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Short communication

Low level determinations of methyl methanesulfonate and ethyl methanesulfonate impurities in Lopinavir and Ritonavir Active pharmaceutical ingredients by LC/MS/MS using electrospray ionization

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ABSTRACT

Methyl methanesulfonate (MMS) and ethyl methanesulfonate (EMS) have been highlighted as potential genotoxic impurities (PGIs). A sensitive LC/MS/MS method is developed and validated for the determination of MMS and EMS impurities in both Lopinavir and Ritonavir Active pharmaceutical ingredient. Method utilizes, Atlantis T3 column with electrospray ionization in multiple reactions monitoring (MRM) mode for quantitation of impurities. The proposed method is specific, linear, accurate and precise. The calibration curves show good linearity over the concentration range of 0.01–0.23 μg/mL for MMS and 0.005–0.23 μg/mL for EMS. The correlation coefficient obtained is >0.99 in each case. Method has very low limit of detection (LOD) and quantification (LOQ). LOD and LOQ of MMS and EMS are as low as ~0.002 μg/mL and ~0.01 μg/mL respectively. Method has accuracy within 80–120% for both the analytes. This method is a good quality control tool for quantitation of MMS and EMS impurities at very low levels in Lopinavir and Ritonavir.

1. Introduction

Lopinavir and Ritonavir are often administered in combination. Lopinavir is an inhibitor of HIV protease. Ritonavir is administered in combination as it inhibits the metabolism of Lopinavir [1], thereby providing increased plasma levels of Lopinavir. Starting materials, intermediates and by-products are often found as impurities in drug substances. Some of these known impurities are potential mutagens or carcinogens, but can be difficult or impossible to eliminate completely from the synthetic scheme. Based on the current regulatory guidances for genotoxic impurities, analytical methods should be developed to meet the required limit of 1.5 μg/day daily intake of individual impurity. Methanesulfonic acid is 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 any 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 [2]. These potential genotoxic impurities (PGIs) are known to induce genetic mutations or chromosomal aberrations and are reported as known carcinogens in rats and mice [3]. 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 [4]. In 2008, US FDA has also come up with the draft guidelines on genotoxic and carcinogenic impurities in drug substances and products [5]. 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/day [acceptable Threshold of Toxicological Concern (TTC)] is recommended in these guidelines [4–6].

Based on the maximum daily dosage of Lopinavir and Ritonavir, MMS and EMS are required to be controlled at a combined limit of 1.4 μg/g and 1.25 μg/g, respectively.

Due to the increasing concern from the regulatory perspective in relation to the potential hazards, there has been a general renaissance and increased numbers of analytical methods are reported. Mainly gas chromatographic (GC) methods utilizing both flame ionization detector (FID) and mass spectrometric detectors are reported in literature for the determination of alkyl methanesulfonate impurities. But these methods have drawbacks of either higher LOQ or LOD [7–10]. Direct analysis methods suffered from the drawback of inlet contamination and degradation due to intro-

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duction of higher amounts of drug substance, leading to recovery issues. To avoid the introduction of non-volatile and reactive material in the GC inlet, extraction methods such as liquid–liquid extraction (LLE), SPME (solid phase micro extraction) and LPME (liquid phase micro extraction) have been reported [11].

However, the extraction based methods are labour-intensive and are prone to interferences from other solvents as well as emulsion formation. Moreover, the resulting sample preparation method requires extra validation. Other than direct analysis, in direct methods involving derivatisation [12,13] are reported for the determination of MMS and EMS, involving derivatisation with aqueous sodium thiosulphate and with pentaflurothiophenol, but these are often cumbersome to perform. In addition to this interferences from derivatisation reagent leading to variable recoveries were observed.

An indirect derivatisation method using LC/MS in SIM mode for determination of alkyl sulfonates using triethylamine and trimethyl amine is reported [14]. This method has drawback of tedious sample preparation time of 60 min for derivatisation. The peak shapes of the analytes were very broad and tailing on reported hydrophilic interaction liquid chromatography (HILIC) column. Reagent interference was observed and required subtraction to improve adequate recoveries.

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 specific analytical methodologies that can adequately monitor potentially genotoxic impurities at very low levels.

Since, no method was reported with multiple reactions monitoring (MRM) mode using LC/MS/MS for the quantitation of MMS and EMS in Lopinavir and Ritonavir, an attempt was made to overcome the shortcoming of the existing methods and in developing a highly sensitive, cost-effective, specific, direct and accurate LC/MS/MS method. In this method, MRM mode used for quantitation which provides better signal to noise ratio and is more specific mode than SIM mode. MRM provides reduced offset of the baseline as compared to SIM mode. MRM mode allows drastic reduction or elimination of matrix effects that limits the accuracy and detection limits of SIM methods. Method is very simple and easy to perform in comparison to indirect derivatization method, making it more practicable. Method is highly reproducible and requires lesser analysis time. In this method both MMS and EMS are stable up to 9 h under ambient conditions. LOD and LOQ of MMS and EMS are as low as ~0.002 μg/mL and ~0.01 μg/mL respectively.

2. Experimental

2.1. Materials

HPLC-grade acetonitrile was purchased from J T backer (Phillipsburg, USA); formic acid was purchased from Fluka (St. Louis, MO 63178, USA), purified water collected through Milli-Q water purification system (Millipore, Bedford, MA, USA). Reference substances, methyl methanesulfonates and ethyl methanesulfonate were purchased from Acros organics (500 American Road, Morris Plains, USA). Lopinavir and Ritonavir drug substance samples were obtained from R&D division of Ranbaxy laboratories Ltd. (India).

2.2. Chromatographic conditions

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,

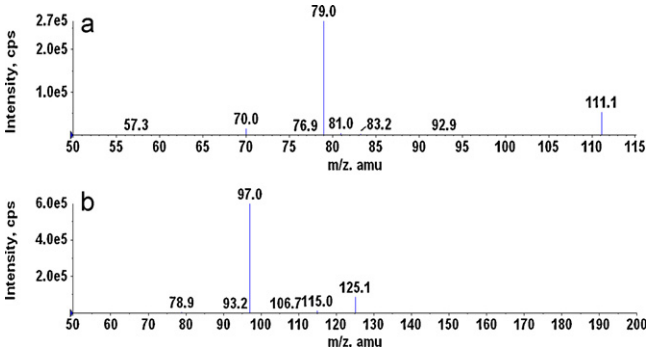

Fig. 1. (a) MS/MS spectrum of MMS and (b) MS/MS spectrum of EMS.

auto sampler and a column compartment. The analytical column was Atlantis T3 (150 × 4.6 mm) 3.0 μm. The mobile phase consisted of eluent A of formic acid 0.1% (v/v in water) and eluent B of acetonitrile. The gradient elution mode was used for analysis with a flow rate of 1.0 mL per min. In gradient program eluent B was kept 40% for 6 min and after 1 min eluent B was changed to 80%, followed by a hold time of 5 min, then in 1 min eluent B was changed to 40% and a re-equilibration period of 7 min was given. Premixed and degassed solution of water and acetonitrile in the ratio of 30:70 was used as diluent. The run time for standard was kept as 6 min and for sample and blank as 20 min. Column oven temperature was maintained at 30 °C. Injection volume was 50 μL. The control of the HPLC system and data collection was done by Empower software. All the solutions were filtered through 0.45 μm nylon filter.

2.3. Mass spectrometer

A Q-trap mass spectrometer (4000 Q-trap of Applied Biosystems, Switzerland) was used with electrospray ionization (ESI) probe in positive polarity. Splitter was used to achieve a flow rate of 0.2 mL/min. The control of the system and data collection was done by PE Sciex Analyst 1.4.1 (Applied Biosystems). Typical operating conditions were as follows: ion spray voltage was kept as 5500 V and source temperature 250 °C. Curtain gas was applied at 20 psi and collision gas at 10 psi. The ion source gas 1 and gas 2 were kept at 45 and 50 psi respectively. Venting was done using valco valve (Valco Instruments Co. Inc., VICI AG International). Venting was given from 6.1 min to 16.0 min and the MRM transitions of m/z 111.1 > 79.0 and 125.1 > 97.0 were selected for quantification of MMS and EMS respectively.

2.4. Validation study

The common method for determination of MMS and EMS in Lopinavir and Ritonavir was validated. The linearity was evaluated by preparing and analyzing eight calibrators of 0.01–0.23 μg/mL for MMS and 0.005–0.23 μg/mL for EMS. The slope, intercept and regression coefficient were determined by the least squares linear regression analysis. System precision of the mass spectrometric response was established by making six injections of the standard solution. The limit of quantitation and detection were determined by showing precision, by making six replicate injections of lower concentration solutions of analytes. The limit of quantitation and limit of detection were calculated on the basis of the lowest concentration of each compound that gives %RSD < 10 (for LOQ) and %RSD < 33 (for LOD). The method precision was evaluated by spiking each analyte and determining the %RSD. In accuracy experiment, known amount of sample was taken sepa-

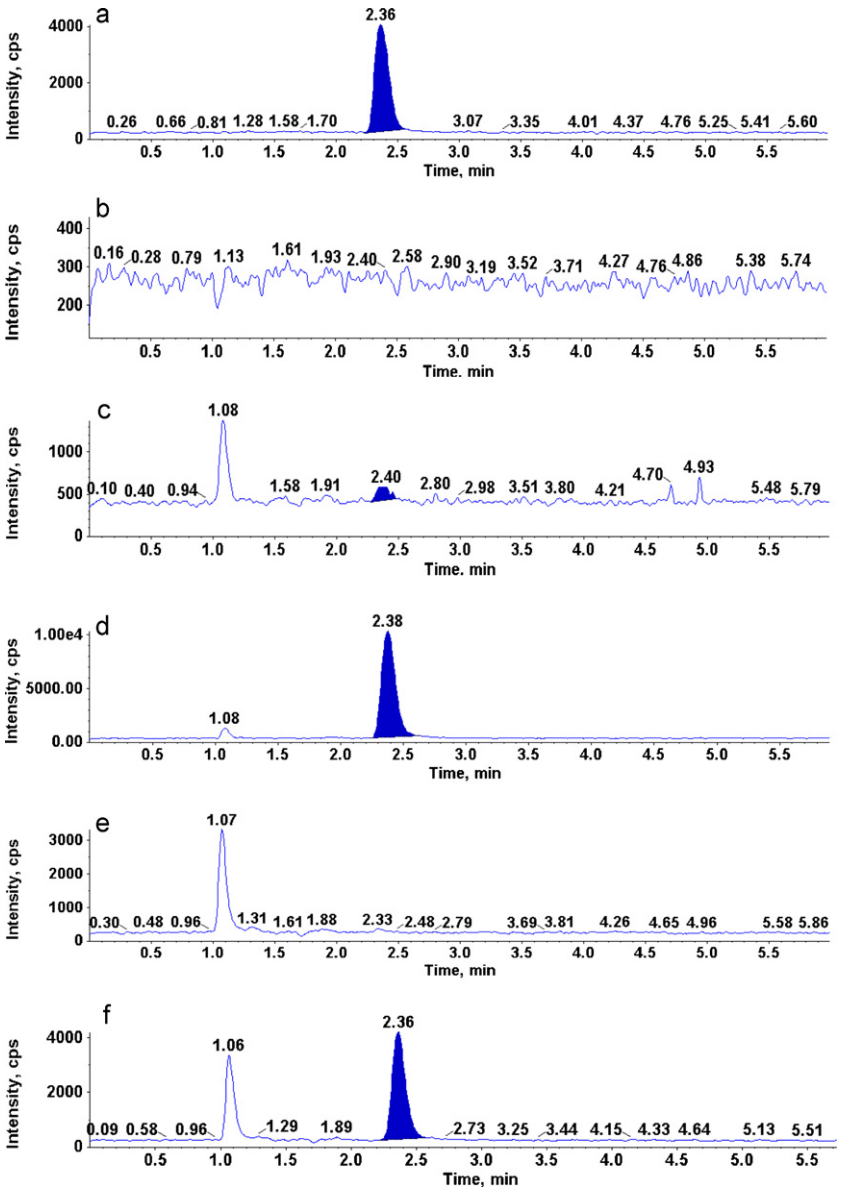

Fig. 2. Chromatograms of MMS using MRM scan. (a) Chromatogram of standard containing 0.125 μg/mL of MMS, (b) chromatogram of blank, (c) chromatogram of MMS in Lopinavir sample, (d) chromatogram of Lopinavir sample spiked with MMS at 0.14 μg/mL, (e) chromatogram of MMS in Ritonavir sample, (f) chromatogram of Ritonavir sample spiked with MMS at 0.125 μg/mL.

rately into six different volumetric flasks and spiked with known quantities of MMS and EMS. Accuracy was calculated after making corrections for the amount already present in the sample. Stability of analytes in sample solution was done by analyzing spiked sample solution at different time intervals at room temperature.

2.5. Standard solutions and sample preparation

2.5.1. Standard solution preparation

For Lopinavir, standard solution of 0.14 μg/mL (for both MMS and EMS) was prepared. In case of Ritonavir the standard concentration was kept as 0.125 μg/mL for both MMS and EMS. Standard preparation was done by preparing stock solution of both the analytes in acetonitrile and then suitability diluting with diluent.

2.5.2. Sample preparation

Sample solution of 100 mg/mL concentration was prepared for Lopinavir and Ritonavir in diluent. Suitable quantity of drug substance were taken and dissolved in diluent using sonication.

3. Results and discussion

3.1. Optimization of sample preparation

Sample preparation is an important part of the GTI analysis, because matrix effects in trace analysis are magnified, causing loss of sensitivity, abnormal recovery and analyte instability. Different diluents were evaluated with respect to extraction efficiency and chromatography. Solubility of both the drug substances and analytes was good in acetonitrile. Acetonitrile alone was not found suitable due to lower responses of the analytes and bad peak shape. Premix of acetonitrile and water in different ratios were evaluated.

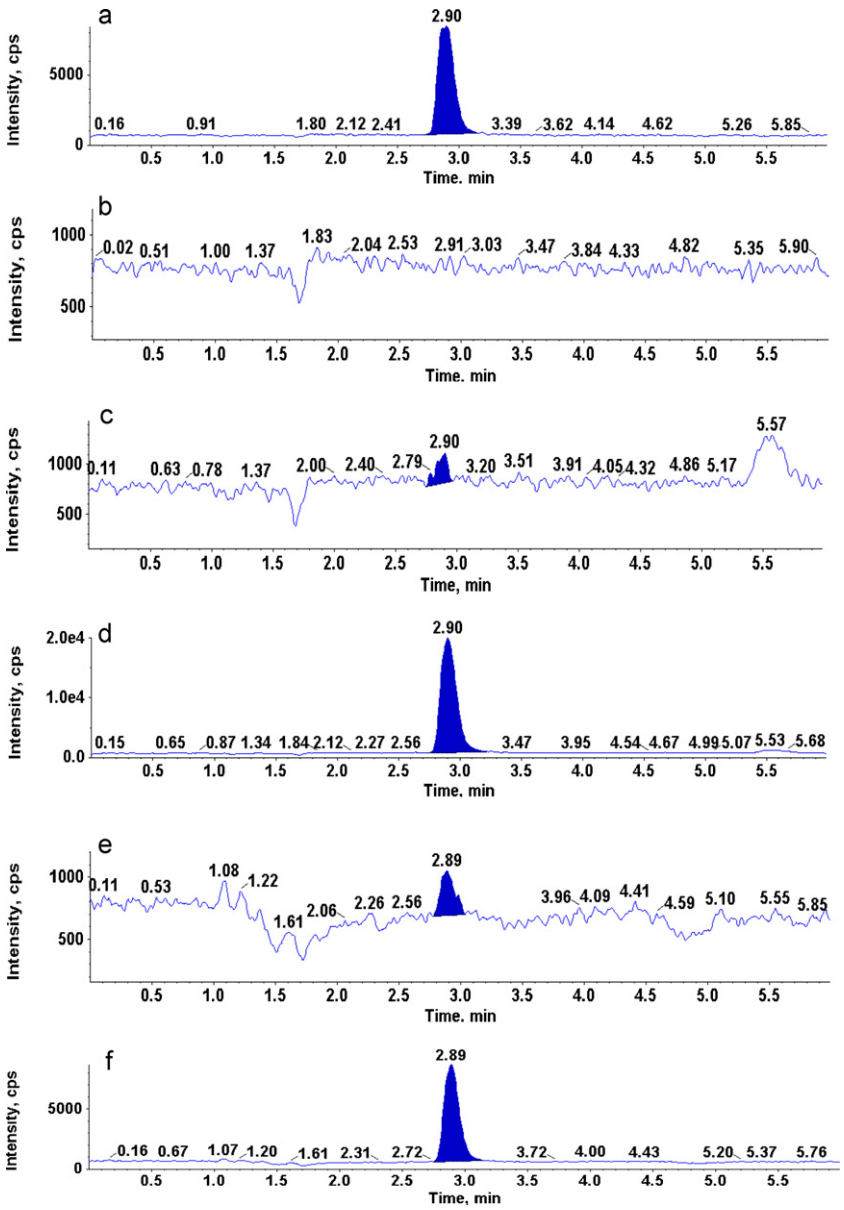

Fig. 3. Chromatograms of EMS using MRM scan. (a) Chromatogram of standard containing 0.125 μg/mL of EMS, (b) chromatogram of blank, (c) chromatogram of EMS in Lopinavir sample, (d) chromatogram of Lopinavir sample spiked with EMS at 0.14 μg/mL, (e) chromatogram of EMS in Ritonavir sample, (f) chromatogram of Ritonavir sample spiked with EMS at 0.125 μg/mL.

In premixed solution of acetonitrile and water in the ratio of 70:30, good response and proper peak shapes were obtained for both the analytes. Good recoveries (90–111%) were observed for both MMS and EMS in both drug substance matrixes in this diluent.

3.2. Column selection and separation

Different stationary phases were evaluated to get proper separation of the analyte peaks from the drug substance peak. It was important to achieve proper separation as the concentration of drug substance was high leading to broad peak. Various columns like Kromasil C18, Atlantis T3 and Zorbax Rx C8 of different dimensions were evaluated. Kromasil C18 and Zorbax Rx C8 were not found suitable as the response of analytes was observed less and analyte peaks were not well resolved from the drug substance peak. On Atlantis T3 column of dimensions 150 mm × 4.6 mm internal diameter, 3.0 μm, separation and response for both MMS and EMS were found to be good. On this column the analytes were well retained and separated from the drug substance peaks of both Lopinavir and Ritonavir. The unique combination of bonding and end capping on Atlantis T3 column (advanced T3 bonding) provided better retention and improved peak shapes. Different composition of mobile phase using ammonium formate, 0.1% acetic acid (v/v in water) and 0.1% formic acid (v/v in water) with acetonitrile were studied. Good separation and responses were observed using formic acid 0.1% (v/v in water). Both isocratic and gradient elution modes were evaluated. Gradient elution was observed to be more efficient in achieving optimum separation of MMS and EMS from the drug substance peaks. The column was thermostated at 30 °C to avoid any shift in retention time. Flow rate of 1 mL/min was used for chromatographic separation using conventional column. However, prior to electrospray ionization flow rate was reduced to 0.2 mL/min using a splitter. Retention time of MMS and EMS were observed to be about

2.5 and 3 min, respectively. Peaks were well separated from the drug substance peaks (both Lopinavir and Ritonavir) and Gaussian.

Peaks corresponding to Lopinavir and Ritonavir eluted at around 10 and 12 min, respectively. Due to adequate separation it was possible to design suitable venting using switching valco valve wherein only the analyte peaks were allowed to enter the mass detector and the drug substance peak was vented. In this way only the peaks of interest were monitored and matrix effect due to high concentration of drug substance was avoided.

3.3. 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 of tandem LC/MS/MS was evaluated in MRM mode. Multiple Reaction Monitoring (MRM) mode has two fundamental advantages over SIM. First, detection is based on secondary "product ion" produced by the collisional dissociation of an analyte "precursor ion". The analyte precursor ion (isolated in first quadrupole by a SIM mechanism) has the same selectivity as SIM, but there is a high probability that at least one of the resultant product ions will be unique to the precursor and not the interference. The increase selectivity of MRM is apparent by the reduced offset of the baseline as compared to SIM. Secondly, during the mass filtering process in first quadrupole, all lower m/z ions from the sample are eliminated. The unique product ions from the collisional dissociation are measured in this "zero" noise region of the spectrum. The combination of a unique product ions (more selectivity) and the elimination of background noise results in consistently low limits of detection even for complex matrices. This mode permits significant enhancement of selectivity and sensitivity for screening and quantification. For MRM quantitation, specific mass transitions (daughter ions) were selected for MMS and EMS by preparing standard solution of the analytes in acetonitrile and directly infusing into the electrospray ionization probe. The major fragments for MMS (m/z 111.1) were observed to be at m/z 79.0 and m/z 70.0. For EMS (m/z 125.1) the major fragment ions were observed at m/z 97.0 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 111.1 (parent mass of MMS) → 79.0 (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 → 97.0 (fragment mass of EMS) was selected on the basis of response. These transitions were observed to be specific and intense (refer Fig. 1). The ion source parameters were optimized to get proper response.

3.4. Validation

The developed method for the determination of MMS and EMS in Lopinavir and Ritonavir drug substances was validated as per ICH guidelines [15]. The linearity experiment conducted for both analytes showed that mass spectrometric responses are proportional to their concentration within the range of 0.01–0.23 μg/mL for MMS and 0.005–0.23 μg/mL for EMS. A rectilinear calibration graph was obtained over the range of 0.01–0.23 μg/mL for MMS and 0.005–0.23 μg/mL for EMS. The correlation coefficient was obtained more than 0.99 for both the analytes. The % relative standard deviation (%RSD) was found to be below 3% for both the analytes in system precision. LOD and LOQ values observed for MMS were 0.0026 μg/mL and 0.010 μg/mL respectively. LOD and LOQ values observed for EMS was 0.0017 μg/mL and 0.0051 μg/mL respectively. Precision of the method was demonstrated by %RSD values of less than 7. % Recovery for MMS was observed in the range of 104.67–108.27% and for EMS in the range of 105.60–110.02 in Lopinavir drug substance and 89.66–111.01% for MMS and 91.57–107.95% for EMS in Ritonavir drug substance (Figs. 2 and 3). Both MMS and EMS were found stable in analytical solution at room temperature for 9 h in presence of Lopinavir and for 11 h in presence of Ritonavir, making the analysis more practicable. This method has been used successfully for determination of MMS and EMS in six different batches of Lopinavir and Ritonavir. In all the batches of Ritonavir and Lopinavir, MMS and EMS were not detected. In one of the batch of Lopinavir both MMS and EMS were observed, however the levels were below LOQ. EMS was observed below the LOQ level in one batch of Ritonavir.

4. Conclusion

The proposed method is a direct tandem mass spectrometric method for screening and quantification of MMS and EMS in the Lopinavir and Ritonavir drug substances. Method utilizes MRM mode for quantitation which provides better selectivity and sensitivity. The described analytical method is cost-effective, direct, accurate and convenient quality control tool for determination of MMS and EMS in both Lopinavir and Ritonavir. The advantage of this method lies in its improved sensitivity and simpler sample preparation technique to those previously reported methods. MRM mode allows drastic reduction or elimination of matrix effects that limits the accuracy and detection limits of SIM methods. This method can be further studied for its application to other drug substances.

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