Liquid chromatography-tandem mass spectrometric method for trace quantification of ethyl methanesulfonate: a genotoxic impurity in dapoxetine hydrochloride

Ravi Kiran Panchakarla¹, Punna Rao Ravi²*, and Venkata Gowri Chandra Sekhar Kondapalli¹*

Abstract
Background: Dapoxetine hydrochloride is a selective serotonin reuptake inhibitor drug for treating premature ejaculation. This study was designed to develop and validate a sensitive and selective LC–MS/MS method for trace analysis of genotoxic impurity ethyl methanesulfonate in Dapoxetine hydrochloride.

Results: Chromatographic separation was achieved on the Shodex RSpak DS-413 column, 150 x 4.6 mm, 3.0 µm using eluent containing a equal volumes of acetonitrile and 0.1% v/v formic acid in water was used in the isocratic elution mode at a pump flow of 1.0 mL/min. No interference was observed at the retention time of ethyl methanesulfonate, indicating that the developed method is specific and selective for trace level quantification. The developed method was found to be linear in the concentration range of 1–50 ppm with coefficient of regression of 0.9997. Detection limit and quantification limit were determined to be 0.6 ppm and 1.0 ppm respectively. Acceptable RSD values (< 10.0%) and recovery results (> 90%) obtained from the accuracy and precision experiments indicate that the developed method is precise and accurate in the concentration range of 1–50 ppm. Ethyl methanesulfonate solutions were stable for two days when stored at room and refrigerated temperatures.

Conclusion: The developed method has the ability to quantify ethyl methanesulfonate in dapoxetine hydrochloride. Thus, the anticipated method has high probability to adopt in the quality testing laboratories of pharmaceutical industry.

Keywords: LC–MS/MS, MRM, Genotoxic impurities, Dapoxetine, Active pharmaceutical ingrediant

Background
Over the last decade, regulatory agencies across the world are increasingly vigilant on the assessment and control of mutagenic impurities in active pharmaceutical ingrediants (API) and drug products. A drug substance can contain different types of trace-level impurities resulting from residues of starting materials, reagents, intermediates, by-products generated during the course of synthesis and degradation products [1]. These trace-level impurities present in the drug substance yield no therapeutic benefit to the patient but in fact have the potential to cause risk to safety of the patient. Some of trace-level impurities present in the drug substance may cause deleterious changes in the genetic material of cells. Therefore, the levels of such potential genotoxic impurities (GTIs) present in the drug substances should be assessed and controlled for ensuring the safety of the patient. The International Council on Harmonization (ICH) covers...
both the safety and quality frameworks for establishing acceptable limits that will assure negligible risk to patients [2]. The ICH M7 recommended limits for daily intake of GTIs are 120, 20, 10 and < 1.5 µg/day, for < 1 month, > 1–12 months, > 1-10 years and > 10 years to lifetime, respectively. Based on the maximum daily dose (MDD) of the drug substance, limit for the quantitation of mutagenic impurities will be established.

Dapoxetine hydrochloride (DAPO) is a novel short acting selective serotonin reuptake inhibitor drug for treating premature ejaculation (PE) and depression [3]. Unlike other selective serotonin reuptake inhibitors, DAPO is absorbed and eliminated rapidly from the body. Due to its absorption, distribution, metabolism, and excretion (ADME) properties, DAPO is recommended and prescribed for the treatment of PE rather than depression. The maximum recommended oral dose of DAPO is 60 mg per day for adults in the age group of 18–65 years. DAPO is available as tablets with 30 mg as well as 60 mg dose strengths. It is reported to have several side effects including diarrhea, anxiety, dry mouth etc. Presence of any GTIs in the DAPO can further aggravate the side effects of the drug in patients receiving drug products containing DAPO. Therefore, it is very important to identify and quantify any GTIs that could be present in DAPO.

In the synthesis of DAPO, methane sulfonylchloride is used to protect alcohol group in the stage three intermediate of the synthesis. During the downstream processing of DAPO ethanol is used as the solvent. It is highly likely that ethanol (used in the downstream processing) can react with residual amount of methane sulfonylchloride (remaining from stage III reaction) or methane sulfonic acid (formed due the hydrolysis of methane sulfonylchloride due its high reactivity with water) to generate EMS. Therefore, in the bulk synthesis of DAPO, it is quite possible that there will be trace levels of EMS in DAPO. Based on the structural alerts [4, 5] and more importantly as per the guidelines issued by European Directorate for the Quality of Medicines and Healthcare (EDQM), EMS is considered to be genotoxic in nature and should be measured at trace levels [2, 6, 7]. The complete synthetic scheme of DAPO

![Synthetic scheme of dapoxetine hydrochloride](image-url)
is shown in Fig. 1. Based on the MDD, EMS should be controlled at 25 ppm in the DAPO drug substance.

There are few methods reported in the literature for determination of EMS using gas chromatography (GC) with flame-ionization detector (FID) and mass analyzer. Some of the reported GC methods involve direct determination of EMS [8–11], while the remaining involve derivatization of EMS using different reagents [12, 13]. The reported GC methods couple with FID or mass analyzer for direct determination of EMS were found to have less sensitivity and higher injection volumes which are not ideal for regular GC analysis. While the GC methods involving derivatization of EMS can suffer from false-positive results due to the possibility of residual alcohols present in the drug substances also undergoing derivitization. The mass spectrometric methods reported [14–17] for the determination of EMS in different drug substances were found to have either higher injection volume or higher LOQ or narrow linearity ranges.

Till date, no method is reported for quantification of EMS in DAPO by LC–MS/MS. In this current work, simple and sensitive LC–MS/MS method was developed and validated for the determination of EMS in DAPO. The developed method was used for trace-level quantification of EMA in three batches of DAPO and its advanced intermediate.

Methods
Safety concerns
All samples of EMS were handled under a fume hood due to its genotoxic properites. Appropriate institutional safety procedures were followed in the collection and disposal of eluent obtained from the liquid chromatographic anlaysis of samples.

Chemicals and materials
DAPO was obtained as gift sample from Herrlich Pharma, Hyderabad, India. EMS (Purity > 99%), formic acid and acetonitrile (ACN) was procured from Merck, Mumbai, India. Reagents and solvents used in this study were of mass spectrometric grade. Chromatographic grade water obtained from water purification system (Merck/Milli-Q® Integral 3 system, MA, USA) was used in the preparation of mobile phase and the diluents.

Instrumentation
Shimadzu Nexera-X2 chromatographic separation unit (Shimadzu Corp, Kyoto, Japan) attached with 8040 triple quadrupole mass analyzer (Shimadzu Corp, Kyoto, Japan) was used for this study. Data acquisition and integration was performed using automated software (Shimadzu Corp, Kyoto, Japan). During the preparation of sample and standard solutions calibrated auto-pipettes were used. Filtration of premixed eluent was performed using 0.45 µm membrane filter (Millipore® MA, USA). Before injection, all the standard and sample solutions were filtered using 0.45 µm polyvinylidene difluoride (PVDF) syringe filter (Millipore® MA, USA).

Liquid chromatographic and mass spectrometric conditions
LC conditions
Separation was performed on Shodex RSpak DS-413 (150 x 4.6 mm, 3.0 µm) column in reverse phase mode. The eluent solution containing a mixture of an organic phase and aqueous phase. Aqueous phase consisted of 0.1% v/v of formic acid in water, while the organic phase contains ACN. Separation between EMS and DAPO was achieved in isocratic elution mode with eluent flow of 1.0 mL min⁻¹. The sample injection volume was 20 µL. Autosampler and column oven temperatures were set at 25 °C (± 2 °C) and 30 °C (± 2 °C), respectively. Method conditions are detailed in Table 1.

MS conditions
Electron-spray ionization (ESI) source in positive mode using multiple reaction monitoring (MRM) was employed for the ionization of EMS. Source and desolvation line (DL) temperatures were set at 150 °C and 320 °C, respectively.

| Parameter | Conditions |
|-----------|------------|
| Eluent    | 0.1% Formic acid in water/acetonitrile—50/50 (v/v) |
| Flow rate | 1.0 mL min⁻¹ |
| Auto-sampler temperature | 25 °C |
| Injection volume | 20 µL |
| Column temperature | 30 °C |
| Elution | Isocratic |
| Run time | 10 min |
| Source | ESI |
| Ionization mode | Positive |
| Capillary voltage | 5 kV |
| Cone voltage | 30 V |
| Acquisition mode | MRM |
| Parent m/z selection for quantification | 125.1 Da |
| Source temperature | 150 °C |
| Desolvation temperature | 320 °C |
| Drying and nebulization gas | Nitrogen |
| Nebulization gas flow | 1.5 mL min⁻¹ |
| Drying gas flow | 15 mL min⁻¹ |
| CID gas | Argon |
| Collision energy | 40 V |

Table 1 Summary of method conditions
respectively. Ultra-high pure nitrogen gas (99.95% purity) was used as nebulizing and drying gas at a flow rate of 1.5 mL min\(^{-1}\) and 15 mL min\(^{-1}\), respectively. Ultra-high pure argon gas was used as collision-induced dissociation (CID) gas to enhance the response. MRM transition of m/z 125.1 > 97.0 was employed for the quantification of EMS.

**Preparation of stock solutions and sample solutions**
Primary stock solution of EMS was prepared at a concentration of 0.125 mg mL\(^{-1}\) using a mixture of water:ACN (10:90 v/v) as diluent and stored under refrigerated conditions. First intermediate stock solution (1.25 µg mL\(^{-1}\)) of EMS was prepared from the primary stock solution (0.125 mg mL\(^{-1}\)). First intermediate stock solution of EMS was further diluted to yield a second intermediate stock solution of EMS with a concentration of 250 ng mL\(^{-1}\). Calibration curve standard solutions were prepared by drawing appropriate aliquots of either first intermediate stock solution or second intermediate stock solution and diluting with a mixture of water:ACN (10:90 v/v). Six different calibration curve standard solutions (10, 125, 250, 500 ng mL\(^{-1}\)) were prepared for EMS in the range of 10–500 ng mL\(^{-1}\) (≈ 1–50 ppm with respect to DAPO amount).

System suitability was established by injecting six replicate injections of second intermediate stock solutions (250 ng mL\(^{-1}\)) of EMS and determining the relative standard deviation [RSD (%)] of response obtained for the replicate injections.

For determining the LOD and LOQ concentration of EMS in the optimized method, different concentrations of EMS standard solutions ranging from 2 to 25 ng mL\(^{-1}\) (≈ 0.2–5 ppm w.r.t 10 mg mL\(^{-1}\) concentration of DAPO) were prepared from the second intermediate stock solution of EMS (250 ng mL\(^{-1}\)).

The precision of the optimized method was established by performing method precision (intra-day precision, repeatability) and intermediate precision (reproducibility) studies using the spiked sample solution. In the method precision study, six different spiked samples were prepared by spiking the EMS at 100% level in the DAPO sample. Reproducibility was performed by repeating method precision experiment by different analyst on different day using different column.

Accuracy of the method was evaluated using un-spiked and spiked samples of DAPO. Unspiked DAPO sample (0% level) and spiked samples at five different levels (LOQ, 50%, 100%, 150% and 200%), with respect to the DAPO concentration (10 mg mL\(^{-1}\)) were prepared in triplicate using the same diluent used for the preparation of primary and secondary stock solutions.

For robustness studies, second intermediate stock solution (250 ng mL\(^{-1}\)) of EMS and spiked samples of DAPO (10 mg mL\(^{-1}\)) with EMS at 100% concentration level were used. The study was done in triplicates. Test samples of DAPO and its process intermediate were prepared in duplicate at 10 mg mL\(^{-1}\).

**Method validation**
The developed method for the low-level quantification of EMS in DAPO was systematically validated as per the regulatory guidelines [18, 19]. As part of method validation, sensitivity, selectivity, accuracy, linearity, precision, solution stability and robustness.

**Results**

**Optimization of liquid chromatographic and mass spectrometric method conditions**
In the development of a LC–MS/MS method for quantification of EMS (a non-volatile GTI) in DAPO, first the LC conditions were optimized followed by the mass spectrometric conditions. Optimization of LC conditions was done based on elution time of EMS and DAPO, peak area of EMS and resolution between EMS and DAPO. Based on the trials, Shodex RSpak DS-413 column (150 mm × 4.6 mm × 3.5 µm) using premixed eluent containing equal ratio of 0.1% v/v formic acid in water and ACN in isocratic elution mode were selected as final LC conditions.

Various experimental runs were performed by changing the mass spectrometric parameters. Finally, better sensitivity was achieved using the conditions mentioned in Table 1.

**Method validation**

**Specificity**
The total ion chromatogram clearly indicate that baseline is clean and free from interference at the elution time of EMS and there is good separation of EMS from DAPO. The MRM transition chromatograms at m/z of 125.1(Q1)/79.1(Q3) for blank, un-spiked DAPO sample (10 mg mL\(^{-1}\)), standard EMS solution (25 ppb), and a spiked sample of DAPO with EMS is presented in Fig. 2.

**Sensitivity**
Limit of detection (LOD) and limit of quantification (LOQ) concentrations for EMS were determined to be 0.6 ppm and 1.0 ppm, respectively, w.r.t. 10 mg mL\(^{-1}\) sample concentration of DAPO. The MRM transition chromatograms at m/z of 125.1(Q1)/79.1(Q3) for blank, LOD solution and LOQ solution is presented in Fig. 3. RSD values of six replicate injections of EMS at LOQ level was found to be 2.58%.
**Linearity**

The calibration curve for EMS in DAPO (Range: 1–50 ppm with respect to DAPO amount) was constructed with concentration of EMS on the x-axis and peak area response on the y-axis. From the regression...
analysis; $r^2$, intercept, slope, and residual plots pattern were calculated and results are briefed in Table 2.

### Precision
RSD values for EMS content from six individual preparations of spiked solution in the repeatability and reproducibility experiments were found to be within the predefined acceptance criteria of $\leq 10.0\%$. Cumulative RSD (%) values for the results obtained from precision (analyst 1) and intermediate precision (analyst 2) were found to be within the satisfactory limit of $\leq 10\%$. Results from the precision studies are detailed in Table 2.

### Accuracy
Percentage recovery values of EMS, at all the five levels in the range of LOQ-200%, were found to be $> 90\%$ with RSD value of $< 7.2\%$. These results indicate the trueness of the method as the percentage recovery and %RSD values were found to be within the satisfactory limits of $80–120\%$ and $\leq 10\%$, respectively. The results from accuracy studies are summarized in Table 2.

### Robustness
Developed method robustness was assessed for the eluent flow rate (0.8 and 1.2 mL min$^{-1}$) and nebulization gas flow rate (1.3 and 1.8 mL min$^{-1}$) and the results are summarized in Table 2. RSD value from six replicate injections of system suitability solution of EMS for the change in eluent flow rate and nebulization gas flow rate was found to be $< 5.3\%$. Percentage recovery values of EMS from triplicate spiked sample preparations for the change in eluent flow rate and nebulization gas flow rate were found to be in the range of 91–103%. Results from different robustness parameters were found to be within the acceptance criteria of $\leq 10\%$ (RSD) and within limit of 80–120% for recovery.

### Table 2 Summary of data obtained from method validation

| Test parameter               | Acceptance criteria                                                                 | Results for EMS                                                                 |
|------------------------------|--------------------------------------------------------------------------------------|---------------------------------------------------------------------------------|
| System suitability           | %RSD for peak area response ($n = 6$) Day-1: 1.6% Day-2: 0.69%                     |                                                                                |
| Specificity                  | Interference from blank                                                               | No interference                                                                 |
| Sensitivity                  | Concentration                                                                        |                                                                                |
|                              | $S/N$ for LOD solution should be $> 3.1$                                             | 4.1                                                                            |
|                              | $S/N$ for LOQ solution should be $> 10.1$                                            | 15.1                                                                           |
|                              | RSD for six replicate LOQ solution injections should be $\leq 15.0\%$                | 2.38                                                                           |
| Linearity                    | Range                                                                                | 1.02–50 ppm                                                                   |
|                              | Calibration Equation $y = 297x + 770.9$                                              | 0.9997                                                                        |
|                              | Residual plots                                                                       | Random scatter                                                                 |
| Precision                    | Average recovery ($n = 6$) from the spiked samples performed at 100% level; RSD should be $\leq 10.0\%$ | 93.6%; 5.4%                                                                   |
| Accuracy                     | Average recovery ($n = 3$) from the spiked samples performed at 5 levels—LOQ—200%; RSD should be $\leq 10.0\%$ | LOQ—90.3%; 7.2% 50%–90.9%; 6.5% 100%–95.8%; 1.5% 150%–105.3%; 1.9% 200%–102.6%; 1.1% |
| Intermediate precision (Ana- | Average recovery ($n = 6$) from the spiked samples performed at 100% level; RSD should be $\leq 10.0\%$ | 96.9%; 3.6%                                                                   |
| lysit 2)                     | Standard and 100% spiked solution stored at ambient laboratory conditions (25 ± 5 °C) and refrigerated conditions (2–8 °C) were studied for 48 h | Stable for 48 h                                                               |
| Solution stability           |                                                                                      |                                                                                |
| Robustness                   | RSD (%) for peak area response ($n = 6$) with 0.8 flow rate 2.4%                     |                                                                                |
|                              | %Recovery ($n = 3$) for 100% spiked solution with 0.8 flow rate 95.6%                  |                                                                                |
|                              | RSD (%) for peak area response ($n = 6$) with 1.2 flow rate 1.8%                      |                                                                                |
|                              | %Recovery ($n = 3$) for 100% spiked solution with 1.2 flow rate 91.4%                  |                                                                                |
|                              | RSD (%) for peak area response ($n = 6$) with 1.3 mL/min gas flow 5.3%                 |                                                                                |
|                              | %Recovery ($n = 3$) for 100% spiked solution 102.8%                                    |                                                                                |
|                              | RSD (%) for peak area response ($n = 6$) with 1.8 mL/min gas flow 2.2%                 |                                                                                |
|                              | %Recovery ($n = 3$) for 100% spiked solution 93.5%                                      |                                                                                |
**Solution Stability**

Solution stability of secondary intermediate stock solution (250 ng mL\(^{-1}\)) of EMS and spiked samples of DAPO (10 mg mL\(^{-1}\)) containing EMS at 100% concentration level were evaluated up to two days at ambient laboratory temperature (25±5 °C) and refrigerated condition (2–8 °C). The percent recoveries of EMS in secondary intermediate stock solution and spiked sample were calculated by matching against the freshly prepared secondary intermediate stock solution of EMS. The data obtained from the stability studies for EMS are presented in Fig. 4.

**Method application**

The level of EMS present in the three batches of bulk samples of DAPO and its advanced intermediate was found to be less than LOD of the method.

**Discussion**

The objective was to develop a capable method for fast and accurate quantification of EMS in the shortest run time with realistic accuracy [20, 21]. Optimization of LC conditions was done based on elution time of EMS and DAPO, peak area of EMS and resolution between EMS and DAPO. During the optimization of LC conditions, first different stationary phases (such as Luna C18, Zorbax C8, Shodex RSPak DS-413, and Atlantis T3) with varied polarity were evaluated with different aqueous and non-aqueous mobile phase compositions. As a part of selecting the LC–MS compatible aqueous mobile phase (mobile phase A), two different solvents, namely, 10 mM ammonium acetate in water and 0.1% v/v formic acid were tried. Methanol and ACN were tried to identify the suitable non-aqueous mobile phase (mobile phase B).

In the optimization of MS conditions, the sample was ionized using electrospray ionization technique and analyzed in scan mode with m/z range of 50–500. The resultant MS signal was found to have good intensity for the parent ion (Q1). Subsequently the parent ion (Q1) was fragmented by providing collision energy in the presence of collision induced dissociation (CID) gas in the second quadrupole (MS2) to yield daughter ion (Q3) with good intensity. The CID gas and collision energy parameters were fine tuned to get stable daughter ion (Q3). Further, instrument dependent parameters like desolvation line (DL) and source temperature, CID and drying gas flows were optimized for quantification of EMS. Two different transitions at m/z values of 125.1(Q1)/79.1(Q3) and 125.1(Q1)/97.0(Q3) were monitored during the optimization studies. The response obtained for the transition 125.1(Q1)/79.1 (Q3) was not intense and therefore it was not considered for further experiments. In the final optimized method, 125.1(Q1)/97.0 (Q3) was selected for sample analysis.

No interference was observed in the MRM transition chromatograms of blank and DAPO at m/z of 125.1(Q1)/79.1(Q3) at the retention time of EMS, indicating that the developed method is specific and selective for EMS. LOQ value found to be lower or equal to the reported LC–MS/MS methods [14, 16, 17]. F\(_{\text{cal}}\) values from the linear regression analysis were found to be significantly greater than their corresponding F\(_{\text{crit}}\) at 5% level of significance, suggesting that the regression model is significant. Lower %Y-intercept and higher r\(^2\) values from the calibration curve standards in the entire range demonstrates that the method is linear. Residual plots obtained from the linear regression analysis have shown a random distribution of the residuals across zero, indicating the absence of a trend/bias in the results.

RSD values and recovery results obtained from the method validation (Table 2) indicate that the developed method is precise and accurate for the low level quantification of EMS in DAPO. The data from the robustness experiments had appreciably indicated that the optimized method is robust to the small deliberate changes in the method parameters.

The data from solution stability experiments indicated that no significant difference in the stability of the samples when stored at room (25±5 °C) and refrigerated (2–8 °C) temperature for a period of two days.
Conclusions
The developed method is rapid, sensitive and selective, accurate and precise for trace-level determination of EMS in DAPO and its advanced intermediate. Standard solutions of EMS as well as the spiked sample solutions of EMS were found to be stable for two days at room temperature and refrigerated conditions. The method was successfully employed in the quantification of EMS in three batches of DAPO and its advanced intermediate. This method can be applied in pharmaceutical testing labs for trace level quantification of the EMS in DAPO and can be easily adopted to other sample APIs.

Abbreviations
ACN: Acetonitrile; ADME: Absorption, distribution, metabolism and excretion; API: Active pharmaceutical ingredient; CID: Collision-induced dissociation; DAPO: Dapoxetine hydrochloride; EDQMH: European directorate for the quality of medicines and healthcare; EMS: Ethyl methanesulfonate; FID: Flame ionization detector; GC: Gas chromatography; GTI: Genotoxic impurity; ICH: International council for harmonization; LC: Liquid chromatography; LC–MS/MS: Liquid chromatography-mass spectrometer; LOD: Limit of detection; LOQ: Limit of quantification; MDD: Maximum daily dose; MS: Mass spectrometer; MRM: Multiple reaction monitoring; PE: Premature ejaculation; PVDF: Polyvinylidene difluoride; RSD: Relative standard deviation.

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Authors’ contributions
RKP designed experiment, carried out the experiment and contributed in framing the article. PRR and KVG assisted during the method development and analysis using LC–MS/MS. All authors read and approved the final manuscript.

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Data can be shared upon request.

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Competing interests
The author declares no competing interests.

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