Time-dependent selected reaction monitoring-based GC-MS/MS method for estimation of genotoxic impurities in new antibacterial agent: alalevonadifloxacin mesylate

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Abstract
Alalevonadifloxacin mesylate (ALA), pro-drug of levonadifloxacin is a new antibiotic approved in India to treat infections caused by Gram-positive bacteria. Alkyl mesylates (AMs) are known genotoxic impurities (GTIs) formed in drug substances isolated as mesylate salts. Time-dependent selected reaction monitoring (t-SRM)-based gas chromatography tandem mass spectrometry (GC-MS/MS) method has been developed for trace estimation of AMs, namely, methyl methane sulfonate (MMS), ethyl methane sulfonate (EMS) and isopropyl methane sulfonate (IMS) in ALA. Liquid-liquid extraction (LLE) procedure using dichloromethane (DCM) as an extracting solvent was employed to extract AMs from the drug substance. Automatic selective reaction monitoring (auto-SRM) tool was applied to identify the most intense SRM pair of the ions to achieve the highest sensitivity. The method was validated in terms of specificity, linearity, sensitivity, precision, and accuracy. The limit of quantitation (LOQ) for the MMS, EMS and IMS were 5, 10, and 20 ng/g of ALA, respectively. For all analytes, the correlation coefficient (R) were greater than 0.9975 in the concentration range of 3.0–260 ng/mL. Mean recovery of all analytes was in the range of 91.77 to 97.65%.

Keywords: Alalevonadifloxacin, GC-MS/MS, Time-dependent SRM, Alkyl mesylates

Introduction
A broad group of fluoroquinolones and quinolones is used as an antibacterial agent in clinics. Alalevonadifloxacin (ALA, WCK 2349) is a mesylate salt of L-alanine ester pro-drug of levonadifloxacin. Chemically, it is S-(−)-9-fluoro-8-(4-L-alaninylxypiperidin-1-yl)-5-methyl-6,7-dihydro-1-oxo-1H, 5H-benzo-[i, j]-quinolizine-2-carboxylic acid methane sulfonic acid salt (Patel et al. 2012). The chemical structure of ALA is shown in Fig. 1. ALA has completed phase I study in US (ClinicalTrials.gov Identifier: NCT02217930) and phase III study in India for the treatment of skin and skin structure infections (Chugh et al. 2015; Niu et al. 2015; Chugh et al. 2016; Rodvold et al. 2018; Mason et al. 2019). Very recently, a marketing approval has been issued by the Drug Controller General of India to commercialize this new drug in India. Synthesis of ALA involves use of methanol, ethanol, and isopropanol as solvents at various stages of synthesis and during purification. Possibility of undesired reactions between the methane sulfonic acid and the alcohols exists resulting in the formation of AMs (Andrew et al. 2010; Snodin and Teasdale 2015; Snodin 2019). These impurities are deoxyribo nucleic acid (DNA) reactive substances that have the potential to directly cause DNA damage, thereby leading to genetic mutations causing cancer. These impurities induce genetic mutations or chromosomal abnormalities in bacterial and mammalian cell systems, as well as in vitro salmonella reverse mutation (Ames) and micronucleus...
tests. ICH guideline M7 (R1) adopted a staged “threshold of toxicological concern (TTC)” concept based on the daily dose and duration of the treatment. Working Party, which has been appointed in 2008 by the European Pharmacopoeia Commission, has elaborated five general chapters on different sulfonates, which have been published in the European Pharmacopoeia Supplement 8.7 that came into force on 1 April 2016 [Muller et al. 2006; EMEA guideline on genotoxic impurities 2006; Recommended approaches on genotoxic and carcinogenic impurities by CDER 2008; Teasdale 2011; ICH guideline M7(R1) 2017].

Literature reveals several chromatographic approaches, including LC-MS and GC-MS for the determination of genotoxic impurities in pharmaceutical products. Alkylsulfonates in capacitabine and imatinib methanesulfonate were estimated using GC-MS/MS-based method (Liu et al. 2019). AMs in lamivudine were determined by using GC-MS in selected ion monitoring (SIM) mode (Ramana et al. 2012). AMs in doxazosin mesylate were determined by using GC-MS in SIM mode (Sitaram et al. 2011). MMS and EMS were estimated in imatinib mesylate by GC-MS (Ramakrishna et al. 2008). Solid phase micro-extraction-based sample preparation was employed for the estimation of AMs in some active pharmaceutical ingredients using GC-MS in SIM mode (Ramana et al. 2012). Trace determination of alkyl sulfonates by derivatization followed by GC-head space analysis was reported (Liu et al. 2018). Derivatization-based LC-MS method was applied to achieve the sensitivity for estimation of alkyl sulfonates (An et al. 2008). MMS and EMS were determined in emtricitabine by LC-MS/MS using electrospray ionization (Kakadiya et al. 2011). Sulfonate esters in imatinib mesylate and vinpocetine were estimated by LC-MS/MS (Guo et al. 2014). Simultaneous determination of mesylates and besylates was carried out by direct injection on GC-MS (Wollein and Schramek 2012). Alkylating agents were estimated using derivatization and headspace GC-MS methodology (Alzaga et al. 2007).

Use of t-SRM acquisition in GC-MS/MS analysis has been proved to be more sensitive and selective than the traditional SIM method. It reduces the matrix effects, which often impact the recovery and sensitivity of the method (Introduction of Auto SRM, Thermo fisher scientific 2012; Dobbeleer et al. 2018).

Herein, we describe t-SRM-based GC-MS/MS method development for quantification of AMs (MMS, EMS and IMS) in new drug substance ALA and its validation. An efficient procedure for extraction of genotoxic impurities from the water soluble drug substance is also elaborated.

Material and methods

Chemicals and reagents

MMS and EMS were purchased from Spectrochem Pvt Ltd. (India). IMS was purchased from Across Organics (USA). Chlorobenzene was procured from Qualigen fine chemicals (India). ALA was synthesized in-house at the Wockhardt Research Centre, India. HPLC grade dichloromethane (DCM) was supplied by Avantor (India). Water passed through Milli-Q (Millipore, USA) was used during analysis. Rtx-6.24 column 30 m × 0.32 mm I.D. with 1.8 μm film thickness (Restec Technologies, USA) was used for GC-MS/MS studies.

GC-MS/MS conditions

The GC-MS/MS analysis was performed on Trace 1300 gas chromatograph coupled with TSQ 8000 Evo triple...
quadrupole mass spectrometer as detector and Al 1310 auto liquid sampler (Thermo Fisher Scientific, USA). Rtx-624, 30 m × 0.32 mm I.D. with 1.8 μm film thickness column was used for analysis. The initial oven temperature was set at 80 °C and held for 1 min, raised to 150 °C at 15 °C/min, for 4 min. High-purity helium (purity 99.999%) was used as the carrier gas at a constant flow rate of 1.5 mL/min. Injector port temperature was set at 180 °C. Injection volume was 2 μL. Injections were made in split mode with split ratio 10:1. Runtime for the analysis was 9.67 minute. Data acquisition and processing was performed using Chromleon 7.2 SR5 chromatographic software.

The mass spectrometer was operated in positive electron impact ionization (70 eV) mode and data was acquired using t-SRM function for quantitative analysis. The MS transfer line and ion source temperature were set at 250 °C and 200 °C, respectively. Quadrupole Q1 and Q3 were set at unit resolution with scanning speed 20 Hz/s for 0.3 s. Analyte-dependent MS parameters are depicted in Table 1.

### Preparation of analytical solutions and extraction procedure
Chlorobenzene was used as an internal standard (IS). IS was dissolved in DCM at a concentration of 40 ng/mL. This solution was used as an extraction solvent. Standard stock solution of MMS, EMS, and IMS was prepared at concentration of 50 ng/mL in an extraction solvent. Test solution was prepared by extracting aqueous solution of ALA (100 mg in 0.5 mL) using 1 mL of an extraction solvent. The mixture was vortexed for 30 s and allowed to separate the two layers. Lower layer (DCM layer) was used for analysis. Standard solution was prepared by adding 0.5 mL of water into 1 mL of standard stock solution and processed in similar way. Area ratio of individual analyte peak and IS peak were considered for quantitation of AMs. During extraction method optimization, each experiment was performed in triplicate.

### Method validation
Method was validated as per the ICH guideline Q2(R1) (ICH 2005). System precision was determined by analyzing standard solution six times and RSD of peak area ratio was reported, RSD of ≤ 15% was considered as acceptable. Method precision was evaluated by analyzing six individual test preparations. RSD of % content of MMS, EMS and IMS were determined. Intermediate precision was determined by performing experiment by different analyst using different column. RSD of ≤ 15% was an acceptable limit. Limit of detection (LOD) and limit of quantitation (LOQ) were determined by “signal to noise ratio (S/N)” method. Standard solutions in the low range of concentrations were prepared, extracted, analyzed, and S/N was determined. S/N of 3:1 and 10:1 was considered acceptable for LOD and LOQ respectively. Six replicate measurements were performed at the determined LOD and LOQ concentrations; RSD of ≤ 33% and ≤ 15%, respectively, was considered as acceptable. Accuracy of the method was determined by standard addition method. Accuracy was determined at three levels viz. 50, 100, and 150% of the proposed specification limit (0.5 μg/g). Method was considered as an accurate when the mean recoveries obtained are in the range of 80 to 120%. Robustness of the method was evaluated by purposely varying the chromatographic conditions like carrier gas flow rate, oven temperature, and injector temperature. The standard solution was analyzed in six replicates at each variable condition, and impact on the chromatographic separation and precision of the determination was evaluated.

### Results and discussion

#### Method development

**Chromatographic separation**
Column selection and chromatographic parameters optimization was carried out using GC with MS as a detector. During method development variety of stationary phase columns like DB-1, DB-624, Rtx-624, and DB-5 were evaluated in terms of the analyte peak resolution, column efficiency and peak shape. DB-5 and DB-1 column showed unsymmetrical peak shapes. DB-624 column also showed unsymmetrical peak shape and low column efficiency (less sensitivity) for IMS. Rtx-624 (cross bonded 6% cyanopropylyphenyl 94% dimethyl polysiloxane) column showed better performance in terms of symmetrical peak shape, resolution and sensitivity. Hence Rtx-624 column was chosen for conducting further experiments. Comparative total ion chromatograms acquired on these columns are presented in Fig. 2 and the chromatographic performance data is shown in Table 2.

### Table 1 Analyte-dependent MS parameters for SRM pairs

| Parameter                  | Chlorobenzene | MMS | EMS | IMS |
|----------------------------|---------------|-----|-----|-----|
| Retention time (min)       | 4.83          | 5.79| 6.71| 7.15|
| RT window (min)            | 0.6           | 0.5 | 0.5 | 0.5 |
| Polarity                   | Positive      | Positive | Positive | Positive |
| Quantifier ion pair        |               |     |     |     |
| Precursor ion mass         | 112           | 80  | 109 | 123 |
| Product ion mass           | 77.1          | 65  | 79  | 79  |
| Collision energy           | 15            | 5   | 5   | 10  |
| Qualifier ion pair         |               |     |     |     |
| Precursor ion mass         | 77.1          | 79.1| 79  | 79  |
| Product ion mass           | 51.1          | 75.6| 78.3| 78  |
| Collision energy           | 10            | 50  | 5   | 5   |
Fig. 2 Comparative total ion chromatograms of alkyl mesylates on various columns a Rtx-624, b DB-5, c DB-1, d DB-624 at identical GC conditions

Table 2 Chromatographic performance data

| Column   | Parameter      | Chlorobenzene | MMS | EMS | IMS | Remark                                             |
|----------|----------------|----------------|-----|-----|-----|----------------------------------------------------|
| Rtx-624  | Retention time (min) | 4.83           | 5.79 | 6.71 | 7.15 | Ideal peak shape                                  |
|          | No. of theoretical plates | 101708         | 190290 | 210354 | 148517 |                                                   |
|          | Tailing factor | 0.96           | 1.07 | 0.98 | 0.97 |                                                   |
|          | Resolution    | –              | 16.91 | 16.58 | 6.63 |                                                   |
| DB-5     | Retention time (min) | 4.77           | 5.80 | 6.65 | 6.98 | Unsymmetrical peaks                               |
|          | No. of theoretical plates | 80486          | 75084 | 135201 | 108511 |                                                   |
|          | Tailing factor | 1.01           | 2.74 | 0.74 | 3.38 |                                                   |
|          | Resolution    | –              | 14.8 | 10.9 | 5.8  |                                                   |
| DB-1     | Retention time (min) | 4.85           | 5.90 | 6.83 | 7.25 | Unsymmetrical peaks                               |
|          | No. of theoretical plates | 110932         | 92035 | 157181 | 105789 |                                                   |
|          | Tailing factor | 0.94           | 2.81 | 1.24 | 3.55 |                                                   |
|          | Resolution    | –              | 15.3 | 15.8 | 6.1  |                                                   |
| DB-624   | Retention time (min) | 4.83           | 5.79 | 6.71 | 7.16 | Unsymmetrical peaks and low sensitivity for IMS   |
|          | No. of theoretical plates | 117254         | 191748 | 196673 | 62078 |                                                   |
|          | Tailing factor | 0.90           | 1.04 | 0.95 | 0.76 |                                                   |
|          | Resolution    | –              | 17.48 | 16.32 | 5.11 |                                                   |
Optimization of extraction solvent and procedure

Selection of an extraction solvent was a critical step in achieving the recovery of the AMs from the drug substance. ALA is freely soluble in water and very slightly soluble or practically insoluble in organic solvents like methanol, DCM, ethanol, and n-hexane. In contrast, MMS, EMS, and IMS are freely soluble in organic solvents like methanol, DCM, and n-hexane and practically insoluble in water. The difference in the solubility of the drug substance and AMs was ideal situation for the application of LLE procedure. LLE procedure made the sample devoid of matrix hence improved the life of GC column; it also helped in improving sensitivity.

ALA (100 mg) was dissolved in minimum quantity (0.5 mL) of water and attempts were made to extract AMs using various water immiscible solvents like n-hexane, ethyl acetate, toluene, chloroform, DCM, and methyl tertiary butyl ether. Extraction efficiency was determined by standard addition method. AMs were spiked in ALA at 0.25, 0.50, and 0.75 μg/g in triplicate and extracted with respective solvents. Extraction efficiency was calculated by using following equation

\[
\text{Extraction efficiency (mean recovery, } n = 3) = \frac{\text{Found amount of AMs in ALA} \times 100}{\text{Added amount of AMs in ALA}}
\]

In ethyl acetate, mean recovery of AMs was in the range of 63.11 to 74.17%, in toluene, it was 76.77 to 79.51%, in n-hexane and methyl tertiary butyl ether, mean recoveries were much below, i.e., < 58%. Mean recovery of AMs in halogenated solvents viz dichloromethane and chloroform were in the range of 92.69 to 96.18% and 72.25 to 77.01% respectively. Thus maximum recovery was achieved in DCM as a solvent. This might be due to different solubility of AMs in the tested organic solvents. The polarity index of the tested solvents is chloroform (4.1) > dichloromethane (3.92) > methyl tertiary butyl ether (2.4) > toluene (2.4) (data obtained from Polarity index of solvents [https://macro.lsu.edu]). Extraction efficiency data is presented in Table 3.

| Matrix solvent | Extraction solvent | Recovery (%) |
|----------------|-------------------|--------------|
|                |                   | MMS | EMS | IMS |
| Water          | Ethyl acetate     | 63.11| 65.02| 74.17|
| Water          | Toluene           | 79.51| 77.71| 76.77|
| Water          | n-Hexane          | 56.55| 52.13| 55.93|
| Water          | Dichloromethane   | 92.69| 95.11| 96.18|
| Water          | Chloroform        | 72.25| 77.01| 73.44|
| Water          | Methyl tertiary butyl ether | 52.11| 57.85| 58.07|

Auto-SRM optimization

Manual SRM method development is time-consuming, it involves selection of one or two precursor ions for each analyte, and subsequently collision energy needs to be optimized to obtain maximum response for product ions for detection. In auto-SRM method development the MS software systematically determines the intense precursor ion and product ion pairs. For auto-SRM method development, solution containing MMS, EMS, and IMS at concentration of 1000 ng/mL was used. Optimization of auto-SRM method involves three step procedure. In first step, precursor ions were identified by acquiring the data in full scan mode. Due to high energy electron ionization, 3 to 4 precursor ions were formed for each analyte. The mass spectra of MMS, EMS, IMS, and IS are provided in supplementary file S1.

In second step, all the precursors were sent for possible product ion formation. SRM pairs of the most intense precursors and product ions were created. In third step, collision induced fragmentation was optimized by ramping the collision energy.

For MMS, quantifier SRM pair was 80 > 65.1 and qualifier SRM pair was 79.1 > 75.6. The MMS possess mass of 110 Da, loss of –OCH3 with transfer of hydrogen generates ion of m/z 80. From m/z 80, loss of methyl produces m/z 65.1. Loss of methoxy from parent molecule (m/z 110) gives rise to the fragment ion of m/z 79.1, which sub-sequentially undergoes carbene formation having m/z 76.1.

For EMS, quantifier SRM pair was 109 > 79 and qualifier SRM pair was 79 > 78.3. EMS possess mass of 124 Da, loss of methyl from EMS generates ion having m/z 109. From m/z 109, loss of –OCH2 produces ion of m/z 79. This ion further fragments to m/z 78.3 with loss of hydrogen.

For IMS, quantifier SRM pair was 123 > 79 and qualifier SRM pair was 79 > 78. IMS possess mass of 138 Da, loss of methyl from IMS leads to give fragment having m/z 123. This ion of m/z 123 losses –CH3CH2 and produces fragment ion of m/z 79, which further fragments to m/z 78 by loss of hydrogen.

More intense SRM pair was used as MS quantifier and second most as MS confirming pair. This strategy is in accordance with common practice for SRM-based quantitation on a triple quadrupole which ensures specificity. Use of this approach helps to overcome the matrix effects (Wang et al. 2016). The optimized auto-SRM transitions data of MMS, EMS, IMS and chlorobenzene is provided in supplementary file S2.

In SRM mode, the mass spectrometer is set to scan all SRM transitions simultaneously. The simultaneous scanning of multiple transitions at all the time causes lowering of sensitivity. However in t-SRM mode, the mass spectrometer scans particular transition during a set retention time.
window, which ultimately effects in increasing the signal to noise ratio.

Overlaid total ion chromatograms of extraction solvent with IS, standard solution, and test solution are shown in Fig. 3. For quantitation, extracted ion chromatograms were processed using the Gaussian smooth integration.

Method validation

The developed method was specific as there was no interference from the extraction solvent at the retention time as well as mass transition of analytes. The retention times of IS, MMS, EMS, and IMS were about 4.8, 5.8, 6.7, and 7.1 minute, respectively. The system was precise with RSD of the peak area ratio of 2.54%, 15.54%, and 5.31% for MMS, EMS, and IMS, respectively.

Because the test batch of ALA did not contain AMs, method precision was determined on the externally spiked solution. The method was precise with RSD of the individual AM content less than 10% determined on six individual measurements in repeatability and intermediate precision experiments.

The detector response was linear for MMS in the range of 3.1 to 255.8 ng/mL with regression equation $y = 22.33x + 0.0247$ and correlation coefficient of 0.9991; EMS in the range of 3.1 to 255.8 ng/mL with regression equation $y = 10.572x - 0.0136$ and correlation coefficient of 0.9986; IMS in the range of 10.2 to 255.8 ng/mL with regression equation $y = 5.118x - 0.0136$ and correlation coefficient of 0.9975. The calibration curves of MMS, EMS, and IMS are provided in supplementary file S3.

LOQ of MMS, EMS, and IMS were 5, 10, and 20 ng/g of ALA, respectively. On column LOQ of MMS, EMS, and IMS were 0.01, 0.02, and 0.04 ng, respectively. RSD of the peak area ratio at the LOQ was 4.40%, 5.44%, and 3.43% for MMS, EMS, and IMS, respectively. RSD of the peak area ratio at the LOD was 4.64%, 14.02%, and 10.70% for MMS, EMS, and IMS, respectively.

Method was accurate with mean recoveries of MMS, EMS, and IMS were 91.77 ± 3.09%, 93.56 ± 2.97%, and 97.65 ± 4.43%, respectively. The developed GC-MS/MS method was found robust as there was no significant effect of the possible variables on the chromatographic performance and system precision. The method validation data is summarized in Table 4.

Advantages and application of the method

The developed method uses a simple, cost effective LLE sample preparation procedure as compared to reported method like filtration (additional cost of filter), solid phase micro-extraction (high cost of extraction
cartridges), and pre-derivatization (require specific chemical, involve various side reactions, sample matrix gets more complex and overall longer analysis time). In LLE, analytes are extracted from one phase to another phase based on the partitioning of the analytes between two immiscible phases. The LLE also help to produce matrix free test solution for trace analysis, which also prevents the clogging of the injector port and column resulting in consistent response and improved GC column life.

Use of SRM scan function made the method more specific and selective as compared to the SIM. In SIM scan an intensity of the precursor ion is considered for quantification purpose whereas in the SRM scan an intensity of the product ion of the precursor ion is measured to quantify, thus the later mode enhances the specificity and selectivity of the measurement. Overall shorter run time of < 10 min for the estimation of AMs facilitate high through screening of the ALA batches in quality control set up.

Based on the therapeutic daily dose and shorter duration of the treatment, acceptable limit for genotoxic impurity in ALA is 40 μg/g. The LOQ achieved by the developed method is well below of this acceptable limit. Thus, the developed and validated method is suitable for determination of AMs in the new drug substance ALA. In comparison to the reported methods for other drugs, the developed method is more sensitive, the comparative LOQ data is summarized in Table 5.

The present method could be broadly applied to any other water soluble drug substances, excipients or drug products for determination of AMs. Sensitivity of the method could be further enhanced by modifying the split ratio, injection volume and test solution concentration.

Developed method was applied for the estimation of AMs in pre-clinical and clinical batches of ALA. All these batches of ALA were found to be free from the AMs proving the capability of the purification process adopted to remove these unwanted genotoxic impurities during manufacturing of ALA.

**Conclusion**

t-SRM-based GC-MS/MS method successfully developed for estimation of AMs in new drug ALA. Auto-SRM capability was employed to identify the apt precursor ion and product ion pair which could provide maximum sensitivity. LLE technique was engaged to isolate the AMs from the new drug substance ALA. The developed method was highly sensitive with LOQ for MMS, EMS, and IMS as 5, 10, and 20 ng/g of ALA, which was very important for investigation of genotoxic impurities in new drug substance. The method was fully validated as per the ICH guideline. The method validation results were well within the acceptance limits.

Overall, the described method is simple, convenient, rapid and cost effective. This research demonstrated application of the auto-SRM and t-SRM in detecting trace level of genotoxic impurities using GC-MS/MS during pharmaceutical development.

| Table 4 Method validation summary |
|----------------------------------|
| Parameter                       | MMS    | EMS    | IMS    |
| System precision (peak area ratio RSD, %) | 2.19   | 1.54   | 5.31   |
| Method precision (RSD, %)        | 1.50   | 1.39   | 4.64   |
| Linearity (R)                    | 0.99957| 0.99932| 0.99877|
| Mean accuracy (%) (n = 3)        | 91.77  | 95.36  | 97.65  |
| LOQ (ng/g)                       | 5      | 10     | 20     |
| LOQ (Absolute on column, ng)     | 0.01   | 0.02   | 0.04   |
| LOD (ng/g)                       | 2.5    | 5.0    | 10     |
| LOD (absolute on column, ng)     | 0.006  | 0.01   | 0.02   |
| Robustness                       | Peak area ratio (RSD, %) |
| Flow rate (+ 10%)                | 1.83   | 1.39   | 3.96   |
| Flow rate (− 10%)                | 1.74   | 1.55   | 4.81   |
| Column temperature (+ 2 °C)      | 1.93   | 1.77   | 4.39   |
| Column temperature (− 2 °C)      | 1.88   | 1.63   | 5.11   |

| Table 5 LOQ comparison of MMS, EMS, and IMS with published methods |
|------------------------|-----------------|-----------------|
| Technique              | LOQ (ng/g)      | Reference       |
|                        | MMS             | EMS             | IMS             |                  |
| GC-MS/MS-direct injection | 40              | 30              | 30              | Liu et al. 2019  |
| LC-MS/MS with ESI       | 400             | 400             | —               | Kakadiya et al. 2011 |
| LC-MS/MS with APCI      | 10000           | 10000           | 10000           | Guo et al. 2014  |
| GC-MS-direct injection  | 2720            | 2240            | 1920            | Wollein and Schramek 2012 |
| GC-MS-headspace derivatization | 310          | 60              | 150             | Alzaga et al. 2007 |
| GC-MS/MS with LLE       | 5               | 10              | 20              | Present work     |
Supplementary information

Supplementary information accompanies this paper at https://doi.org/10.1186/s40543-020-00214-w.

Additional file 1: Supplementary file S1. Mass spectrum of Chlorobenzene (IS), MMS, EMS and IMS with putative fragmentation pathway.

Additional file 2: Supplementary file S2. Auto-SRM optimization data.

Additional file 3: Supplementary file S3. Calibration curves of MMS, EMS and IMS.

Abbreviations
ALA: Allevonafloxacin mesylates; AMs: Alkyl mesylates; GTs: Genotoxic impurities; t-SRM: Time-dependent selected reaction monitoring; GC-MS/MS: Gas chromatography tandem mass spectrometry; MMS: Methyl methane sulfonate; EMS: Ethyl methane sulfonate; IMS: Isopropyl methane sulfonate; LLE: Liquid-liquid extraction; Auto-SRM: Automatic selective reaction monitoring; DNA: Deoxyribonucleic acid; ICH: International Conference on Harmonization; TTC: Threshold of toxicological concern; LC-MS/MS: Liquid chromatography tandem mass spectrometry; SIM: Selected-ion monitoring; USA: United States of America; DCM: Dichloromethane; IS: Internal standard.

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Authors’ contributions
Vinod K Ahirrao: Conceptualization, Writing-Original draft, Visualization, Rajiv A Jadhav: Methodology, Investigation, Vipul P Rane: Writing-Review and Editing. Harshal Bhmare: Validation, Ravindra D Yeole: Supervision, Project administration, Writing-Review and Editing. All authors read and approved the final manuscript.

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Availability of data and materials
All data generated during this study are included in the present manuscript and its supplementary information files.

Ethics approval and consent to participate
This article does not contain any studies with human participants or animals performed by any of the authors.

Competing interests
The authors declare that there is no conflict regarding publication of this manuscript.

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