl Ketone (MVK), a Potential Genotoxic Impurity (PGI), in Ivabradine HCl. A limit level concentration of 0.5 ppm for MVK was calculated by applying the concept of Threshold of Toxicological Concern (TTC). The developed HPLC method employs a Phenyl analytical column (150 mm x 4.6 mm, 3.5 µ), 5-µL injection volume, mobile phase of buffer (pH 2.5)-acetonitrile [gradient elution], a column temperature of 50°C and a flow rate of 1.0 mL/min. The retention time of MVK obtained was approximately 4.0 minutes in a program with run time of 22 minutes. The Limit of Detection was 0.06 ppm and Limit of Quantitation was 0.2 ppm. The method proved to be sensitive, linear, accurate and precise in the specified range (LOQ to 150 % of limit level concentration). The validated method was applied for identification and quantification of MVK in different batches of the API.

INTRODUCTION: Pharmaceutical analysis with sophisticated techniques has immensely contributed to the development of the pharmaceutical industry.[1] It is not confined to the analysis of active pharmaceutical ingredients (API) or its formulations but also plays a pivotal role in the analysis of trace-level impurities. [2]

1.1 Potential Genotoxic Impurities (PGIs): PGIs are chemical entities that could potentially damage DNA.[3] An API synthesis involves multiple reaction steps for the conversion of basic starting materials to the final API.

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Trace-levels of reagents, catalysts or by-products may be present in the final API as impurities. These reactive impurities may have severe toxicities like genotoxicity and carcinogenicity and hence may react with DNA bases leading to mutations. [4, 5] Hence it is essential for process chemists to try avoiding such genotoxic impurities in the synthetic process. The analyst must also identify and characterize genotoxic impurities and control them at the stages of formation. However, it may not always be possible to eliminate PGIs completely from the manufacturing scheme. Therefore, it is a major challenge for analysts to develop suitable analytical methods to accurately quantify the impurities present at parts-per-million or parts-per-billion levels and control their levels in APIs. [6, 7]

1.2 Sources: PGIs that may be present in the API commonly fall into the following categories:

- Unreacted intermediates or contributory chemicals with alerting substructures that have survived processing.
- Structures closely related to intermediates, or the API itself which contains a known structural alert.
- Unrelated structures formed by combinations of reagents and solvents with each other or with intermediates. [8]

1.3 Regulation: Regulations insist on the analysis and control of impurities in drug substances as well as drug products to ensure the identity, strength, potency and quality of drug products. [9]

- International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) documents Q3A (R2) and ICH Q3B (R2): Provides guidance on limits for majority of the less toxic impurities in new drug substances and drug products respectively.[10, 11]
- The European Medicines Agency (EMA) guideline: Recommends a Threshold of Toxicological Concern (TTC) of 1.5 µg per day for genotoxic impurities. The TTC indicates a threshold exposure level to compounds that do not pose a significant risk for carcinogenicity or other toxic effects. [12]
- ICH M7 guideline 'Assessment and Control of DNA Reactive (Mutagenic) Impurities in Pharmaceuticals to Limit Potential Carcinogenic Risk': Presents a structured framework for the identification, categorization, qualification, and control of the mutagenic impurities to limit potential carcinogenic risk. [13]

1.4 Methyl Vinyl Ketone (MVK):

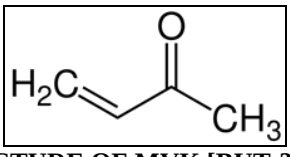

FIG. 1: STRUCTURE OF MVK [BUT-3-EN-2-ONE] [14]

MVK is a known PGI which belongs to the structural class of α,β–unsaturated carbonyl compounds (Class 3). These carbonyl compounds, which possess a double bond between carbons 2 and 3 (α and β), are highly reactive. Due to their reactivity with cellular nucleophiles (for example, Michael adduct formation with DNA bases and with glutathione), they are likely to represent a potential health risk, resulting in mutagenicity and carcinogenicity. Thus, α,β -unsaturated carbonyl group is a reactive moiety that represents a potential structural alert for genotoxicity.[15, 16]

1.5 Rationale: Ivabradine is an effective antianginal, anti-ischemic drug indicated for the treatment of chronic stable angina pectoris. The maximum daily dose is 7.5 mg twice daily. [17, 18] The synthesis of this drug involves the use of ketone based solvents which may be a probable source for MVK formation. Hence, an analytical method for trace level quantification of MVK in Ivabradine HCl needs to be developed.

Because of the increasing concern from regulatory bodies with respect to PGIs, a number of sophisticated analytical techniques are available for the analysis of Genotoxins in drug substances and drug products. [19] Literature survey revealed that few analytical methods using GC [20], LC-MS [21] and GC-MS [22] techniques are available for the estimation of MVK in bulk drugs; either individually or in combination with analysis of other impurities. However, no HPLC method was found for MVK analysis. Hence, there was a need to develop a simple and sensitive HPLC method for the trace level quantification of MVK in the drug substance namely Ivabradine HCl.

2. MATERIALS AND METHODS:

2.1 Chemicals and Reagents: Methanol and Acetonitrile (ACN) of HPLC Grade was purchased from S D Fine-Chem Limited (SDFCL). The Milli-Q Purification system was used to purify water. Sodium perchlorate and Perchloric acid were purchased from Merck KGaA and Fisher Scientific respectively. The PGI MVK was purchased from Merck Millipore and the drug substance Ivabradine HCl was received as a gift sample. All chemicals were of analytical grade and used as received.

2.2 Instrumentation: All the analytical studies were performed on a Shimadzu (LC-2010A HT) HPLC system driven by Chromeleon Software (Version 6.8, Dionex). This system is composed of a quaternary pump, a mobile phase degasser, an autosampler and a diode array detector.

2.3 Preparation of solutions:

2.3.1 Preparation of buffer solution (pH 2.5, 10 mM): About 1.40 gm of Sodium perchlorate was accurately weighed, transferred in a mobile phase glass bottle containing 1000 mL of HPLC grade water and sonicated to dissolve the buffer. It was adjusted to pH 2.5 ± 0.05 units with perchloric acid, filtered through 0.45 µ nylon membrane filter and degassed using an ultrasonicator.

2.3.2 Selection of diluents: Methanol was selected as the diluent.

2.3.3 Reference (standard) preparation for the PGI (Limit level - 0.5 ppm)

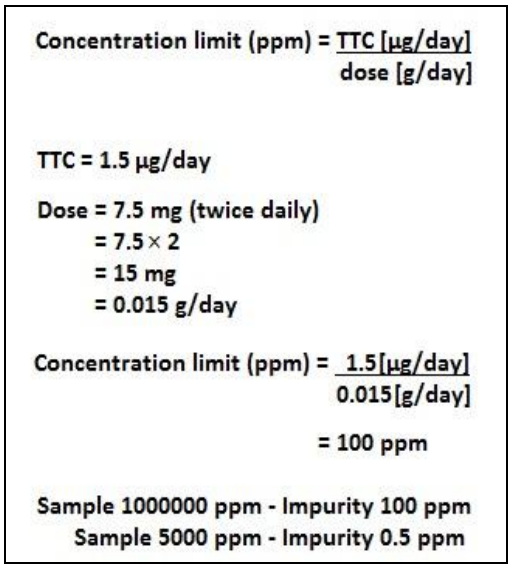

FIG. 2: TTC BASED CALCULATION FOR LIMIT LEVEL CONCENTRATION

2.3.3.1 Reference stock solution (10 ppm): About 50 mg of MVK was accurately weighed and transferred in a 50 mL volumetric flask. Approximately 25 mL of the diluent was added, sonicated to dissolve and then diluted upto the mark with the diluent and mixed well. 1 mL of the above solution was diluted to 100 mL with the diluent and mixed well.

2.3.3.2 Reference solution (0.5 ppm): 2.5 mL of the above stock solution (10 ppm) was diluted to 50 mL with the diluent and mixed well.

2.3.4 Sample preparation for the drug (5000 ppm): About 250 mg of Ivabradine hydrochloride was accurately weighed and transferred in a 50 mL volumetric flask. Approximately 25 mL of the diluent was added, sonicated to dissolve and then diluted upto the mark with the diluent and mixed well.

2.4 Final Optimized Method:

- Mobile phase - Buffer (pH 2.5) : ACN [Gradient Elution - **Table 1**]
- Buffer - 10 mM Sodium perchlorate, pH 2.5 using Perchloric acid
- Column - Zorbax SB Phenyl column (150 mm x 4.6 mm, 3.5 µ)
- Column temperature - 50°C
- Detection wavelength - 210 nm
- Injection volume - 5 µL
- Flow rate - 1.0 mL/min
- Run time - 22 minutes

TABLE 1: GRADIENT ELUTION PROGRAM FOR THE DEVELOPED HPLC METHOD

Time	% Buffer	% ACN
00.10	90	10
03.00	90	10
07.00	80	20
10.00	70	30
12.00	30	70
15.00	30	70
15.10	90	10
22.00	90	10

2.5 Method Validation: Method Validation is the procedure used to confirm that the analytical process employed for a specific test is suitable for its intended use. [23] Analytical method development and validation is required by regulatory authorities like ICH, USP, US FDA for marketing authorizations and guidelines have been published by these respective authorities. [24-26]

2.5.1 System Suitability: Reference solution of the PGI was prepared in duplicate at 100 % test concentration (0.5 ppm) namely standard A and standard B. Initially, blank was injected followed by standard A (injected six times) and standard B (once). % Relative Standard Deviation (RSD) of retention time and peak area for standard A was calculated. Similarity factor, theoretical plates, tailing factor and symmetry factor was also determined.

2.5.2 Specificity: Specificity was demonstrated by injecting the following sequence of solutions: Blank [Diluent - methanol], Identification (ID) solutions [MVK standard (0.5 ppm, Ivabradine HCl

(Sample) (5000 ppm)], Sample (Drug - 5000 ppm) spiked with the genotoxic impurity MVK - 0.5 ppm. The chromatograms were checked for interference at the retention time of MVK.

2.5.3 Limit of quantitation (LOQ): For the preparation of LOQ Level (0.2 ppm) solution, 4 mL of the reference solution of the PGI (0.5 ppm) was transferred in a 10 mL volumetric flask and diluted up to the mark with diluent. The prepared solution was injected six times, and an optimum signal-to-noise ratio (SNR) was obtained. Thus the method's LOQ Level was set at 0.2 ppm. **Fig. 3** depicts the chromatogram of the LOQ Level for MVK.

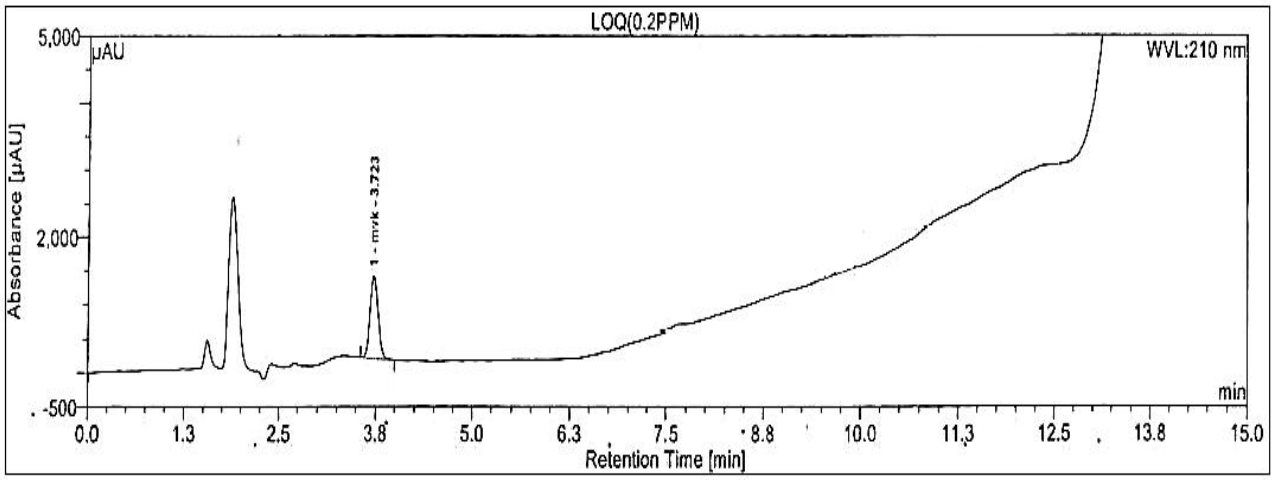

FIG. 3: LOQ LEVEL CHROMATOGRAM FOR MVK

2.5.4 Limit of detection (LOD): For the preparation of LOD Level (0.06 ppm) solution, 3 mL of the LOQ Level solution (0.2 ppm) was transferred in a 10 mL volumetric flask and diluted up to the mark with diluent. The prepared solution was injected thrice, and an optimum SNR was obtained. Thus the methods LOD Level was set at 0.06 ppm.

2.5.5 Linearity: Linearity was evaluated in the range of LOQ (40 %) to 150 % of the limit level concentration. The limit level concentration for MVK was set at 0.5 ppm. The linearity response was determined by preparing six different concentrations of standard solutions corresponding to six different linearity levels namely 40 %, 50 %, 80 %, 100 %, 120 % and 150 % of the limit level concentration as detailed in **Table 2**. Each level was injected in duplicate except for the levels LOQ and 150 %, which was injected 3 times. The average peak area was calculated for each level. % RSD of retention time and peak area was calculated for the levels LOQ and 150 %. A graph of concentration (ppm) v/s peak area was plotted and the regression coefficient (R^2), slope of the regression line and % Y intercept (as shown in **Fig. 4**) was determined.

$$\% \text{ Y intercept} = \frac{\text{Y intercept}}{\text{Peak area at Level 100 \% (i.e. limit level concentration)}} \times 100$$

FIG. 4: FORMULA FOR CALCULATION OF % Y INTERCEPT

TABLE 2: PREPARATION OF LINEARITY LEVEL SOLUTIONS

Linearity Level	Volume of Reference Stock Solution (10 ppm) Pipetted (mL)	Final Volume (mL)	Concentration (ppm)
Level I (LOQ)	1.00	50	0.20
Level II (50 %)	1.25	50	0.25
Level III (80 %)	2.00	50	0.40
Level IV (100 %)	2.50	50	0.50
Level V (120 %)	3.00	50	0.60
Level VI (150 %)	3.75	50	0.75

2.5.6 Accuracy (By Recovery): The accuracy of the procedure was demonstrated by recovery studies which were carried out by spiking the sample (drug) solution (5000 ppm) with MVK at four different levels namely 0.2 ppm (LOQ), 0.25 ppm (50 %), 0.5 ppm (100 %) and 0.75 ppm (150

%) as detailed in **Table 3**. Three replicates were injected for each level and % recovery was calculated using the formula depicted in **Fig. 5**. Further, mean (% recovery) for each level was calculated.

$$\% \text{ Recovery} = \frac{\text{Area (Sample spiked with the genotoxic impurity)} - \text{Area (Sample as such)}}{\text{Area (Genotoxic impurity in Reference solution)}} \times 100$$

FIG. 5: FORMULA FOR CALCULATION OF % RECOVERY FOR THE SPIKED GENOTOXIC IMPURITY

TABLE 3: PREPARATION OF ACCURACY SPIKED SAMPLE SOLUTIONS

Impurity Level Spiked in the Sample	Amount of Sample added (mg)	Volume of Reference Stock Solution (10 ppm) added (mL)	Final Volume (mL)
LOQ (40 %)	250	1.00	50
50 %	250	1.25	50
Limit level (100 %)	250	2.50	50
150 %	250	3.75	50

2.5.7 Precision: To determine precision, the reference solution for the PGI - 0.5 ppm (Level 100 % i.e. limit level concentration) was prepared and injected six times into the chromatographic system. % RSD of retention time and peak area for the six injections was calculated.

3. RESULTS AND DISCUSSION:

3.1 Method Development: MVK is a known PGI belonging to the structural class of α,β–unsaturated carbonyl compounds. Due to the common solubility of Ivabradine HCl and MVK in methanol, it was selected as the diluent. As per ICH M7 Guideline, MVK belongs to 'Class 3' Mutagenic impurities which needs to be controlled below TTC limits. Thus, its limit was set as 0.5 ppm with respect to Sample (5000 ppm). Method development was initiated on an Inertsil C18 column (250 mm x 4.6 mm, 5.0 µ) using Water: Acetonitrile (90:10, v/v) as the mobile phase. A flow rate of 1.0 mL/min, column oven temperature of 30°C and detection wavelength of 210 nm were set as the initial chromatographic conditions. The PGI was poorly retained as well as the peak showed typical tailing. To reduce peak tailing, 0.1 % TEA was incorporated in the mobile phase. The Inertsil C18 column was replaced by Inertsil Phenyl column to improve the retention of the analyte.

However, the baseline appeared to be noisy. So, to reduce peak tailing without baseline disturbance, the column was replaced by an endcapped Zorbax SB Phenyl column as well as the mobile phase was replaced back to Water: ACN (90 :10). But the retention time of MVK was delayed. The following trial introduced a gradient elution program with a run time of 20 minutes. The observation was that MVK was poorly retained and also the peak shape was not appreciable. Consequently, in the next trial, the gradient elution program was further optimized to retain MVK without increasing the run time significantly. To improve the peak shape appreciably, Sodium perchlorate buffer (adjusted to pH 2.5 with perchloric acid) was incorporated in the mobile phase. Finally, an appreciable peak shape with a run time of 22 minutes was achieved with an optimum retention time for MVK (3.7 minutes).

3.2 Method Validation:

3.2.1 System suitability: System suitability parameters of method validation were found to be within limits, thus the system was suitable for the method. The results obtained are summarized in **Table 4**.

TABLE 4: SYSTEM SUITABILITY RESULTS

System Suitability Parameters	Results obtained for MVK	Acceptance Criteria
% RSD of Retention time (six replicates)	0.02	NMT 1 %
% RSD of Peak area (six replicates)	0.60	NMT 5 %
Theoretical plates	5677	NLT 2000
Tailing factor	1.2	Between 0.8 and 2.0
Symmetry factor	1.2	Between 0.8 and 2.0
Similarity factor	1.01	Between 0.98 and 1.02

3.2.2 Specificity: The retention time of MVK and Ivabradine HCl were 3.725 minutes and 13.852 minutes respectively. The specificity of the method was validated by absence of interference from blank and sample at the retention time of the main peak namely MVK.

Identification of the peaks in the spiked sample was done by comparing their retention time with the retention time of the identification (ID) solutions. Thus, the method was found to be specific for the analysis of MVK in Ivabradine HCl sample matrix.

3.2.3 LOD AND LOQ: The LOD level should have a SNR ≥ 3 and the LOQ level should have a SNR ≥ 10. Thus, LOD level was set at 0.06 ppm (SNR = 46) and LOQ level was set at 0.2 ppm (SNR = 155).

3.2.4 Linearity: A Linearity curve of concentration (ppm) v/s peak area was plotted for MVK as shown in **Fig. 6** respectively. Results are summarized in **Table 5** and **Table 6**. The linearity parameters (R^2, % Y intercept, % RSD of retention time and peak area for the levels LOQ and 150 %) of method validation for the analysis of MVK were found to be within the acceptance criteria. Thus, the results obtained in the specified range for intended application, i.e. LOQ (40 %) to 150 % of working level concentration, proved linearity.

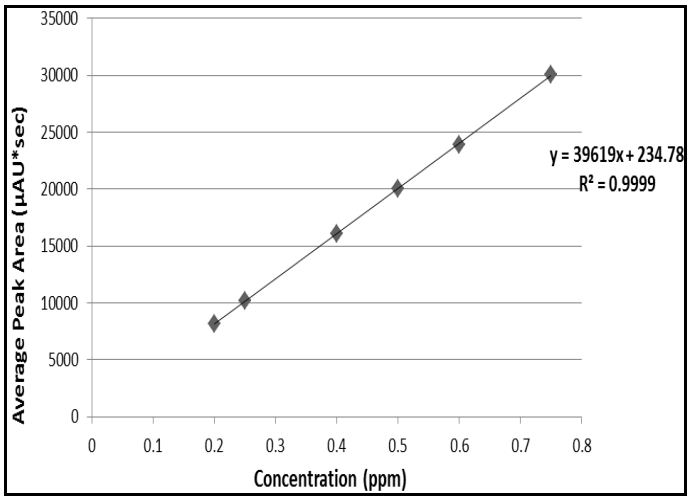

FIG. 6: LINEARITY CURVE OF METHYL VINYL KETONE (LOQ TO 150 %)

TABLE 5: RESULTS OBTAINED FOR THE AVERAGE PEAK AREA FOR LINEARITY LEVELS OF MVK

Linearity Level	Concentration (ppm)	Average Peak Area (µAU*sec)
Level I (LOQ)	0.20	8183
Level II (50 %)	0.25	10163
Level III (80 %)	0.40	16050
Level IV (100 %)	0.50	20059
Level V (120 %)	0.60	23888
Level VI (150 %)	0.75	30036

TABLE 6: SUMMARY - LINEARITY RESULTS OF MVK

Linearity Parameters	For MVK	Acceptance Criteria
% RSD of RT (LOQ level)	0.1	NMT 1 %
% RSD of Peak Area (LOQ level)	0.8	NMT 5 %
% RSD of RT (Level 150 %)	0.02	NMT 1 %
% RSD of Peak Area (Level 150 %)	0.1	NMT 5 %
R square	0.9999	≥ 0.999
% Y intercept	1.2	± 2.0 %

3.2.5 Accuracy (By Recovery): The mean % recovery for MVK at LOQ level as well as at 50 %, 100 % and 150 % levels were found to be within the acceptance criteria. The results for accuracy are summarized in **Table 7**.

TABLE 7: SUMMARY - ACCURACY RESULTS FOR MVK

Level Spiked	Mean % Recovery	Acceptance
LOQ	97.55	80-120
50 %	94.78	85-115
100 %	95.84	
150 %	96.69	

3.2.6 Precision: The % RSD of retention time and peak area for six replicates of standard solution at limit level (0.5 ppm) was found to be 0.02 % and 0.60 % respectively. It was found to meet the acceptance criteria of NMT 1% (for Retention time) and NMT 5 % (for Peak Area) respectively.

3.3 Application of the method - Sample analysis: The developed HPLC method was used to analyze three different batches of the bulk drug Ivabradine hydrochloride to demonstrate that MVK was controlled below TTC level in the bulk drug. It was observed that MVK was found to be not detected (ND) in the three different batches of the bulk drug.

CONCLUSION: This article is based on the development and validation of a simple and sensitive HPLC procedure suitable for the analysis of PGI MVK in the drug substance Ivabradine HCl. It was demonstrated that the procedure developed is specific, linear, accurate and precise. This Reverse Phase HPLC method has significant application in the identification and trace level quantification of the potential genotoxic impurities.

CONFLICT OF INTEREST: Corresponding author Deepali Gangrade and co-author Jessica D'Souza declare that they have no conflict of interest.

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