Asian Journal of Chemistry; Vol. 36, No. 3 (2024), 683-689

ASIAN JOURNAL OF CHEMISTRY

https://doi.org/10.14233/ajchem.2024.30736

# *In silico* Toxicity Assessment and Quantification of Potential Genotoxic Impurity in Olmesartan Medoxomil Drug Substance using GC-MS/MS

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*Received*: 20 October 2023; *Accepted*: 5 February 2024; *Published online*: 28 February 2024; AJC-21562

This study was designed to assess the *in silico* toxicity of 4-chloromethyl-5-methyl-1,3-dioxol-2-one (4-CMMD) in olmesartan medoxomil (OLM) using sophisticated advanced analytical GC-MS/MS method. The developed GC-MS/MS method is more sensitive as well as selective, for trace level analysis of genotoxic impurity 4-chloromethyl-5-methyl-1,3-dioxol-2-one (4-CMMD) in olmesartan medoxomil (OLM). *In silico* genotoxicity of 4-CMMD have been confimed by ICH M7 guidelines and tested to be "POSITIVE" in both knowledge and statistical based approaches. The conditions of gas chromatographic separation and mass spectrometry were optimized on stationary phase DB-35MS, helium carrier gas as at a flow rate of 1.5 mL/min. Quantification was performed in multiple reaction monitoring (MRM) mode. The absence of interference at the retention time of 4-CMMD indicates that the newly developed approach is very specific and selective for accurately measuring trace levels of impurities. Additionally, this method provided linear results which were validated by linearity solutions, with concentrations ranging from 3.74 to 45.12 ppm and an observed coefficient of regression of 0.9981. Sensitivity results shows this method is more sensitive detection limit (DL) achieved at 1.23 ppm and quantification limit (QL) achieved at 3.74 ppm. The developed method is precise and accurate according to the precision results, which show RSD values < 10% and recovery > 90%, both of which are within acceptable standards. The solution stability of the samples was assessed at both room temperature and refrigerated settings, and it was found to be stable for a period of 48 h. As a result, this method has been employed for the intented purpose of quantifying 4-CMMD at the trace levels in testing laboratories, pharmaceutical analytical laboratories, and quality control laboratories.

**Keywords: Genotoxic impurities, GC-MS/MS, Multiple reaction monitoring, Olmesartan medoxomil, Drug substance.**

## INTRODUCTION

In the commercial synthesis of drug substance, trace-level genotoxic impurities are originated from a variety of sources such as starting materials, reagents, intermediates, byprodcuts and catalysts [1]. Regulatory requirements for identifying and controlling genotoxic impurities (GTIs) have become more rigorous due to their toxic character. This is reflected in the increased number of literature on the subject in the past decade. In order to safeguard the paitent's safety, GTIs in the active pharmaceutical ingridents should be assessed and controlled using sensitive analytical methods [2-4]. To minimize the risk of GTI's, International Council on Harmonization (ICH) provides safety and quality frameworks for the determination of the allowable limits [5,6]. The limit for the genotoxic impurity is determined by the maximum daily dosage (MDD) of the drug substance and the duration of treatment.

Olmesartan medoxomil (OLM) (Fig. 1a) is a novel angiotensin II receptor blocker drug used for the treatment of hypertension [7] and it was 139$^{th}$ most prescribed medication in United States [8]. The maximum recommended oral dose of OLM is 40 mg per day in adults. During the synthesis of OLM, 4-CMMD (Fig. 1b) is used as an alkylating intermediate. There is a scarcity of genetic toxicology data in the literature, therefore, it is feasible

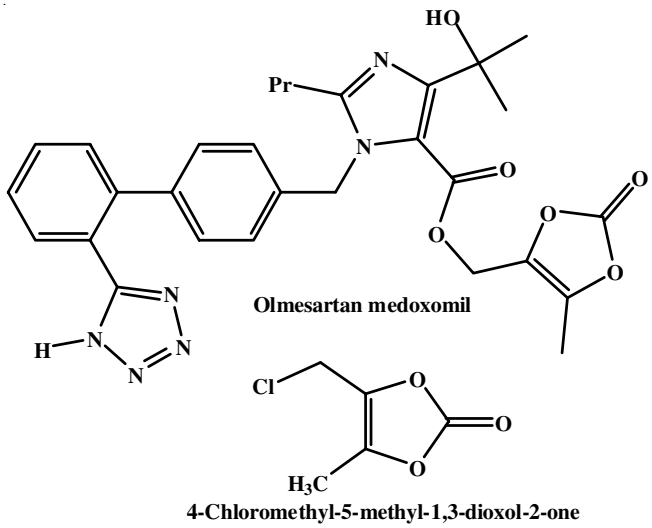


Fig. 1. Structure of olmesartan medoxomil (OLM) and 4-chloromethyl-5-methyl-1,3-dioxol-2-one (4-CMMD)

that OLM contains impurities containing residual quantities of 4-CMMD. Hence, *in silico* assesment study was performed for 4-CMMD as per the ICH M7 guide-lines using expert and statistical based approach. Based on MDD (highest dose that a patient may be administrated in one day), 4-CMMD should be regulated at 37.5 ppm in the OLM drug substance.

Various analytical methods have been reported for both qualitative and quantitative analysis of OLM using HPLC-UV and mass spectrometer (MS) detection [9-16]. These methods are applicable for analyzing both bulk drug and formulation samples. The reported methods are neither sensitive nor specific for the low-level quantification. In recent days, *in silico* toxicity studies gaining attention for the prediction of genetic toxicity of the impurities [17]. Currently, no *in silico* toxicology assessment performed or analytical technique has been reported by using GC-MS/MS technique for trace level quantification of 4-CMMD in OLM. This work aims to validate and optimize a suitable approach the GC-MS/MS method for trace level detection in OLM and assess the *in silico* toxicity of 4-CMMD.

## EXPERIMENTAL

Olmesartan medoxomil (OLM, purity > 99%) was obtained as a gift sample from a local pharmaceutical company of Hyderabad, India. GC grade 4-Chloromethyl-5-methyl-1,3-dioxol-2-one (4-CMMD, purity > 99%) and dichloromethane were procured from Merck, India). Gas chromatographic grade gases, which include nitrogen, hydrogen and helium as well as zero air gases, were purchased from Siddi Vinayaka Industrial Gases Pvt. Ltd., Hyderabad, India. The DB-35MS column (30 m × 0.25 mm) with a film thickness of 0.25 µm (Agilent Inc. USA) was used.

**Instrumentation:** Gas chromatograph (Shimadzu Corp, Kyoto, Japan) coupled with TQ8050 NX triple quadrupole mass analyzer (Shimadzu Corp, Kyoto, Japan), semi-micro analytical balance (Mettler Toledo®, OH, USA) was used for this study. Automated software (Shimadzu Corp, Kyoto, Japan) was used for the data collection and processing of the analyte peak. The calibrated auto-pipettes were employed to dilute the standard and sample solutions.

**GC-MS conditions:** In this method, a DB-35MS column (30 m × 0.25 mm, film thickness 0.25 µm) was used for the chromatographic separation process. The initial oven temperature program began at 65 ºC and held isothermal for 2 min. After that, it ramped up to 170 ºC at a rate of 15 ºC per min and held isothermal for 5 min. The split ratio was 1:6 and the injection volume was 1.0 µL. Helium carrier gas was employed, with a 1.5 mL min flow rate. Injector temperature was maintained at 120 ºC. Electron impact ionization (EI) source is in positive mode using multiple reaction monitoring (MRM) was employed to ionization of 4-CMMD. Interface temperature was adjusted to 230 ºC, whereas the ion source temperature was adjusted to 220 ºC. The MRM transition of *m/z* 148→113 (major fragment for quantification), *m/z* 148→69 (minor fragment for qualification) was employed for the quantification of 4-CMMD. Table-1 contains the parameters that are used in the GC-MS technique.

TABLE-1
SUMMARY OF GC-MS/MS METHOD CONDITIONS

| Parameter | Conditions |
|---|---|
| Column | DB-35 MS, 30 m × 0.25 mm, 0.25 µm |
| Carrier gas | Helium |
| Flow rate | 1.5 mL/min |
| Injector temperature | 120 °C |
| Injection volume | 1 µL |
| Split ratio | 1:6 |
| Oven program | Initial 65 °C hold for 2 min<br>Ramp@ 15 °C/min to 170 °C hold for 5 min |
| Elution | Gradient |
| Run time | 14 min |
| Source | EI |
| Ionization mode | Positive |
| Detector voltage | 2.0 KV |
| Source temperature | 220 °C |
| Interface temperature | 230 °C |
| Acquisition mode | MRM |
| MRM transitions | *m/z* 148→113 (major fragment used for quantification)<br>*m/z* 148→69 (major fragment used for qualitative analysis) |
| Collision energy | 6 V for *m/z* 148→113 and<br>12 V for *m/z* 148→69 |

**Preparation of solutions (stock and sample):** Prepared a 4-CMMD standard stock solution at a concentration of 0.375 mg/mL by diluting it with dichloromethane (DCM). A solution at 3.75 µg/mL of 4-CMMD prepared from the primary stock solution (0.375 mg/mL), labeled as first intermediate stock. First intermediate stock solution was further diluted to in the concentration range of 3.75-45.00 ng/mL and labeled as suitability solution. The intermediate stock solution was used for the preparation of calibration curve standard solutions and diluted with dichloromethane. Seven different calibration curve standard solutions (3.75, 11, 30, 33.5, 37.5, 40.0, 45.0 ng/mL) have been prepared for 4-CMMD in the range of 3.75-45 ng/mL (≈ 3.75-45 ppm with respect to OLM amount).

As a system suitability run, given six replicate injections of 4-CMMD into chromatographic system at 37.5 ng/mL concentration and calculated % RSD for response obtained for the

six replicate injections. In the finalized method, to establish the LOD (limit of detection) and LOQ (limit of quantification), tried with different concentrations of 4-CMMD ranging from 1-6 ng/mL (with respect to 1 mg/mL test concentration of OLM) were prepared from the primary stock solution of 4-CMMD (3.75 µg/mL).

Repeatability (intraday precision) and reproducibility (intermediate precision) were performed as part of precision for the optimized method conditions on the spiked sample solution. For precision study, six different spiked samples which were prepared at 100% concentration level by spiking 4-CMMD in the OLM sample. As part of the reproducibility method precision experiment repeated with different day, with different analyst using different column.

In optimized method, the established recovery (accuracy) by using unspiked (4-CMMD at 0% level) and spiking of 4-CMMD at LOQ, LOQ, 80%, 100%, 120% and 200% levels to OLM sample, total 5 different levels were used for the accuracy study. The above spiked solutions were prepared with respect to OLM 1 mg/mL test concentration, which were prepared in triplicate. Throughout the analysis, only one type of diluent was utilized to prepare the primary and secondary stock solutions in order to avoid diluent impact and undesirable results. As part of a study on robustness, the procedure parameters were systematically modified in order to gain a better understanding of their impact on the results.

**Method validation:** The newly developed method was validated according to the regulatory guidelines [18,19]. Linearity was established by performing the analysis over a range of six various concentrations, ranging from 1.5-10.0 ppm for 4-CMMD. The calibration data was evaluated using a statistical parameter least-square regression analysis, which was carried out across the linearity range. The limit of detection and limit of quantification were determined with 0.2-2.0 ppm of 4-CMMD containing solutions, which should be injected to achieve a signal-to-noise (S/N) ratio of at least 3:1 and 10:1, respectively.

By comparing the percentage recovery values and RSD (%) values for 4-CMMD, the accuracy and precision of the optimized method was also evaluated. The obtained responses from unspiked and spiked samples prepared in triplicate determination at five different levels ranging from LOQ-200% of the specification limit was evaluated and compared. To ensure accuracy, the percentage recovery values must be within 100 ± 20%, with an RSD (%) value of less than 10%. At room temperature and in refrigerators, the solution stability was established for the sample and spiked sample solutions.

## RESULTS AND DISCUSSION

***In silico* prediction of mutagenicity and carcinogenicity of 4-CMMD:** No investigations on the toxicological potential of 4-CMMD are available in the literature database. As a result, ICH M7-based knowledge-based (DEREK Nexus) and statistically-based (SARAH Nexus) techniques were used to conduct *in silico* toxicity investigations. For 4-CMMD, the DEREK Nexus forecast is "Plausible". The structure of 4-CMMD exhibits structural alerts for both allyl and alkyl halides, which correlates to allylating and alkylating chemicals and matches examples 022 and 027 from the DEREK data set. According to the DEREK knowledge base 2022 2.0, *in vitro* mutagenicity in bacteria and *in vitro* damage to mammalian chromosomes are both possible and certain based on alert structure 027 and 022, respectively. Statistical based (SARAH) prediction was carried out using Sarah model 2.0 and 4-CMMD was predicted to be positively mutagenic *in vitro* (positive on the Ames test) with 100% confidence. The hypothesis which is supporting and contains similar examples from the training set. The DEREK and SARAH forecasts led to the classification of 4-CMMD as an ICH M7 class-3 impurity. Findings from the DEREK and SARAH Nexus for 4-CMMD are shown in Table-2.

**Optimization of the chromatographic and mass spectrometric method conditions:** Gas chromatographic separation module coupled with flame-ionization and or mass detection persists as a key analytical tool for quantifying impurities that are volatile [20,21]. The MDD of OLM were used to compute the 4-CMMD limit in accordance with the ICH M7 guidelines. A maximum of 37.5 ppm should be the limit for 4-CMMD control, with a threshold of toxicological concern (TTC) of 1.5 µg/day and an MDD of 40 mg/day.

During the optimization of the GC conditions, initially evaluated method development trial on the different polarity of stationary phases, such as DB-FFAP (30 m × 0.32 mm, film thickness 0.25 µm), DB-WAX (30 m × 0.32 mm, film thickness 0.5 µm), DB-35MS (30 m × 0.25 mm, film thickness 0.25 µm) and DB-1 (30 m × 0.32 mm, film thickness 1.0 µm) were assessed using helium as carrier gas using flame-ionization detection. Good separation (Resolution-USP; Rs > 1.5) was achieved with DB-35MS (30 m × 0.25 mm, film thickness 0.25 µm) column from the known solvent peaks, because of very stringent low level specification limit for 4-CMMD, the attempts with flame-ionization detector were not successful to achieve desired level of sensitivity. Hence, mass detection was opted for this study. As presented in Fig. 2, the total ion chromatogram for 4-CMMD contains three major transitions at *m/z* 148 (molecular ion),

TABLE-2
*In silico* TOXICITY PREDICTION RESULTS FOR 4-CMMD USING DEREK AND SARAH NEXUS SOFTWARE

| Impurity | DEREK prediction | SARAH prediction |
|---|---|---|
| Veratryl chloride | • Chromosome damage *in vitro* in mammal is "PLAUSIBLE"[a]<br>• Alert matched to 27 alkylating agent<br>• Alert matched to 22 allylating agent<br>• Mutagenicity *in vitro* in mammal is "PLAUSIBLE" | • The compound is predicted to be "POSITIVE"[b] with 100% confidence for the "mutagenicity *in vitro*"<br>• Hypotheses analysis was found to be "POSITIVE" with structure ID# H-33 and H-211 |

[a]As per DEREK prediction outcome definition, "PLAUSIBLE" indicate that the weight of evidence supports the proposition.
[b]As per SARAH prediction outcome definition, "POSITIVE" indicate that the query structure is predicted to be positive in a bacterial reverse mutation assay (Ames test).

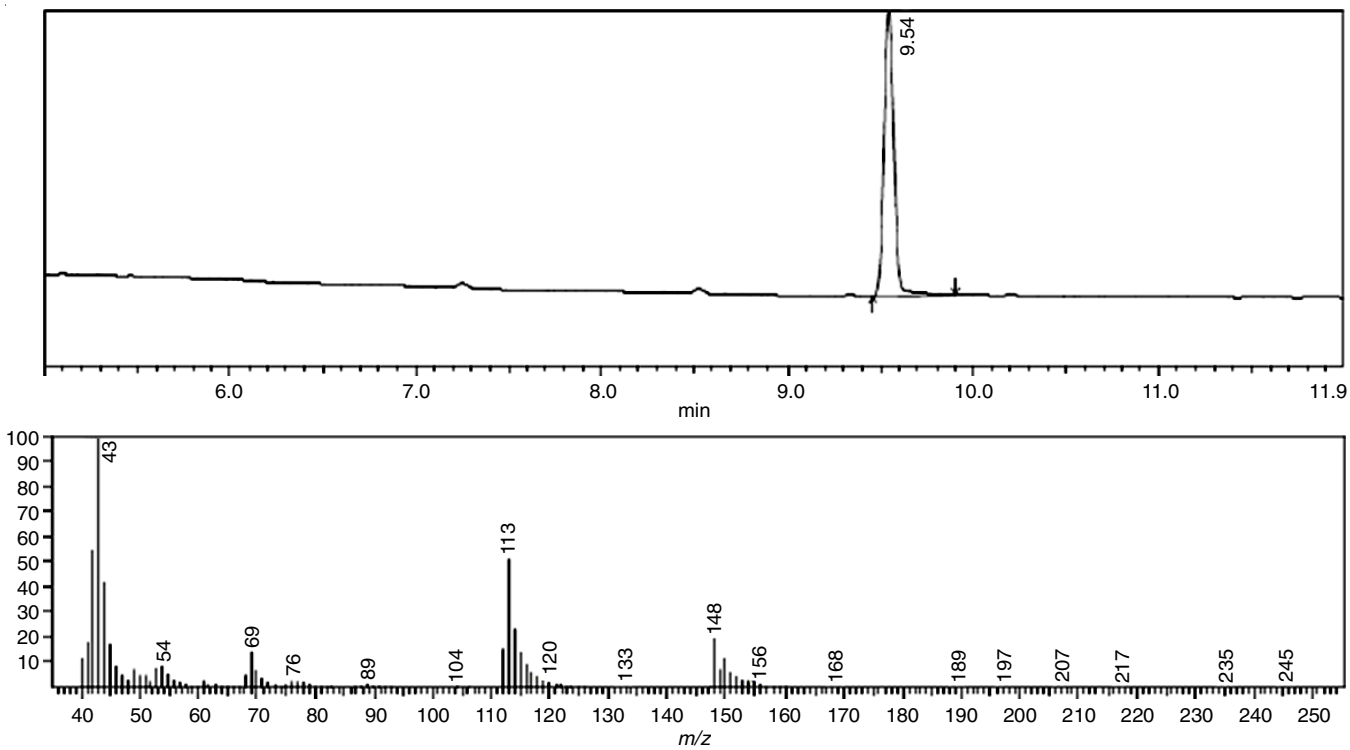


Fig. 2. Total ion chromatogram and mass spectrum for 4-chloromethyl-5-methyl-1,3-dioxol-2-one (4-CMMD)

*m/z* 113 (product ion) and *m/z* 69 (product ion). MRM transition of *m/z* 148→113 (major fragment for quantification), *m/z* 148→69 (minor fragment for qualification) was employed for the quantification of 4-CMMD.

The 4-CMMD and sample were ionized using the electron impact ionization technique under ideal MS conditions and the analysis was done in scan mode with a 10-to 300 *m/z* range. The resultant MS signal was found to have good intensity for the ions with *m/z* 148, *m/z* 113 and *m/z* 69. Two different MRM transitions of *m/z* 148→113 and *m/z* 148→69 were monitored during the optimization studies of MS conditions. The response obtained for the transition and *m/z* 148→69 was not intense and therefore it was not considered for the quantification. In the final optimized method, *m/z* 148→113 was selected for sample quantification of 4-CMMD in the samples.

Mass method parameters that were examined were collision energy, detector voltage, temperature program, injector temperature and column flow rate and the final optimized conditions are summarized in Table-1.

## Method validation parameters

**Specificity:** The retention time of 4-CMMD was found to be 9.499 min, the MRM chromatograms for the transition *m/z* 148→113, for blank, unspiked OLM sample (1 mg $mL^{-1}$), standard 4-CMMD solution (37.5 ppm), unspiked sample and a spiked sample of OLM with 4-CMMD is presented in Fig. 3.

**Sensitivity:** The LOD and LOQ values for 4-CMMD were determined to be 1.23 ppm and 3.74 ppm, respectively, with regard to 1 mg $mL^{-1}$ OLM sample concentration. The MRM transition chromatograms for LOD and LOQ solutions are shown in Fig. 4. RSD for six replicate injections of 4-CMMD at LOQ level was found to be 4.8%.

**Linearity:** The calibration curve was constructed with *x*-axis as concentration of 4-CMMD and peak response as area counts under the cureve at *y*-axis. Correlation coefficient ($r^2$), slope, intercept and residual plot pattern were computed using the regression analysis and results are tabulated in Table-3.

**Precision:** For content of 4-CMMD in OLM, six individual spiked solutions prepared for repeatability and reproducibility experiments, results found to be within the predefined acceptance criteria of ≤ 10.0%. A satisfactory results within ≤ 10% variation were obtained for the cumulative RSD (%) values which were obtained from precision (analyst 1) and intermediate precision (analyst 2), the results from the precision studies are tabulated in Table-3.

**Accuracy:** Accuracy performed from LOQ to 120%, the percentage recovery values of 4-CMMD were found to be > 90%. The findings demonstrated that the method works as intended under ideal conditions, with percentage recovery and percent RSD values falling within the acceptable limits of 80-120% and < 10%, respectively. The results from accuracy studies are tabulated in Table-3.

**Robustness:** In this method uni-variations were assessed for flow rate at 1.3 and 1.7 mL/min oven temperature at 63 and 67 ºC obtained results are tabulated in Table-3, for six replicate injections of system suitability solution of 4-CMMD, found the %RSD < 10.0%,where altered in carrier gas, flow rate and oven temperature. The recovery values for 4-CMMD in OLM from triplicate sample preparations by varying carrier gas flow rate and oven temperature were found to be in the range of 90-110%. Results found to be within acceptable criteria of ≤ 10% (RSD) and within limit of 80-120% for recovery.

**Solution stability:** For solution stability, injected system suitability standard solution at 37.5 ng/mL concentration of

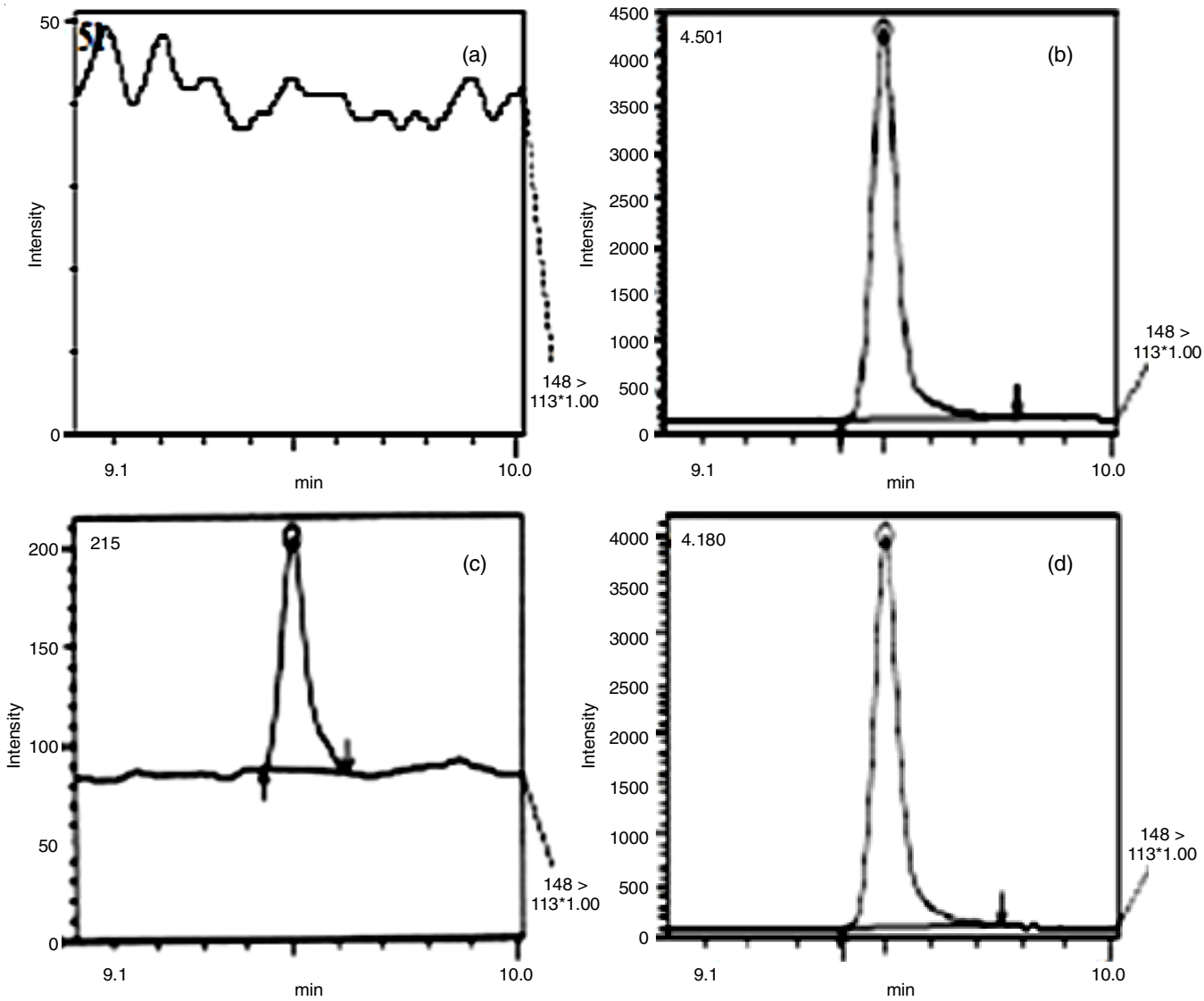


Fig. 3. MRM chromatograms of (a) blank, (b) standard 4-CMMD solution, (c) un-spiked OLM sample and (d) spiked sample of OLM with 4-CMMD

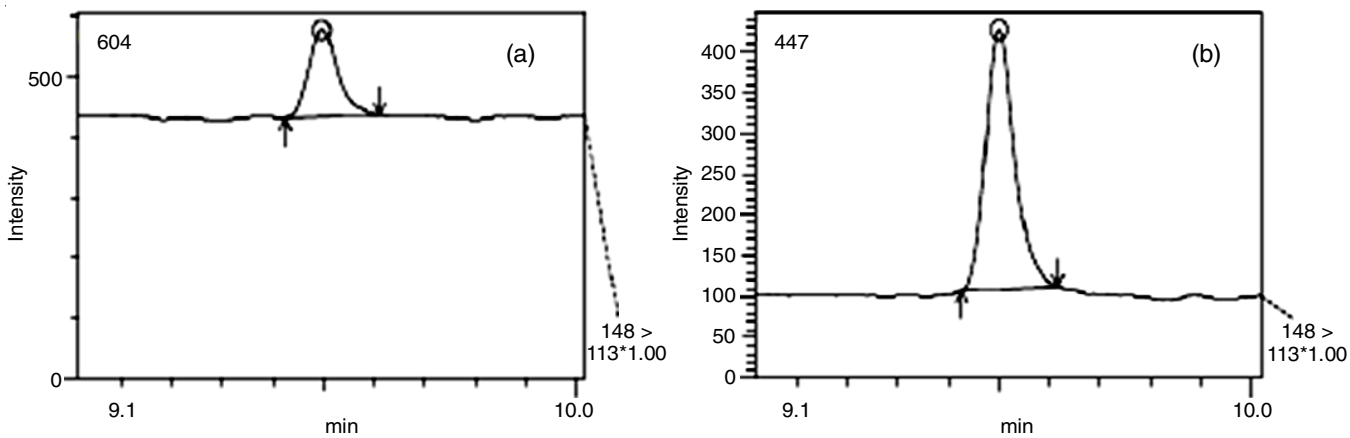


Fig. 4. MRM chromatograms of (a) LOD solution, (b) LOQ solution

4-CMMD and also prepared spiked samples of OLM at 1 mg/mL test concentration which is containing 4-CMMD at 100% concentration level were evaluated up to 48 h at ambient laboratory temperature (25 ± 5 ºC/room temperature) and refrigerated condition (2-8 °C). The percent recoveries of 4-CMMD were calculated by comparing against the freshly prepared system

TABLE-3
SUMMARY OF DATA OBTAINED FROM METHOD VALIDATION

| Test parameter | Acceptance criteria | Results for 4-CMMD |
|---|---|---|
| System suitability | %RSD for peak area response (n = 6) | Day-1: 3.32%; Day-2: 4.50% |
| Specificity | Interference from blank | No interference at 9.49 |
| Sensitivity | Concentration | LOD-1.23 ppm; LOQ-3.74 ppm |
| | S/N for LOD solution should be > 3:1 | 6:1 |
| | S/N for LOQ solution should be > 10:1 | 14.1:1 |
| | RSD for six replicate LOQ solution injections should be ≤ 15.0% | 13.7 |
| Linearity | Range | 3.74-45.12 ppm |
| | Calibration Equation | y = 422.61x + 204.72 |
| | $r^2$ | 0.9971 |
| | Residual plots | Random scatter |
| Precision | Average recovery (n = 6) from the spiked samples performed at 100% level; RSD should be ≤ 10.0% | 92.7%; 2.07% |
| Accuracy | Average recovery (n = 3) from the spiked samples performed at 5 levels-LOQ-200%; RSD should be ≤ 10.0% | LOQ-103.0%; 5.2%<br>80%-95.3%; 4.3%<br>100%-91.2%; 2.9%<br>120%-11.2%; 1.9% |
| Intermediate precision (Analyst 2) | Average recovery (n = 6) from the spiked samples performed at 100% level; RSD should be ≤ 10.0% | 94.7%; 2.6% |
| Solution stability | 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 24 h |
| Robustness | RSD (%) for peak area response (n = 6) with 1.3 flow rate | 2.6% |
| | %Recovery (n = 3) for 100% spiked solution with 1.3 flow rate | 94.6% |
| | RSD (%) for peak area response (n = 6) with 1.7 flow rate | 5.7% |
| | %Recovery (n = 3) for 100% spiked solution with 1.7 flow rate | 99.4% |
| | RSD (%) for peak area response (n = 6) with 63C Oven temperature | 3.1% |
| | %Recovery (n = 3) for 100% spiked solution | 97.7% |
| | RSD (%) for peak area response (n = 6) with 67C Oven temperature | 2.6% |
| | %Recovery (n = 3) for 100% spiked solution | 95.4% |

suitability standard solution. The data obtained from the stability studies for 4-CMMD are tabulated in Table-3.

**Method Application:** The levels of 4-CMMD present in the three bilk batch samples of OLM was found to be less than LOD of the method.

## Conclusion

In current work, the GC-MS/MS method was developed for the trace-level determination of 4-chloromethyl-5-methyl-1,3-dioxol-2-one (4-CMMD) in olmesartan medoxomil (OLM). Toxicity studies confirmed that 4-CMMD is genotoxic and as per ICH, it is classified as a class 3 impurity. The finalized method was sensitive, specific, accurate and precise for the quantification of 4-CMMD in OLM. The developed method can be implemented in the quality control lab, for routine analysis and can be adapted for analysis of 4-CMMD present in other drug substances with minimal sample preparation alteration.

## ACKNOWLEDGEMENTS

For completion of this work, the inputs were taken from Central Analytical Facilities of Jawaharlal Nehru Technological University, Hyderabad Campus, is gratefully acknowledged.

## CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this article.

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