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Research Article

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Method development and validation for quantitative determination of 2-acetoxy methyl-4-methoxy-3,5-dimethyl pyridine, an impurity, in esomeprazole magnesium (API) active pharmaceutical ingredient by LC-ESI-MS/MS

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ABSTRACT

A simple, sensitive and rapid LC- ESI-MS/MS method has been developed and validated for the trace analysis (>1 ppm level) of 2-acetoxy methyl-4-methoxy-3,5-dimethyl pyridine, a genotoxic impurity, in esomeprazole magnesium drug. The chromatographic separation was achieved on a hypersil BDS (150 x 4.6 mm, 5 µm) column using a mobile phase consisting of 5 mM ammonium acetate buffer (pH 4) and acetonitrile (60:40, v/v) at flow rate of 0.7 mL/min and elution was monitored at 305nm. The API-4000 LC-MS/MS was operated on an electrospray ionization equipped with an ESI interface operated in positive ionization (single reaction monitoring) mode and it is able to quantitate up to 0.3 ppm of 2- acetoxy methyl-4-methoxy-3,5-dimethyl pyridine. The newly developed method was validated as per ICH guidelines.

Keywords: Esomeprazole magnesium, Method Validation, LC-ESI-MS/MS, Trace analysis.

INTRODUCTION

Esomeprazole is a proton pump inhibitor which reduces acid secretion through inhibition of ATPase in gastric parietal cells. By inhibiting the functioning of this enzyme, the drug prevents formation of gastric acid. Its chemical name is 5-methoxy-2-[[(4-methoxy-3, 5-dimethyl-2-pyridinyl) methyl] sulphinyl]-1H-benzimidazole. It is a substituted benzimidazole compound and prototype anti-secretary agent. It is the first of the "proton pump inhibitors" widely used for the prophylaxis and treatment of gastro-duodenal ulcers and symptomatic gastro-esophageal reflux [1]. Helicobacter pylori infection is the main cause of gastritis, gastroduodenal ulcer disease and gastric cancer. Esomeprazole provides better controle of intragastric pH than omeprazole, lansoprazole, pantoprazole and rabeprazole. Consequently, esomeprazole produces higher healing rates of erosive oesophagitis and better symptom control than omeprazole in patients with gastro-oesophgeal reflux disease. Esomeprazole has a higher degree of activity against H.pylori than other PPIs as omprazole the increased antimicrobial activity in vitro of esomeprazole against H. pyroli could contribute to improving the outcome of the eradication treatment of such an infection [2].

Pharmaceutical genotoxic impurities (PGIs) may induce genetic mutations, chromosomal breaks (rearragements) and they have potential to cause cancer in human [3-4]. Therefore exposure to even low levels of such impurities present in final active pharmaceutical ingredient (API) may be of significant toxicol importance [5]. Hence it is

significant for process chemists to avoid such genotoxic impurities in the manufacturing process [6]. However it would be difficult or impossible to eliminate PGIs completely from the synthetic scheme. Therefore it is a great challenge to analytical chemists to develop an appropriate analytical method to quantify the impurity accurately and control their levels in APIs. According to the European Medicines Evaluation Agency (EMEA) and feedback from US Food and Drug Administration (USFDA) the proposed use of a threshold of toxicological concern (TTC), it is accepted that genotoxic impurities will be limited to a daily dose of 1.0-1.5 µg/day [7, 8].

2-acetoxy methyl-4-methoxy-3,5-dimethyl pyridine is often used during the manufacture of active pharmaceutical ingredient (APIs), either as counter ion or form salt or as the result of protecting group removal during the synthesis [9]. In fact 2-acetoxy methyl-4-methoxy-3,5- dimethyl pyridine is a known genotoxic impurity and carcinogen in rats and mice [10]. The potential occurrence of these genotoxic impurities has attracted the attention of regulatory authorities. 2-acetoxy methyl-4-methoxy-3, 5-dimethyl pyridine is a known carcinogen; this data would determine that the regulatory authorities may be expected to control the levels of this impurity to be 0.3 ppm in the drug substance (assuming a 1.5 µg/day daily dose). A method that accomplishes of such a lower level of detection is great challenge for analytical method development for controlling these genotoxic impurities. Ideally conventional analytical instruments in pharmaceutical industries such as HPLC with UV detection and GC with FID detection should be employed as the standards in first attempt for PGIs analysis [11, 12], but there are some drawbacks with above mentioned techniques because HPLC retention times can vary, uncertainty can arise as to whether a peak at a new retention time is a new impurity. When impurity standards are not available some method is needed to characterize the impurities online [13]. Therefore for accurate determination at ppm levels the above mentioned techniques are insufficient consequently there is a great need to develop better analytical method for the analysis of such genotoxic impurities in pharmaceutical industries. As a result various kinds of chromatographic techniques and methodologies have been explored as useful approaches [14, 15].

Although there are different methods including UV [16], HPLC [17-25] and UPLC [1, 26], are available in the literature there is no single method to determination of 2-acetoxy methyl-4-methoxy-3, 5-dimethyl pyridine in esomeprazole (API). In the present paper, a simple, sensitive and rapid LC-MS/MS validated method has been proposed for determination of 2-acetoxy methyl-4-methoxy-3, 5-dimethyl pyridine in esomeprazole active pharmaceutical ingredient. Because of its higher sensitivity and selectivity LC-MS/MS has been adopted for quantification of 2-acetoxy methyl-4-methoxy-3, 5-dimethyl pyridine in esomeprazole which is used for the prevention and treatment of gastric acid related diseases

EXPERIMENTAL SECTION

2.1. Chemicals and reagents

Methanol and acetonitrile of HPLC grade were purchased from J.T Baker (Phillipsburg, USA). Analytical grade ammonium acetate, formic acid and HPLC grade water were purchased from Merck, (Mumbai, India). Water used for the LC-MS/MS analysis was prepared from Milli Q water purification system procured from Millipore (Bangalore, India). Reference substance of 2-acetoxy methyl-4-methoxy-3,5-dimethyl pyridine was obtained from Sigma- Aldrich (St. Louis, USA).

2.2. Preparation of stock and standard solutions

Primary stock solutions of 2-acetoxy methyl-4-methoxy-3,5-dimethyl pyridine and esomeprazole were prepared in methanol (1 mg/mL). Another set of working stock standard solution of 0.001 mg/mL was achieved on further dilution with mobile phase. The stock solutions stored at 2-8°C were found to be long term stability for 20 days (data not shown), consecutively diluted with methanol to final concentration (7.5 ng/mL) to get working solutions for obtaining calibration curve.

2.3. HPLC operating conditions

A Shimadzu LC-20 AD Series HPLC system (Shimadzu Corporation, Kyoto, Japan) was used to inject 20 µL aliquots of the processed samples on a Hypersil BDS column (150x 4.6 mm, 5 µm), which was kept at 40 ± 2°C temperature. The isocratic mobile phase, a mixture of 5 mM ammonium acetate (pH 4): acetonitrile (60:40, v/v) was filtered through a 0.45 µm membrane filter (XI5522050) (Millipore, USA or equivalent), then degassed ultrasonically for 5 min and delivered at a flow rate of 0.7 mL/min into the mass spectrometer electrospray ionization chamber.

2.4. Mass spectrometry operating conditions

Quantitation was achieved with MS-MS detection using a MDS Sciex API-4000 mass spectrometer (Foster City, CA, USA) equipped with Turboionspray ™ interface at 400 ºC. The MS/MS method consists of positive ionization mode. The ion spray voltage was set at 5000 V. The source parameters viz., the ion source gases GS1, GS2 and curtain gas were set at 30, 25, and 12 psi, respectively. The compound parameter viz. the declustering potential (DP) was set at 52. Detection of the ions was carried out in the selective ion monitoring mode (SIM) was considered to get better selectivity, by monitoring the 2- acetoxy methyl-4-methoxy-3,5 dimethyl pyridine of $[M+H]^+$ *m/z* 210.3 precursor ion and the $[M+H]^+$ *m/z* 346.3 precursor ion for esomeprazole. The analytical data obtained were processed by Analyst software™ (version 1.4.2).

RESULTS AND DISCUSSION

3.1. Method development and optimization

Optimization of chromatographic conditions was performed, particularly the composition of mobile phase, through several trials to achieve symmetric peak shapes of the analytes peaks, as well as short run time. Resolution positive mode esomeprazole was achieved by using acetonitrile as an organic content in the mobile phase. Separation was attempted using various combinations of acetonitrile and buffer with varying contents of each component on different columns like C_8 and C_{18} of different makes like Chromolith, Hypersil, Hypurity advance, Zorbax, Kromasil and Inertsil. Hypersil BDS column was found to give the best chromatographic resolution with a flow rate of 0.7 mL/min and total run time of 15 min. The 2- Acetoxy methyl-4-methoxy-3,5 dimethyl pyridine and esomeprazole were eluted at 6.9 min and 9.1min with selective ion monitoring (SIM) mode. The inclusion of 5 mM ammonium acetate instead of pure water enhanced the response and improved the reproducibility.

3.2. Method validation

3.2.1. Specificity and selectivity

Specificity is the ability of the method to assess unequivocally the analyte response in presence of components that may be expected to be present in the sample. Esomeprazole and 2-acetoxy methyl-4-methoxy-3,5-dimethyl pyridine solutions were prepared individually at a concentration of about 0.01mg/mL in the diluents and a solution of esomeprazole spiked with 2-acetoxy methyl-4-methoxy-3,5-dimethyl pyridine was also prepared. Specificity was established by injecting esomeprazole spiked with its impurity where in no interference was observed. Blank and specificity chromatograms are shown in Fig. 1.

3.2.2. Robustness

The robustness of the developed method was studied with slight and deliberate changes in experimental conditions. The effect of changes in flow rate of mobile phase (-10% to +10%), % of organic modifier in mobile phase (-2% to +2%) while the amounts of the other mobile phase components were held constant, column oven temperature (-2°C to +2°C).i.e at 38 ºC and 42 ºC and pH of the buffer (-0.2 units to +0.2 units) was studied. For all the above deliberately varied experimental conditions, that these changes do not impact on chromatographic performance.

3.2.3. Determination of LOD and LOQ

The LOD and LOQ, as a measure of method sensitivity, were calculated from S/N (signal to noise) ratios. To determine LOD and LOQ values for a 2-acetoxy methyl-4-methoxy-3,5 dimethyl pyridine concentrations were reduced sequentially such that they yield S/N ratio as 3.2 and 10.1 respectively. The determined LOD and LOQ chromatograms were shown in Fig.2. Data generated from six injections of 2-acetoxy methyl-4-methoxy-3,5-dimethyl pyridine (without API) containing 0.3 ppm of each 2-acetoxy methyl-4-methoxy-3,5-dimethyl pyridine with respect to an API sample concentration 10 mg/mL. The LOQ of 0.3 ppm is typical for the 2- acetoxy methyl-4-methoxy-3,5-dimethyl pyridine, with a LOD approximately three times less than LOQ. In addition, the relative efficiency of SIM versus MRM (SRM) modes in sensitivity improvement was also evaluated [13]. We found that, in SIM mode the LOD was 0.1 ppm, whereas with SRM/MRM was 0.3 ppm, the corresponding chromatograms are not shown.

3.2.4. Recovery studies

The recovery studies by the standard addition method were performed to evaluate accuracy and specificity, accordingly the accuracy of the method was determined in triplicate at LOQ level in bulk drug sample. The recoveries were calculated. Excellent recovery values of 2-acetoxy methyl-4-methoxy-3,5-dimethyl pyridine (100-102%) was obtained. At such a low levels these recoveries and %RSD is <1.0 was satisfactory. Sample and

accuracy at LOQ chromatograms are shown in Fig.3, and the relative standard deviation, %RSD were calculated from the average of triplicate analysis, which were shown in Table1. Further, the stability of 2-acetoxy methyl-4-methoxy-3,5-dimethyl pyridine was found as 48 hr and the stability of this impurity at different time intervals is presented in Table 3.

3.2.5. Linearity and range

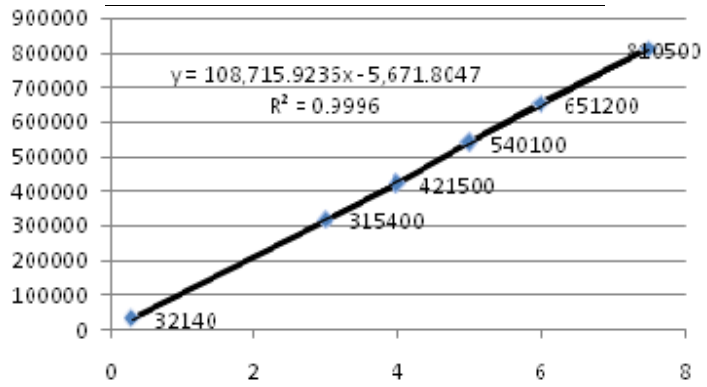
The linearity test for the method was performed according to the guidelines laid by ICH. This method was evaluated at six different concentrations of analytes with in the range of 0.3 – 7.5 ng/mL. These standard solutions were prepared by suitable dilution of stock solution with mobile phase. The linearity of the plot was evaluated using least squares linear regression analysis by selective ion monitoring (SIM). The linearity of 2-acetoxy methyl-4-methoxy-3,5-dimethyl pyridine was satisfactorily established with a six point calibration curve between LOQ and 150% of analyte concentrations (60, 80, 100, 120 and 150 %). The calibration curve was produced by plotting the average of triplicate 2-acetoxy methyl-4-methoxy-3,5-dimethyl pyridine injections against the concentrations expressed in percentage. The slope, intercept and correlation coefficient values were derived from linear least-square regression analysis and the data were presented in Table 2. It reveals that good correlation existed between the peak areas concentration of 2-acetoxy methyl-4-methoxy-3,5-dimethyl pyridine. Repeatability was checked by calculating the relative standard deviation (%RSD) of six determinations by injecting six freshly prepared solutions containing 0.3 ppm of 2-acetoxy methyl-4-methoxy-3,5-dimethyl pyridine on the same day. The low %RSD values confirm the good precision of the developed method.

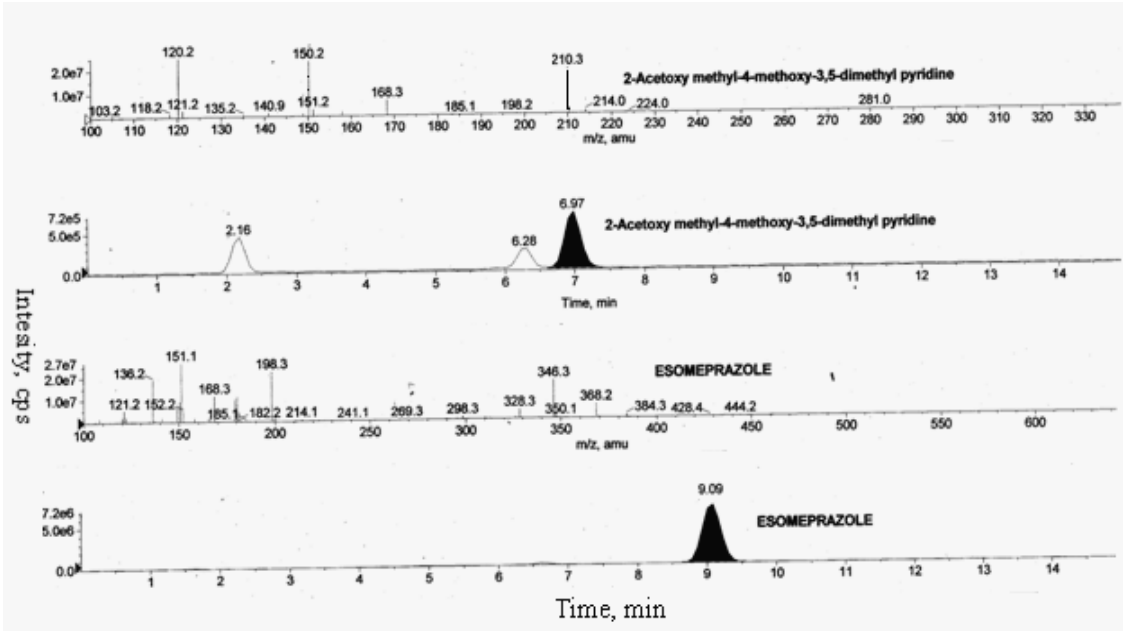
Table 1: Accuracy/recovery of 2- Acetoxy methyl-4-methoxy-3,5 dimethyl pyridine at 0.3 ppm concentration

Sample area	Standard area	Spiked area	Theoretical concentration	Measured concentration	%Recovery
0	32,300	32,2100.30	0.3002		100.08
0	32,300	32,1700.30	0.3041		101.37
0	32,300	35,2500.30	0.3026		100.86
				Average	100.77
				Std dev	0.651
				%RSD	0.65

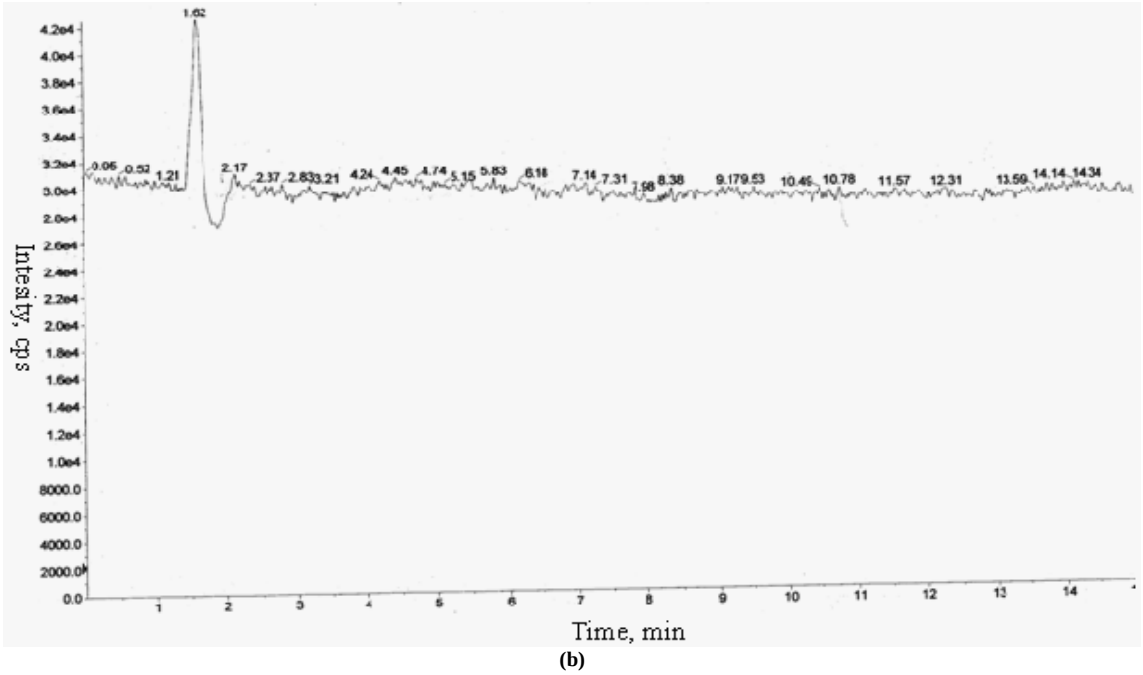
Table 2: Linearity

Concentration (ppm)	Peak area
0.3	32140
3	315400
4	421500
5	540100
6	651200
7.5	810500
Correlation	0.9998
Slope	108715.9236
Intercept	-5671.8047





(a)



(b)

Fig.1. (a) Specificity and (b) blank chromatograms of 2- Acetoxy methyl-4-methoxy-3,5-dimethyl pyridine

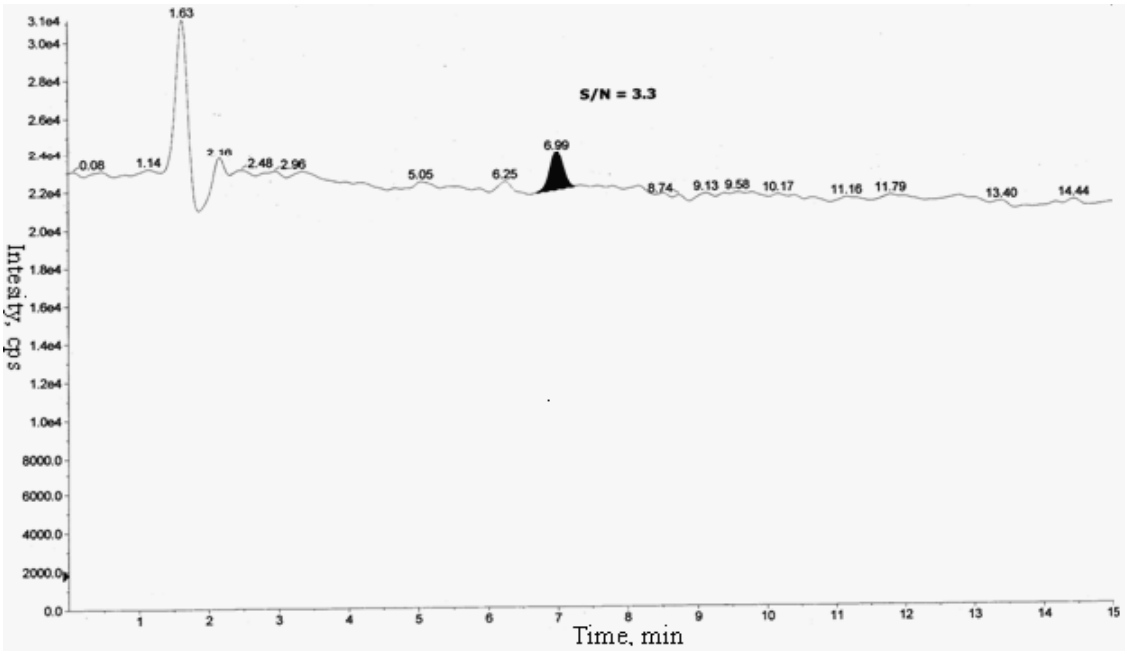


Fig.2. (a) LOD chromatogram

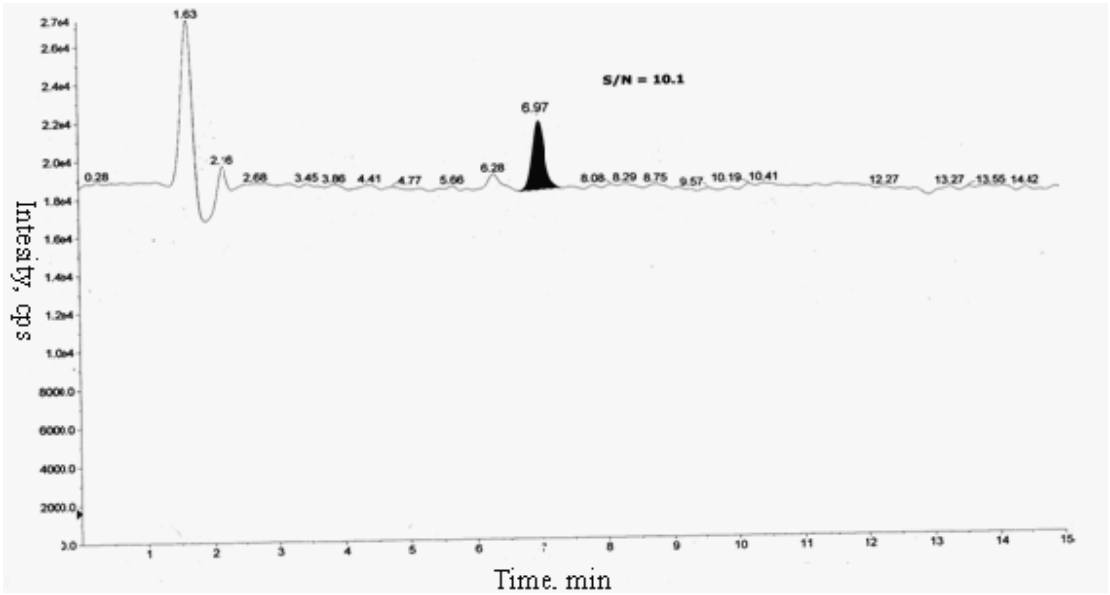
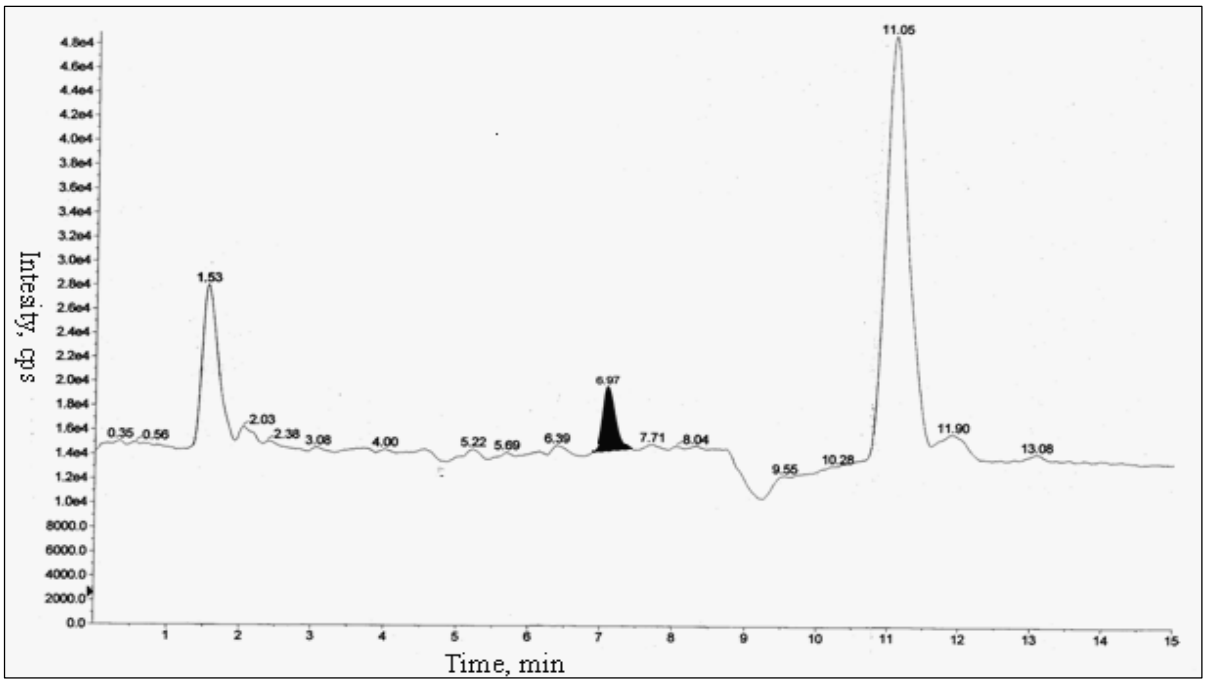


Fig.2. LOD and LOQ chromatograms of 2- Acetoxy methyl-4-methoxy-3, 5 dimethyl pyridine

Table 3: Solution stability data of 2- Acetoxy methyl-4-methoxy-3,5 dimethyl pyridine in diluents

Sample area, Injection time (hr)	Standard area	Spiked area	Theoretical concentration	Measured concentration	%Recovery
Level-I 0	32527	32010	0.3	0.2952	98.41
Level-II 0	32527	31990	0.3	0.2950	98.35
12hrs					
Level-I 0	33450	33110	0.3	0.2970	98.98
Level-II 0	33450	32980	0.3	0.2958	98.59
24hrs					
Level-I 0	31450	31020	0.3	0.2959	98.63
Level-II 0	31450	30980	0.3	0.2955	98.51
48hrs					
Level-I 0	32180	32240	0.3	0.3006	100.19
Level-II 0	32180	31980	0.3	0.2981	99.38



3. (a) Sample Chromatogram of esomeprazol

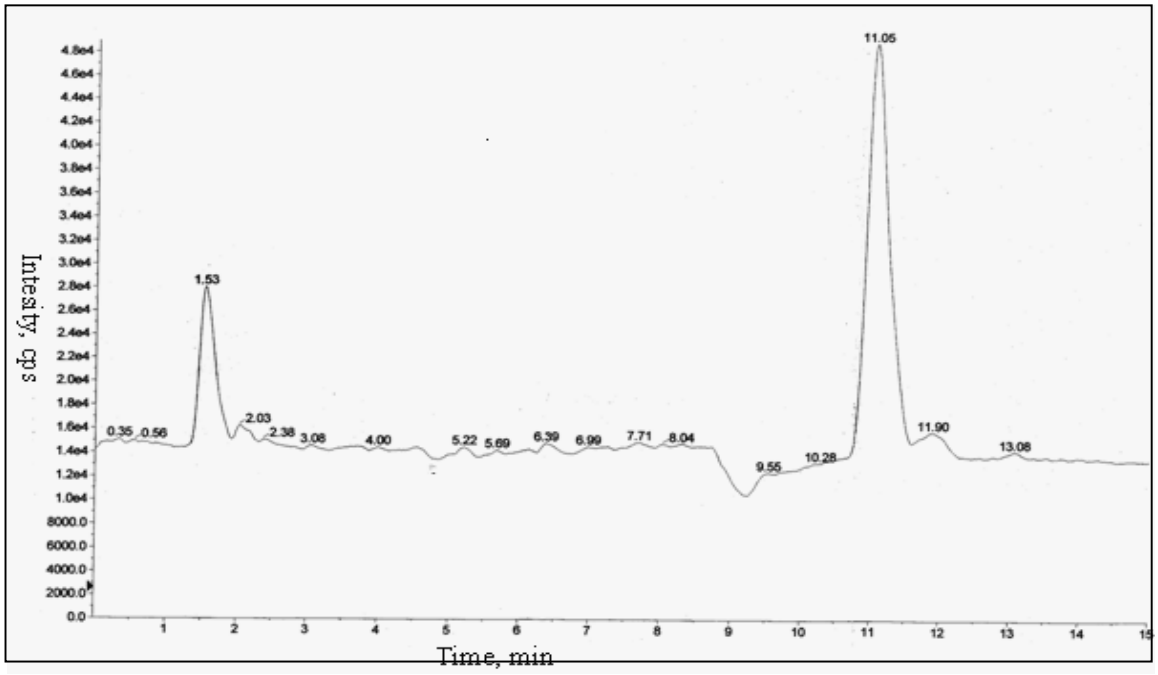


Fig.3. (a) Sample chromatogram of esomeprazole and (b) accuracy chromatogram of 2- Acetoxy methyl-4-methoxy-3, 5 dimethyl pyridine

CONCLUSION

The present development study is based on validation of a highly sensitive, specific, reproducible and high-throughput LC-MS/MS method to quantification of 2-acetoxy methyl-4-methoxy-3,5-dimethyl pyridine in APIs. It has been established that it is highly sensitive with a limit of detection (LOD) of 0.1ppm. Trace level ammonium

acetate is added to the mobile phase to enhance ionization and detection. Selected sample solvents were assessed for the effect on standard stability with and without presence of API. As a systematic approach, it is very important to utilize the comprehensive chromatographic knowledge gained throughout the lifecycle of the development of a drug candidate based on continuous understanding of the API manufacturing process. The method which is able to quantify them at ppm level is developed and validated. We can conclude that the developed method could be very useful for monitoring of 2- acetoxy methyl-4-methoxy-3,5-dimethyl pyridine in esomeprazole in its pure and tablet form.

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