Analytical Chemistry Insights

ORIGINAL RESEARCH

Low Level Determinations of Methyl Methanesulfonate and Ethyl Methanesulfonate Impurities in Emtricitabine Active Pharmaceutical Ingredient by LC/MS/MS Using Electrospray Ionization

P.R. Kakadiya[1], T.G. Chandrashekhar[1], S. Ganguly[1], D.K. Singh[2] and V. Singh[1]

[1]Ranbaxy Research Laboratory, Udyog Vihar Industrial Area, Gurgaon-122015, Haryana, India. [2]Analytical Research Laboratory, Department of Chemistry, Harcourt Butler Technological Institute, Kanpur-208002, Uttar Pradesh, India.
Corresponding author email: vandana.singh@ranbaxy.com

Abstract: Alkyl methanesulfonates have been highlighted as potential genotoxic impurities (PGIs). A sensitive LC/MS/MS method was developed and validated for the determination of Alkyl methanesulfonate impurities in Emtricitabine API (active pharmaceutical ingredient). LC/MS/MS method on Zorbax SB C_{18} column (150 × 4.6 mm i.d.), 3.5 µm, with electrospray ionization (ESI) in multiple reaction monitoring (MRM) mode was used. The proposed method was specific, linear, accurate, rugged and precise. The calibration curves showed good linearity over the concentration range of 0.0025 µg/ml to 0.3 µg/ml the correlation coefficient was >0.999 in each case. Method had very low limit of detection (LOD) and limit of quantification (LOQ) as 0.3 µg/g and 0.4 µg/g respectively for both the analytes. Accuracy was observed within 80%–120% for both the analytes. This method can be further extended a good quality control tool for low level quantitation of Alkyl methanesulfonate impurities in other API.

Keywords: methyl methanesulfonate, ethyl methanesulfonate, LC/MS/MS, Emtricitabine, genotoxic impurities, trace analysis

Analytical Chemistry Insights 2011:6 21–28

doi: 10.4137/ACI.S6471

This article is available from http://www.la-press.com.

Introduction

Emtricitabine is a nucleoside reverse transcriptase inhibitor for the treatment of HIV (Human immunodeficiency virus) infection in adults. The drug works by inhibiting reverse transcriptase enzyme which copies HIV RNA (Ribonucleic acid) into new viral DNA (Deoxyribonucleic acid). Emtricitabine is often administered in combination with Tenofovir disoproxil fumarate, the maximum daily dose of Emtricitabine is 0.2 g per day.

Synthetic starting materials and intermediates are reactive by design and may occur as impurities in the final API. The nature of this chemical reactivity can often translate into biological reactivity and these materials can often be mutagens or carcinogens. Many times it has been established that due to high chemical reactivities the fate of the several alkylating agents precluded their retention within the final API especially if their formation was separated from the final API by several synthetic steps.

Methyl methanesulfonate (MMS) and ethyl methanesulfonate (EMS) are often used during manufacture of pharmaceuticals, either as a counter-ion to form a salt, as acid catalyst or as a result of protecting group removal during the synthesis. However, the presence of alcohol either in any of the stages of synthesis, or the crystallization stage of the salt may cause the formation of sulfonic acid esters which are considered to be potential genotoxic agents.[1] These potential genotoxic impurities (PGIs) are known to induce genetic mutations or chromosomal aberrations and are reported as known carcinogens in rats and mice.[2] The potential presence of these genotoxins has attracted the attention of regulatory authorities. European Medicines Agency's (EMEA) Committee for Medicinal products for Human use (CHMP) has published guidelines regarding limits of genotoxic impurities.[3] Recently, in 2008 US FDA (United States Food and drug administration) has also come up with the draft guidelines on genotoxic and carcinogenic impurities in drug substances and products.[4] These guidelines describe ways to reduce the potential lifetime cancer risk associated with patient exposure to genotoxic and carcinogenic impurities and the ways to reduce them. A maximum daily exposure target of 1.5 µg per day [acceptable Threshold of Toxicological Concern (TTC)] is recommended in these guidelines.[3–5]

Based on the maximum daily dosage of Emtricitabine MMS and EMS are required to be controlled at a combined limit of 7.5 µg/g in the API.

Due to the increasing concern from the regulatory perspective in relation to the potential hazards, there has been a general renaissance and increased number of analytical techniques, mainly gas chromatographic methods utilizing both flame ionization detector and mass spectrometric detectors are reported in literature for the determination of alkyl sulfonate impurities. But these methods have drawbacks of either higher limit of quantitation (LOQ) or limit of detection (LOD). Capillary GC/MS (Gas chromatography-mass spectrometry) method[6] for the determination of EMS has been reported within the linear range of 50–200 µg/g. GC-FID (gas chromatography-flame ionization detector) method[7] have been reported for the determination of alkyl methanesulfonates but this method had the drawback of higher limit of detection. Moreover, GC-FID not being a very selective and specific technique. Direct injection techniques both on GC and HPLC (high performance liquid chromatography) are also reported in the literature. Quantitative GC/MS method involving DB-WAX column, using single ion-monitoring mode (SIM) have been reported using m/z of 78.98 as the common peak. GC-FID method using DB-WAX column[8] for determination of residual MMS and EMS suffered from the drawback of matrix interference due to build up of residual API and required frequent replacement of injection liner after 20 min, further the method had higher LOD and LOQ of 1 µg/g and 5 µg/g respectively.

Extraction based studies have also been reported to deal with the matrix related issues and aimed to clean up the matrix. The frequently used sample preparation technique being the liquid-liquid extraction (LLE)[9] for pre-concentration and matrix removal. LLE preconcentration with GC/MS detection have been reported for determination of MMS and EMS but these methodologies are labour-intensive and are prone to interferences from other solvents are well as emulsion formation. Moreover, the resulting sample preparation method requires extra validation. Limit test method using sample preparation techniques like SPME (solid phase micro extraction), LPME (liquid phase micro extraction) and SPE (solid phase extraction) followed by GC/MS have been reported,[9] with a very high limit of detection of 5 µg/g.

Other than direct analysis, derivatization methods[10,11] are also been reported for the determination of MMS and EMS involving derivatization with aqueous sodium thiosulphate and with pentaflurothiophenol but these are often cumbersome to perform. Most of the GC/FID and GC/MS method reported had drawbacks of matrix interferences and had higher LOD and LOQ. The required low tolerance of these impurities presents a major challenge for the pharmaceutical industry. Although there are a number of different detection techniques available, these have to be chosen carefully on a case-by-case basis. HPLC with Ultra violet (UV) detector is not useful in many cases for the low level quantitation of the analytes. The major issues are sensitivity, selectivity and the problems related to matrix interferences in the APIs. Appropriate controls need to be built into the analytical procedures to ensure confidence in the results generated and methods should be such that they are easily transferred to the quality control environment. It is therefore imperative that the analytical methodology must be robust. In view of these practical issues inherent with the reported methods and increasing concern from the regulatory perspective in relation to the potential hazards of alkyl sulfonate impurities, the biggest challenges facing the pharmaceutical industry is the need for development of extremely sensitive and robust analytical methodologies that can adequately monitor potentially genotoxic impurities at very low levels.

The proposed LC/MS/MS (liquid chromatography/mass spectrometery/mass spectrometery) method for determination of MMS and EMS is a direct, sensitive and robust involving no laborious sample preparation steps. This method has many advantages over the method reported in the literature in terms of specificity, accuracy and reproducibility involving direct analysis and compared to laborious sample preparation techniques. Matrix interference in dealt in method by utilizes a diluent in which Emtricitabine API is insoluble and the analytes are exactable from the API matrix. The proposed method involves MRM mode for quantification of MMS and EMS with electrospray ionization to achieve very low LOD and LOQ. Further, this method does not require switching-valve as the API is insoluble in the selected and avoid the introduction of matrix into the mass detector.

Experimental

Reagents and chemicals

HPLC-grade acetonitrile was purchased from J T backer (Phillipsburg, USA); formic acid was purchased from Sigma-Aldrich (Milwaukee, WI), purified water collected through Milli-Q water purification system (Millipore, Bedford, MA, USA). Reference substances MMS and EMS were purchased from Sigma-Aldrich (Milwaukee, WI) and Emtricitabine API samples were obtained from Ranbaxy Laboratories Ltd (India).

Instrumentation

Chromatography

The LC system used was an Agilent 1100 series LC system (Agilent Technologies, Waldbronn, Germany) consisting of a 1100 series pump with a degasser, a temperature controlled micro-well plate, auto sampler and a column compartment. The analytical column was a Zorbax SB C_{18} (150 × 4.6 mm) 3.5 µm. The mobile phase consisted of premixed and degassed solution of Formic acid 0.1% (v/v in water) and acetonitrile in the ratio of [70:30] [v/v]. The flow rate was 0.2 ml/min and the run time was 20 minutes. Column oven temperature was maintained at 50 °C. Injection was 50 µL. The control of the HPLC system and data collection was by Empower software.

Mass Spectrometer

An ion trap mass spectrometer (4000 Q-trap of Applied Biosystems, Switzerland) equipped with positive ion electospray ionization probe was operated in split less mode. The control of the system and data collection was by Analyst 1.4.1 (Applied Biosystems). MRM transitions m/z 110.9 > 78.8 and 125.1 > 97.1, were selected for quantification of MMS and EMS respectively. Typical operating conditions were as follows: Ion spray voltage was kept as 5500V and source temperature 250 °C. Declustering potential applied was 45V for MMS and 25V for EMS. Collision energy and Collision cell exit potential were 15, 15 and 12, 10V respectively.

Validation Study

The developed mass method for the determination of MMS and EMS in one API was validated. The linearity was evaluated by preparing and analyzing nine calibrators of each analytes in the concentration

range 0.0025–0.3 µg/ml using the appropriate MRM transition. The slope, intercept and regression coefficient were determined by the least squares linear regression analysis. System precision was done by injecting six replicate injections of the standard preparation. The limit of quantitation was calculated on the basis of the lowest concentration of each analytes that gives percentage relative standard deviation (%RSD) <10%. The precision and accuracy were evaluated by spiking each analytes and determining the %RSD < 10%. Stability of analytical solution was evaluated at 10 °C for 5 hours. Ruggedness study was performed by different scientist using different column by spiking each analytes and determining the %RSD < 10%.

Standard and Sample Preparation

Standard preparation

Stock standard solution of MMS and EMS was prepared in acetonitrile of concentration 0.75 µg/ml. Final standard solution was prepared in mobile phase of 0.15 µg/ml concentration.

Sample preparation

API was prepared 100 mg/ml (accurately weighed) in acetonitrile and filtered this sample solution through 0.45 µ polytertafluoroethylene (PTFE) filter. 1 ml of this filtrate's transferred to a 5 ml volumetric flask and made up to the mark with mobile phase.

Sample Analysis

Accurately weighted API powder was carefully transferred in a 20 ml volumetric flask. The powder was mixed with 10 ml of acetonitrile and sonicated for 5 minutes. The mixture was filtered through 0.45 µ PTFE filter and the filtrate solution was collected in a 5 ml volumetric flask. Mixed 1 ml of this filtrate and 1 ml of mobile phase for final sample concentration. A 50 µL aliquot was injected into the mass spectrometer.

Results and Discussion

Method optimization parameters

Optimization of sample preparation

Sample preparation is an important part of the genotoxic impurity analysis analysis, because matrix effects in trace analysis are magnified, causing loss of sensitivity, abnormal recovery and analyte instability. In order to avoid complexity in the sample preparation procedure, diluent selection was done in such a way in which Emtricitabine had no or minimum solubility and at the same time analytes were completely extractable from the matrix. As the quantitation was required to be done at very trace, sample concentration was required to be increased in order to achieve lower detection levels. Sample preparations were done in acetonitrile and in mobile phase as diluent. It was observed that in acetonitrile API was not soluble however MMS and EMS has very good solubilities. In diluent as mobile phase both API and MMS and EMS were soluble, but due to matrix interference of the API low recoveries were observed for MMS and EMS. Acetonitrile alone was also evaluated as diluent but was found to be not suitable due to lower responses of the analytes and bad peak shape. Therefore, during sample preparation acetonitrile was added initially followed by

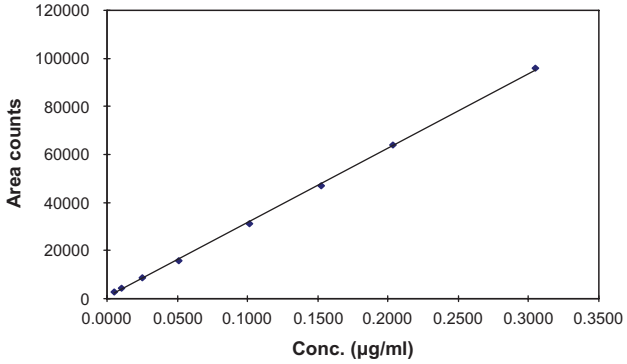

Figure 1. Linearity plot of MMS in the concentration range of 0.0025 µg/ml to 0.3048 µg/ml.

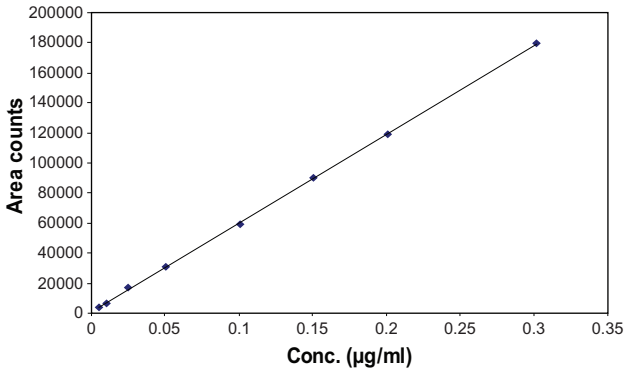

Figure 2. Linearity plot of EMS in the concentration range of 0.0025 µg/ml to 0.3015 µg/ml.

Table 1. Limit of Quantitation (LOQ) and Limit of Detection (LOD) for MMS and EMS.

Conc.	MMS		EMS	
	LOQ	LOD	LOQ	LOD
µg/mL ppm or µg/g	0.0080 0.4	0.0060 0.3	0.0081 0.4	0.0061 0.3
Injection no.	Area counts			
1	3411	3031	5687	5414
2	3467	2730	5233	5709
3	3476	2440	6682	5378
4	3040	3308	5219	4574
5	4090	1623	5397	4773
6	3564	3449	5647	4433
Mean	3508	2764	5644	5047
SD	339	670	546	521
RSD (%)	9.66	24.24	9.67	10.32

dilution in the mobile phase. The purpose of adding acetonitrile was to remove matrix effect of the API. In acetonitrile, Emtricitabine was insoluble and the analytes (MMS and EMS) had very good solubilities. This resulted in removal of the matrix and at the same time optimum extraction of the analytes, resulting in proper peak shapes and recoveries in the range of 83.50 to 101.22% and 80.57 to 90.04% for MMS and EMS respectively.

Column Selection and Separation

For adequate retention and separation of MMS and EMS different columns like ACE C18, Kromasil C18 and Zorbax C18 of different dimensions were evaluated. On ACE C18 and Kromasil C18 early elution, blank interference and inadequate separation of analytes was observed (refer Table 3). However, on Zorbax C18 column separation and response for both MMS and EMS was found to be suitable. Different composition of mobile phase using ammonium formate and formic acid with acetonitrile were studied. Good separation and responses were observed using formic acid 0.1% (v/v in water) and acetonitrile in the ratio of 70:30 (v/v) with column oven temperature of 50 °C at a flow rate of 0.2 mL per min. Under these conditions the retention time of MMS and EMS were observed to be about 9 and 12 min respectively.

Optimization of Mass Spectrometric Parameters

Choosing a detection method is the most important part of pharmaceutical analysis. From the instrument simplicity, stability and availability point of view HPLC-UV and GC-FID were first evaluated. However, on these techniques sufficient sensitivity for the trace level analysis of MMS and EMS was not achieved. In view of this, sensitive and specific mass spectrometric detection was evaluated in MRM mode. MRM technique relies on measurement of the parent/daughter couple of an analyte in the mixture. This mode permits significant enhancement of selectively and sensitivity for screening and quantification. For MRM quantitation, specific mass transitions (daughter ions)

Table 2. Accuracy of MMS and EMS at different spiking concentrations.

Recovery level	MMS			EMS		
	Amount added (µg/g)	Amount recovered (µg/g)	% Recovery	Amount added (µg/g)	Amount recovered (µg/g)	% Recovery
50% Rec-1	3.751	3.132	83.50	3.670	2.957	80.57
50% Rec-2	3.777	3.513	93.01	3.696	3.101	83.90
50% Rec-3	3.761	3.303	87.82	3.680	3.105	84.38
100% Rec-1	7.568	7.018	92.73	7.405	6.432	86.86
100% Rec-2	7.496	6.958	92.82	7.335	6.255	85.28
100% Rec-3	7.543	6.873	91.12	7.381	6.221	84.28
150% Rec-1	11.272	10.614	94.16	11.030	9.547	86.55
150% Rec-2	11.268	10.868	96.45	11.026	9.723	88.18
150% Rec-3	11.338	11.476	101.22	11.094	9.989	90.04
Mean			92.54			85.56
SD			4.995			2.747
RSD (%)			5.40			3.21

Table 3. Comparison of various HPLC columns for separation and elution of MMS and EMS.

Name of HPLC columns	Retention time, separation and response behavior of MMS and EMS
ACE C18	Early elution (peak eluting at the void volume)
Kromasil C18	Blank interference and broad peak shapes for MMS and EMS
Zorbax C18	Separation, good retention and response for both MMS and EMS

were selected for MMS and EMS by preparing standard solution of the analytes in acetonitrile and directly infusing into the ESI probe using Harvard syringe pump. The ion source temperature and ion spray voltage were optimized as at 250 °C and 5500V respectively. Declustering potential and collision energy used for collision induced dissociation were optimized as 45V and 25V for both MMS and EMS. The Collision cell exit potential value was optimized as 15V. The MRM experiment was accomplished by specifying the parent mass of the analytes (both MMS and EMS) for MS/MS fragmentation and then specifically monitoring for a single fragment ion. The major fragments for MMS (m/z 110.9) were observed to be at m/z 78.8 and m/z 69.9. For EMS (m/z 125.1) the major fragment ions were observed at m/z 97.1 and m/z 115.0. For MRM quantitation combination of precursor ion and product ion were selected for both MMS and EMS on the basis of response. For MMS the MRM transition selected was 110.9 (parent mass of MMS) → 78.8 (fragment mass of MMS), as this was the most intense transition. In case of EMS the MRM transition of 125.1 (parent mass of EMS → 97.1 (fragment mass of EMS) was selected on the basis of response.

Validation

Validation was necessary before the application of the developed mass procedure to the commercial products. The developed mass method for the determination of MMS and EMS in Emtricitabine API was validated. The linearity was established by plotting the peak area counts of an individual analytes versus concentration of each analytes in the concentration range 0.0025 µg/ml to 0.3 µg/ml. The slope, intercept and regression coefficient were determined by the least squares linear regression analysis. Linearity correlations of the peak area counts and concentration of both the analytes was

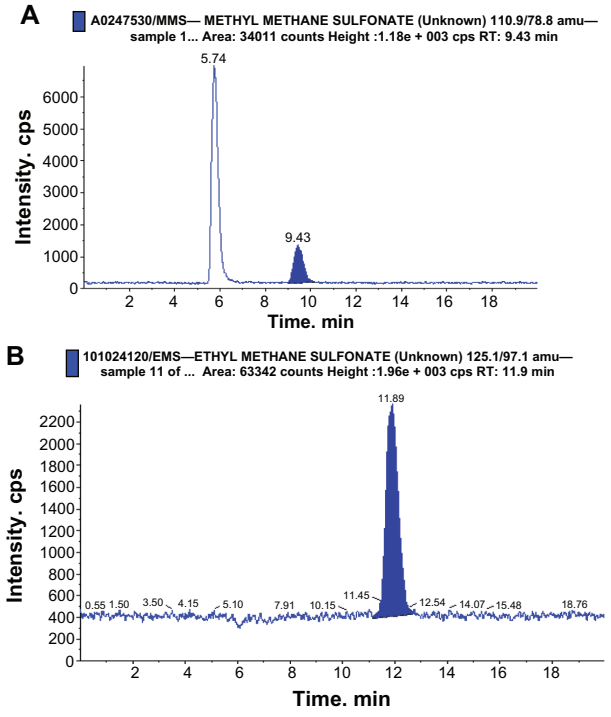

Figure 3. A) MRM scan of MMS in standard preparation. **B**) MRM scan of EMS in standard preparation.

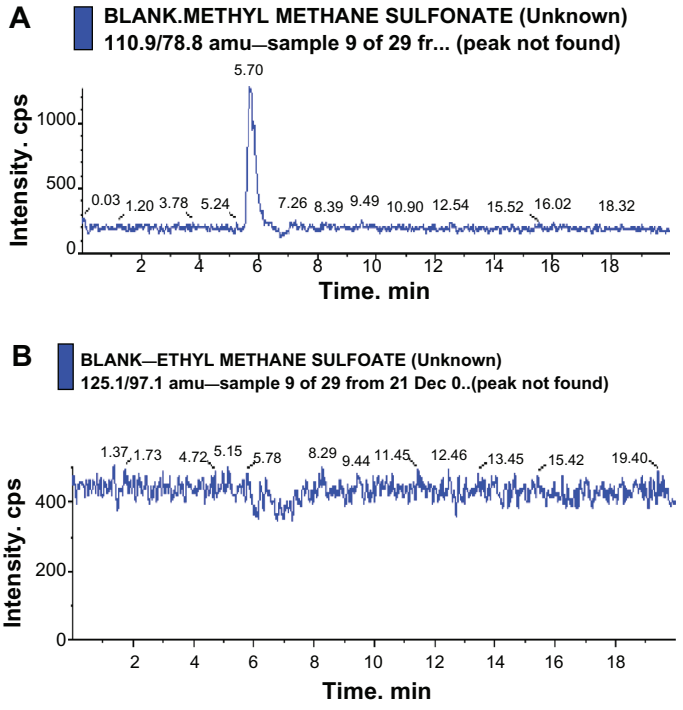

Figure 4. A) MRM scan of MMS in Blank. **B**) MRM scan of EMS in Blank.

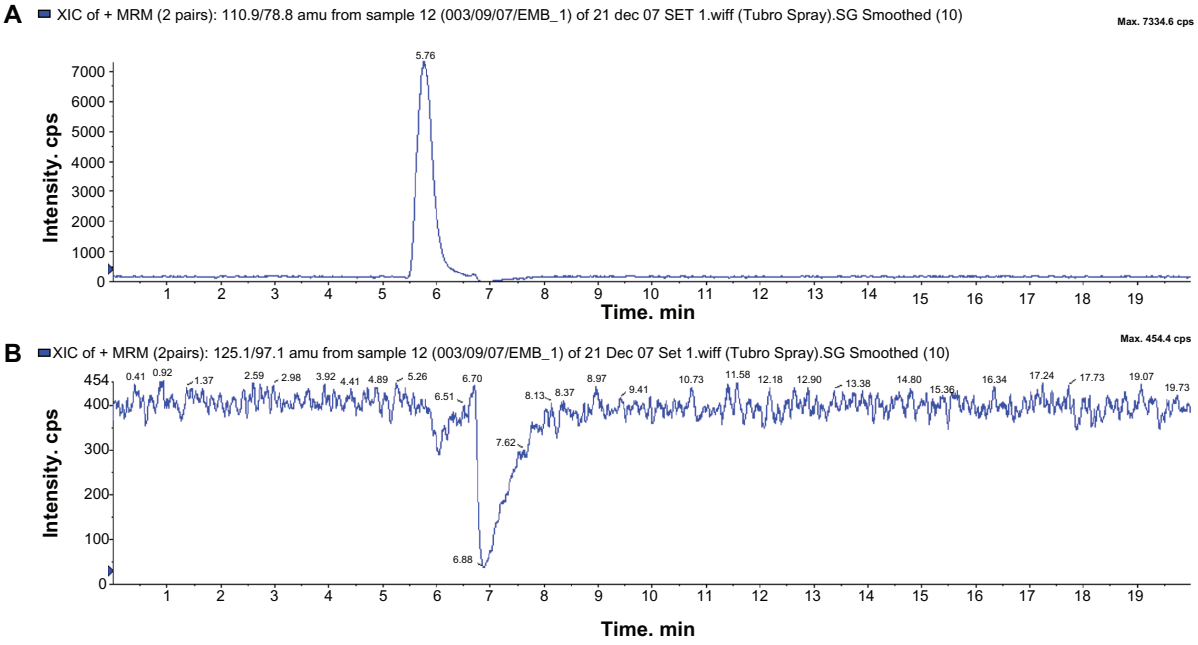

Figure 5. A) MRM scan of MMS in sample solution. **B**) MRM scan of EMS in sample solution.

achieved r^2:0.999 as represented graphically in Figures 1 and 2. The LOQ was calculated on the basis of the lowest concentration of each analytes that gives %RSD <10%. The LOD 0.3 µg/g and LOQ was 0.4 µg/g for both the analytes. Data summarized in Table 1. System precision was 3.82% for MMS and 5.12% for EMS. MMS and EMS were not found in the Emtricitabine sample hence method precision and accuracy experiments were performed by spiking each analyte and determining the %RSD. The %RSD for six replicate preparations was observed as 2.39% and 3.83% for MMS and EMS respectively. Recovery of the spiked amounts of analytes were calculated, the mean recovery percentages were observed to be in the range of 88.11–97.28 for MMS and 82.95–88.26 for EMS indicating good correlation of the calculated and added concentrations. Data summarized in Table 2. Stability of analytical solution was evaluated at 10 °C for 5 hours and the solution was observed stable during this period. Ruggedness study was performed by different scientist using different column. The %RSD for

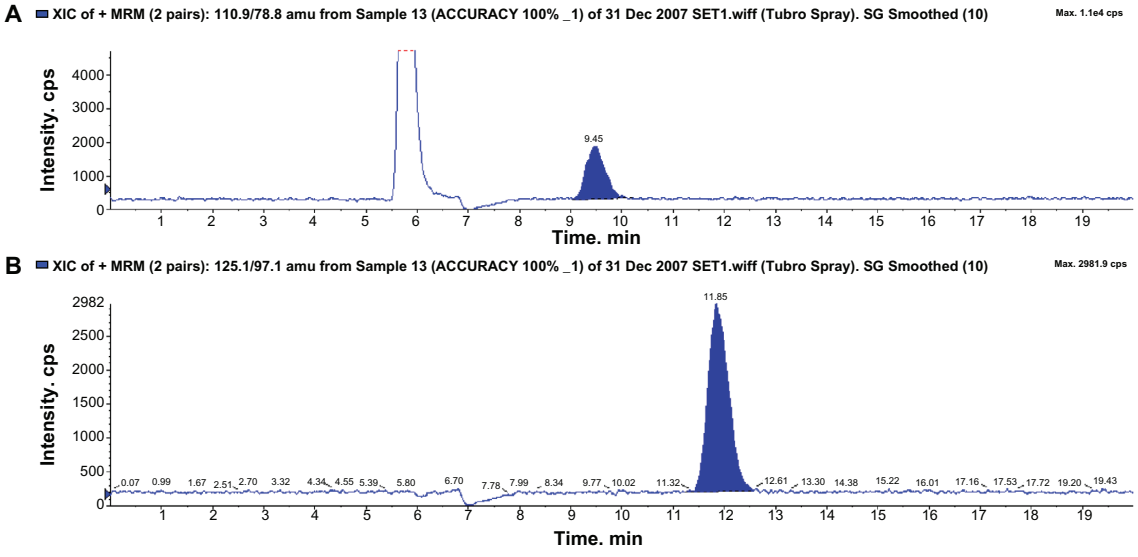

Figure 6. A) MRM scan of MMS, spiked at 100% level in sample solution. **B**) MRM scan of EMS, spiked at 100% level in sample solution.

ruggedness study was observed as 2.66% for MMS and 2.16% for EMS. The derived values indicate good reproducibility and sensitivity of the method.

Conclusion

A direct tandem mass spectrometric method was described for screening and quantification of MMS and EMS in the API. The MS-MS profile was more sensitive and specific than MS profiles for the detection of any undeclared MMS and EMS in the API. Furthermore, the method was accurate and reproducible for measurement of MMS and EMS detected in the API. The high levels of MMS and EMS in the API might be dangerous if this product was not properly tested by drug quality control laboratories. The described method presents a highly reliable technique for rapid detection of Genotoxic impurities in the API with accurately and precisely.

Acknowledgment

Author is thankful to the management of Ranbaxy Research Laboratories for providing research facilities to carryout the present work and for permission to publish this article.

Disclosures

This manuscript has been read and approved by all authors. This paper is unique and is not under consideration by any other publication and has not been published elsewhere. The authors and peer reviewers of this paper report no conflicts of interest. The authors confirm that they have permission to reproduce any copyrighted material.

References

1. Dobo KL, Greene N, Cyr MO, Caron S, Ku WW. The application of structure-based assessment to support safety and chemistry diligence to manage genotoxic impurities in active pharmaceutical ingredients during drug development. *Reg Toxicol Pharm*. 2006;44:282–93.
2. IARC Monographs Program on the Evaluation of Carcinogenic Risks to Humans (2004) for methyl methanesulfonate. 1999;71:1059 and IARC Monographs Program on the Evaluation of Carcinogenic Risks to Humans (2004) for ethyl methanesulfonate. 1974;7:245.
3. Guidelines on the Limits of Genotoxic Impurities. Committee for Medicinal Products for Human Use [CHMP]. European Medicines Agency [EMEA]. London, 2006 Jun 28:28. (CPMP/SWP/5199/02, EMEA/CHMP/QWP/251344/2006).
4. Genotoxic and Carcinogenic Impurities in Drug Substances and Products: Recommended Approaches; US Department of Health and Human Services, Food and Drug Administration, Center for Drug Evaluation and Research (CDER): Silver Spring, MD, USA, 2008 Dec.
5. Kroes R, Renwick AG, Cheeseman M, Kleiner J, Mangelsdorf I, Piersma A, et al. The Threshold of Toxicological Concern Concept in Risk Assessment. *Food Chem Toxicol*. 2004;42:65–83.
6. Miller SA, Ju J, Cucolo M, et al. Poster at the 52nd ASMS Conference, 2004.
7. Filby WG, Gunther K, Penzhorn RD. Relative molar response of a flame ionization detector to some organic compounds containing sulfur. *Anal Chem*. 1975;47:2283–5.
8. Li W. Trace analysis of residual methyl methanesulfonate, ethyl methanesulfonate and isopropyl methanesulfonate in pharmaceuticals by capillary gas chromatography with flame ionization detection. *J Chromator A*. 2004;1046:297–301.
9. Colon I, Richoll SM. Determination of methyl and ethyl esters of methanesulfonic, benzenesulfonic and *p*-toluenesulfonic acids in active pharmaceutical ingredients by solid-phase microextraction (SPME) coupled to GC/SIM-MS. *J Pharm Biomed Anal*. 2005;39:477–85.
10. Lee CR, Guivarch F, Van Dau CN, Ressier D, Krstulovic AM. Determination of polar alkylating agents as thiocyanate/isothiocyanate derivatives by reaction headspace gas chromatography. Analyst, 2003;128:857–63.
11. Alzaga R, Ryan RW, Taylor-Worth K, Lipczynski AM, Szucs R, Sandra P. A generic approach for the determination of residues of alkylating agents in active pharmaceutical ingredients by in situ derivatisation-headspace-gas chromatography-mass spectrometry. *J Pharm Biomed Anal*. 2007;45:472–9.