Identification and Quantification of Trace Level Potential Multiple Genotoxic Impurities in Aripiprazole Monohydrate by using Sensitive GC–MS chromatographic Method with a Regulatory Approach for Method Validation

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Four potential genotoxic contaminants – bis(2-chloroethyl)amine, 3-aminophenol, 1,4-dibromobutane, and 2,3-dichloronitrobenzene in the drug substance Aripiprazole Monohydrate were identified and quantified using a sensitive and selective, developed and validated gas chromatography–mass spectrometry (GC–MS) analytical method. Chromatographic separation of the impurities was accomplished using a DB-624 capillary column (60 m length × 0.32 mm diameter, 1.8 µm film thickness), helium as the carrier gas, and a stationary phase composed of 6% cyanopropylphenyl and 94% polydimethylsiloxane. For quantification using distinctive fragment ions, a triple quadrupole GC/MS system operated in SIM mode was employed at m/z 92 for bis(2-chloroethyl)amine, m/z 80 for 3-aminophenol, m/z 55 for 1,4-dibromobutane, and m/z 109.1 for 2,3-dichloronitrobenzene. The method's specificity, linearity, sensitivity, precision, and accuracy were assessed in accordance with regulatory criteria. The genotoxic impurities' detection limits (LOD) and quantification limits (LOQ) were found to be between 0.18 and 0.36 PPM. The method showed excellent linearity, with correlation values ranging from 0.9979 to 0.9999. The developed method demonstrated satisfactory accuracy with average recoveries ranging from 74.0% to 98.9%, within the acceptable limits of 70–130%. The developed GC-MS method was sensitive, reliable, and suitable for routine monitoring of trace-level genotoxic impurities in Aripiprazole Monohydrate.

Keywords: aripiprazole monohydrate, genotoxic impurities, gas chromatography mass spectrometry, method validation, ICH guidelines

Introduction

Pharmaceutical medicinal substances are produced using reactive chemicals, reagents, solvents, catalysts, and other processing aids. Drug ingredients and final pharmaceutical products may contain impurities due to chemical production or subsequent degradation processes. Some of these impurities may possess genotoxic or carcinogenic potential, posing a possible risk to human health. Therefore, regulatory agencies have established strict guidelines to control such impurities in pharmaceutical products. To reduce the risk of cancer, the ICH M7 (R2) guideline offers a framework for the evaluation and management of DNA-reactive (mutagenic) contaminants in pharmaceuticals [1]. Accordingly, it is essential to identify, evaluate, and control mutagenic impurities at acceptable levels to ensure pharmaceutical safety.

Even at extremely low concentrations, DNA-reactive chemicals have the capacity to directly damage DNA, resulting in mutations and maybe cancer. The Ames test, a bacterial reverse mutation assay, is often used to identify these mutagenic carcinogens [2]. At the quantities commonly found as impurities, other genotoxicants that are not mutagenic typically show threshold mechanisms and do not create carcinogenic hazards in people. Bacterial mutagenicity assays are frequently used to assess mutagenic potential and identify the need for suitable control techniques in order to reduce the possible human cancer risk linked with exposure to mutagenic contaminants. In addition, structure-based assessments are commonly applied to forecast the results of bacterial mutagenicity using existing toxicological knowledge [3]. These assessments may involve literature evaluation and/or methods used in computational toxicology, including quantitative structure–activity relationship (QSAR) modeling.

In the case of Aripiprazole Monohydrate (ARPM), four potential genotoxic impurities — bis(2-chloroethyl) amine, 3-aminophenol, 1,4-dibromobutane, and 2,3-dichloronitrobenzene were identified using the CASE Ultra computational toxicology software. This tool identifies structural alerts associated with toxicity using (Q)SAR models [4]. The predicted probabilities

of mutagenicity for these impurities were found to be 79.5%, 48.9%, 80.8%, and 71.5%, respectively, based on the statistical model for bacterial mutagenicity based on the OECD's bacterial reverse mutation test guidelines (OECD 471) [2]. The structures of these impurities predicted using the CASE Ultra software are presented in Figure 1.

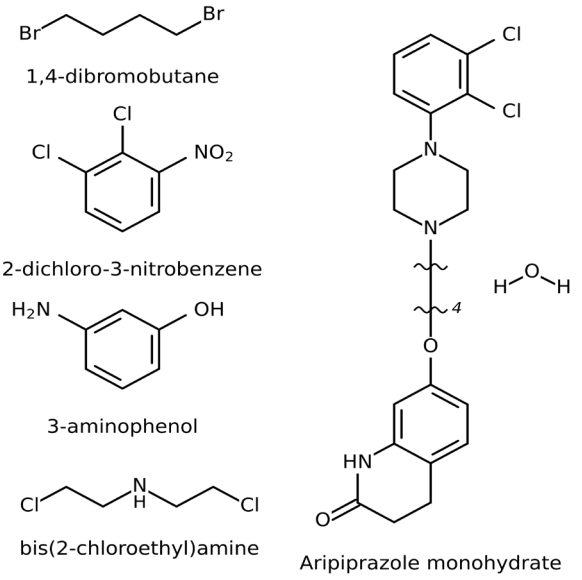


Figure 1. Chemical structures of identified genotoxic impurities and drug substance.

In the ARPM synthetic route, these impurities originate from key starting materials (KSM-I, KSM-II, and KSM-III), and 2,3-dichloronitrobenzene is also generated from KSM-I. These contaminants are categorized as Class 2 contaminants, which are recognized mutagenic carcinogens, in accordance with the ICH M7 (R2) standard [1]. The threshold of toxicological concern (TTC) is a concept that was designed to identify an acceptable intake level for unstudied compounds that have a negligible carcinogenic risk to the OECD guideline for the bacterial reverse mutation test. A TTC value of 1.5 PPM per day for mutagenic contaminants in pharmaceuticals is equivalent to a theoretical extra lifetime cancer risk of 10^{-5} (one in 100,000) [5]. Considering that 830 mg/day is the maximum daily dosage of ARPM, the 100 % specification limit for the developed analytical method was established as 1.8 PPM.

Based on literature reports, ARPM exhibits a boiling point of 137–140 °C and is soluble in dimethyl sulfoxide (DMSO), propylene glycol, and n-hexane. However, based on performancedriven solvent screening, methanol was selected as a diluent because of its ability to provide reproducible peak shape, stable response, and compatibility with the GC–MS system. Conventional detection methods like Flame ionization detection (FID) and Electron Capture Detection (ECD) have been tried for impurity analysis; however, these techniques may lack the required selectivity for trace-level genotoxic impurities (Table S1). In contrast, by detecting substances according to their mass-to-charge ratio (m/z), gas chromatography–mass spectrometry (GC–MS) offers increased sensitivity and selectivity [6-14].

Numerous analytical techniques for pharmaceutical component impurity profiling have been documented; however, extremely sensitive and selective analytical methods are needed to identify trace-level genotoxic impurities [15-25]. The analysis was performed using GC–MS with a triple quadrupole GC/MS system operated in SIM mode, which is inherently suited to single quadrupole mass spectrometers. Unlike MRM mode, which is applicable to tandem mass spectrometry (MS/MS) systems, SIM mode allows selective monitoring of predefined fragment ions, providing enhanced sensitivity and improved signal-to-noise ratio.

This approach enabled reliable trace-level quantification of multiple genotoxic impurities in Aripiprazole by monitoring compound-specific characteristic ions for each analyte, ensuring adequate selectivity and regulatory compliance. The triple quadrupole GC/MS system operated in SIM mode was selected to enhance sensitivity and selectivity for trace-level detection of the identified genotoxic impurities in the GC–MS system.

Therefore, the present study focuses on the creation and verification of a sensitive GC-MS technique to identify four possible genotoxic contaminants — bis(2-chloroethyl)amine, 3-aminophenol, 1,4-dibromobutane, and 2,3-dichloronitrobenzene in Aripiprazole Monohydrate in compliance with ICH Q2 (R2) and USP <1225> validation guidelines requirements [26,27].

Materials and methods

Chemicals and Reagents

The Active Pharmaceutical ingredient Aripiprazole Monohydrate (ARPM) was procured from Merck, India. Reference standards of the genotoxic impurities bis(2-chloroethyl)amine, 3-aminophenol, 1,4-dibromobutane, and 2,3-dichloronitrobenzene were obtained from JS Labs, Hyderabad, India. Methanol (HPLC-grade) was procured from J.T. Baker, while acetonitrile was obtained from Merck, India. The certified purities of the reference standards were 99.7% for bis(2-chloroethyl)amine, 100% for 3-aminophenol, 99.9% for 1,4-dibromobutane, and 99.9% for 2,3-dichloronitrobenzene.

Equipment, Software and Column

The gas chromatography–mass spectrometry apparatus used for the analysis was made of an Agilent 7010D GC/MS Triple Quadrupole System equipped with an electron ionization (EI) source and controlled by MassHunter Workstation software (version 10). Samples and standard solutions were accurately weighed using a Mettler Toledo XPR205DR Analytical Balance, and sample solutions were mixed using a

Genei Vortex Mixer. The genotoxic contaminants were separated chromatographically using an Agilent DB-624 Capillary Column (60 m length × internal diameter of 0.32 mm, 1.8 µm film thickness; Part No. 123-1364).

Gas Chromatographic Conditions

Chromatographic separation of the analytes was conducted on a DB-624 capillary column (60 m length × internal diameter of 0.32 mm, 1.8 µm film thickness). Helium (99.999% purity) was used as the carrier gas at a constant flow rate of 1.2 mL min^{-1}. The analytes were introduced using splitless injection mode with an injection volume of 2 µL. The oven temperature program was configured as follows: it started at 100 °C, ramped up to 240 °C, and then held for 25.5 minutes. The total chromatographic run time was 30 min.

Conditions for Mass Spectrometry

Electron ionization (EI) mode was used for mass spectrometric detection. The MSD transfer line temperature was initially kept at 250 °C, while the ion source temperature was kept at 230 °C. A triple quadrupole GC/MS system operated in SIM mode was used to collect data for the determination of the genotoxic impurities bis(2-chloroethyl) amine, 3-aminophenol, 1,4-dibromobutane, and 2,3-dichloronitrobenzene in Aripiprazole Monohydrate. The selected m/z values for quantification were 92, 80, 55, and 109.1, respectively.The mass acquisition window was set from 6.0 to 13.0 min. The detailed chromatographic and mass spectrometric conditions used in the study are summarized in Table S2.

Impurity specification limits

The specification limits for the genotoxic impurities were established in accordance with regulatory recommendations from the European Medicines Agency and the ICH M7 (R2) guidelines for the management of pharmaceutical contaminants that are DNA-reactive (mutagenic). The threshold of toxicological concern (TTC) concept states that a potential lifetime cancer risk of 10^{-5} corresponds to a tolerable consumption of 1.5 PPM per day (1500 ng/day). Taking into account the 830 mg daily maximum dosage of Aripiprazole Monohydrate, the corresponding specification limit for each genotoxic impurity was calculated. Based on this calculation, the 100% specification limit for bis(2-chloroethyl)amine, 3-aminophenol, 1,4-dibromobutane, and 2,3-dichloro-nitrobenzene was established as 1.8 PPM in the drug substance.

Preparation of solutions

Diluent. Methanol was used as the diluent throughout the study.

Blank solution. A blank solution was prepared by transferring 2.0 mL of methanol into a chromatographic vial.

Standard stock solution 1. Accurately weighed amounts (5.0 mg each) of bis(2-chloroethyl) amine, 3-aminophenol, 1,4-dibromobutane, and 2,3-dichloronitrobenzene were transferred into a single 10 mL volumetric flask. The compounds were dissolved in acetonitrile, and the solution was diluted to volume with the same solvent and mixed thoroughly.

Standard stock solution 2. An accurately weighed amount (25.0 mg) of 1,4-dibromobutane was transferred into a 50 mL volumetric flask, dissolved in acetonitrile, diluted to volume with the same solvent, and mixed thoroughly.

Intermediate stock solution. Aliquots (1.8 mL each) of standard stock solutions 1 and 2 were transferred into a 100 mL volumetric flask. The solution was diluted to volume with methanol and mixed thoroughly.

Standard solution. An aliquot (1.0 mL) of the intermediate stock solution was transferred into a 50 mL volumetric flask, diluted to volume with methanol, and mixed thoroughly.

Sample solution. Approximately 200 mg of the sample was accurately weighed and transferred into a 15 mL centrifuge tube. Subsequently, 2.0 mL of methanol was added, and the mixture was vortexed for 2 min. After standing for an additional 2 min, the solution was filtered through two sequentially connected 0.22 µm polyvinylidene fluoride (PVDF) membrane filters. The standard and sample solutions were stored at 2–8 °C until analysis.

Spiked sample solution. An accurately weighed sample (200.82 mg) was transferred into a 15 mL centrifuge tube, followed by the addition of 2.0 mL of the intermediate stock solution. The mixture was vortexed for 2 min and subsequently filtered through two sequentially connected 0.22 µm PVDF membrane filters.

Results and discussion

Analytical technique development and optimization

The current study's goal was to create an analytical technique that is sensitive, specific, linear, accurate, and exact for the determination of four potential genotoxic impurities—bis(2-chloroethyl)amine,3-aminophenol, 1,4-dibromobutane, and 2,3-dichloronitrobenzene—in the drug substance Aripiprazole Monohydrate. Initial experiments were performed using dichloromethane as the diluent and an Agilent HP5MS GC Column (30 m length× 0.25 mm diameter × 0.25 µm thickness of film). Under these conditions, the target impurities were successfully eluted and their corresponding mass fragments were identified. However, the detector responses were relatively low and not adequate for reliable quantification at trace levels. Therefore, further method optimization was undertaken with particular emphasis on minimizing matrix interference and improving analyte response. Subsequent trials were conducted using a Agilent DB624 Capillary Column (60 m length × 0.32 mm diameter× 1.8 µm thickness of film). Initially, the system was operated in full scan mode to ascertain the typical mass spectra and retention periods of the target impurities. Inlight of these results, the method was further optimized using triple quadrupole GC/MS system operated in

SIM mode for increased selectivity and sensitivity. The optimized SIM parameters are summarized in Table 3. During method development, a matrix interference effect was observed in the sample solution. To overcome this issue, the diluent was initially changed from dichloromethane to acetonitrile. However, this modification did not significantly improve peak shapes. Further optimization involved increasing the column length from DB-624 (30 m) to DB-624 (60 m) and evaluating ethyl acetate as the diluent. Although this configuration improved peak shapes and produced satisfactory detector responses, the matrix effect persisted, resulting in gradual variability in peak areas.To resolve this limitation, methanol was finally selected as the diluents, due to its adequate solubilizing capacity, volatility, compatibility with GC-MS system and superior analytical performance. Methanol was selected through performance driven solvent screening. The use of methanol resulted in stable baseline conditions, consistent peak shapes, and reproducible responses for all four impurities. In addition, comparable impurity peak areas were obtained in both standard solutions and spiked samples, indicating minimal matrix interference and confirming the development of analytical method. All standard and sample solutions were prepared freshly for each analytical run, transferred into sealed vials with minimal headspace, stored briefly at 2–8 °C, and injected immediately to prevent any potential analyte loss due to volatilization or delayed analysis. Representative chromatographic profiles corresponding to the different optimization trials are available in Figures S1–S5 in the Supplementary Information. The final representative chromatogram of developed method is shown in Figure 2.

Validation of Analytical Method

The ICH Q2(R2) requirements were followed in the validation of the developed analytical technique. System suitability, specificity, repeatability, accuracy, linearity, limit of detection (LOD), and limit of quantification (LOQ) were among the validation criteria that were assessed.

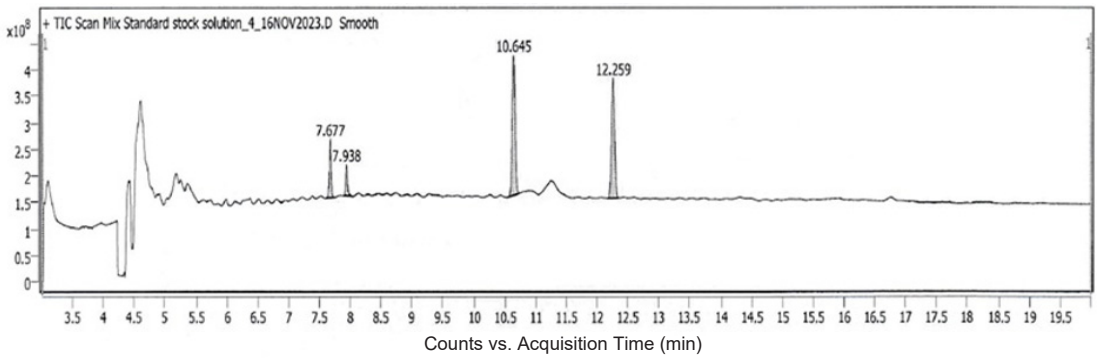
System Suitability

System suitability was assessed at the beginning of the analysis to ensure the proper performance of the ALS-GCMS system. The standard solution was injected six times containing Bis-2-chloroethylamine, 3-aminophenol, 1,4-dibromobutane, and 2,3-dichloro-nitrobenzene at the 100% specification level were analyzed. The acceptance criterion for system suitability was a percentage coefficient of variation (%CV) not exceeding 15%. The observed %CV values were 8.6%, 5.3%, 6.4%, and 8.7% for Bis-2-chloroethylamine, 3-aminophenol, 1,4-dibromobutane, and 2,3-dichloronitrobenzene, respectively.

These results demonstrate satisfactory system performance. The comprehensive findings are presented in Table 1.

Specificity

The ALS-GCMS method was used to analyze blank, standard, sample, and spiked sample solutions in order to assess specificity. The standard solution was injected six times along with injections of blank, sample, and spiked samples. According to the acceptance criteria, Blank interference shouldn't be more than 1% of the six typical injections' average response. At the analyte retention times, no interference peaks were seen. The retention times for Bis-2-chloroethylamine, 3-aminophenol, 1,4-dibromobutane, and 2,3-dichloronitrobenzene were 7.6, 10.6, 7.9, and 12.2 minutes, respectively. Chromatograms of the blank and standard solutions are shown in Figures 3 and 4, confirming that no significant interference was observed and demonstrating the specificity of the method.



Peak	Peak Name	RT	Height	Area	Area%
1	Bis(2-ChloroEthyl)amine	7.677	109323028	232076119	11.03
2	1,4-Dibromobutane	7.938	57701459	128163083	6.09
3	3-Aminophenol	10.645	263958461	919593614	43.69
4	2,3-Dichloro nitrobenzene	12.259	226288115	8247966988	39.19

Figure 2. The final representative chromatogram of developed method.

Table 3. Method validation summary for aripiprazole monohydrate genotoxic impurities.

Impurity Name	Bis-2-Chloro Ethylamine	3-Amino Phenol	2,3-Dichloro Nitrobenzene	1,4 Dibromo Butane	Acceptance Limit
Retention time (minutes)	7.68	7.94	10.65	12.25	Should be less than 13 minutes
System Suitability	8.6	5.3	8.7	6.4	%RSD NMT 10.0%
Linearity correlation coefficient (R)	0.9999	0.9979	0.9993	0.9999	NLT 0.99
Intercept	86542.4	22346.7	12737.2	57446.7	For information
Slope	616099.9	363468.2	251458.1	470197.6	For information
Detection limit, LOD (s/n ratio)	27.119	82.0250	49.8679	14.1311	NLT 3
Quantification limit, LOQ (s/n ratio)	36.8719	156.1257	78.5765	21.9620	NLT 10
LOQ Precision, % RSD (n = 6)	4.1	4.4	4.4	3.3	NLT 15.0%
Accuracy at LOQ level, % average recovery	89.4	84.7	101.4	87.0	Between 70.0% -130.0%
Accuracy at 100% level, % average recovery	84.8	87.3	98.9	91.8	Between 70.0% -130.0%
Accuracy at 150% level, % average recovery	74	80.9	91.4	84.8	Between 70.0% -130.0%
System precision (n=6), area	1486003	800236	571514	1022602	For information
Method precision (n=6), area	1252714	709930	514976	1003590	For information

Limits of Quantification (LOQ) and Detection (LOD)

As recommended by ICH Q2(R2), the signal-to-noise (S/N) ratio was used to calculate the LOD and LOQ. An S/N ratio of more than 3 for LOD and more than 10 for LOQ is required by the acceptance criteria. For LOQ determination, six replicate injections of 20% standard solution were analyzed. The %CV values obtained for Bis-2-chloroethylamine, 3-aminophenol, 1,4-dibromobutane, and 2,3-dichloronitrobenzene were 4.1%, 4.4%, 3.3%, and 4.4%, respectively. The corresponding signal-to-noise ratios were 36.87, 156.13, 21.96, and 78.58. For LOD determination, a 10% standard solution was injected in triplicate. The observed signal-to-noise ratios were 27.11, 82.02, 14.13, and 49.87, respectively. The signaltonoise ratios observed at the declared LOD and LOQ levels (ranging from 14 to 82) exceed the conventional ICH Q2(R2) criteria of approximately 3:1 for LOD and ≥10:1 for LOQ. This outcome indicates that the instrument is capable of detecting and quantifying at concentrations lower than those reported. However, for regulatory purposes, the LOQ was deliberately established at 20% of the specification limit as a practical reporting threshold, ensuring reliable quantitation relative to impuritycontrol requirements. Thus, the reported LOQ represents a regulatoryrelevant control threshold rather than the absolute instrumental quantitation limit, and the higher S/N values reflect conservative reporting consistent with impuritycontrol practices. These results confirm that the developed method provides adequate sensitivity for detecting the target impurities. The LOD and LOQ results are given in Table 1, and representative chromatograms are shown in Figures S3 and S4.

Repeatability

Repeatability (method precision) was demonstrated by analyzing six independently prepared 100% spiked samples, along with blank and standard injections. The concentrations of Bis-2-chloroethylamine, 3-aminophenol, 1,4-dibromobutane, and 2,3-dichloro-nitrobenzene in the spiked samples were determined, and the %CV values were calculated. The %CV values obtained were 3.0%, 3.7%, 2.0%, and 4.1%, respectively, demonstrating good method precision. Chromatograms of the control sample and spiked samples are presented in Figures S5 and S6.

Accuracy

By introducing known quantities of contaminants into the sample matrix at three concentration levels—LOQ (20%), 100%, and 150% of the specification limit—accuracy was assessed by recovery studies. Three replicates were analyzed at the LOQ level, and

six replicates were analyzed at the 100% and 150% levels. The acceptable recovery range was 70%–130%. The observed recovery ranges were:

Bis-2-chloroethylamine: 74.0% – 89.4%

3-aminophenol: 80.9% – 84.7%

1,4-dibromobutane: 91.4% – 101.4%

2,3-dichloronitrobenzene: 84.8% – 87.0%

For bis(2chloroethyl)amine, recovery at the 150% spike level was observed to be 74%. Although this value is within the regulatory acceptance criteria of 70–130%, it is notably lower than recoveries at the LOQ and specification levels (100%). This outcome can be attributed to matrix effects at elevated concentrations and adsorptive losses associated with the alkylating nitrogen mustard structure of the compound. Importantly, routine QC monitoring is performed at or near the specification limit, where recoveries were consistently higher and well within acceptance criteria. Thus, the observed lower recovery at 150% does not impact the reliability of the method for regulatory impurity control.

The accuracy of the approach was confirmed by the fact that every result fell within the permissible limits. The results are shown in detail in Table 3.

Linearity

Linearity was evaluated for Bis-2-chloroethylamine, 3-aminophenol, 1,4-dibromobutane, and 2,3-dichloro-nitrobenzene across a concentration range from 20% (LOQ) to 150% of the specification limit. The calibration curves demonstrated linearity with correlation coefficients (R^2) of 0.9999, 0.9979, 0.9999, and 0.9993, respectively. The linearity results are summarized in Table 3, and the matching calibration curves are presented in Figures S6–S9.

Conclusion

The existence of genotoxic contaminants (GTIs) in pharmaceutical medication ingredients is a major concern due to their potential mutagenic and carcinogenic effects, necessitating the creating and of highly sensitive and reliable analytical methods. In the present study a quick, accurate, and repeatable ALS-GC-MS technique was effectively developed and approved for the determination of bis(2-chloroethyl) amine, 3-aminophenol, 1,4-dibromobutane, and 2,3-dichloronitrobenzene in Aripiprazole monohydrate drug substance in accordance with ICH Q2(R2) guidelines. Chromatographic separation was achieved using a DB-624 column (60 m length × 0.32 mm diameter, 1.8 µm thickness of film), providing good peak resolution without interference from the blank matrix. The developed method demonstrated adequate sensitivity with LOD values ranging from 0.18–0.19 PPM and LOQ values ranging from 0.36–0.38 PPM, while precision at the LOQ level showed %CV values below 5%. The method exhibited excellent linearity ($R^2 > 0.99$) and acceptable accuracy with recoveries within 70–130%. All standard and sample solutions were prepared freshly, handled in sealed vials with minimal headspace, and injected immediately; therefore, separate solutionstability studies were not required under ICH Q2(R2) guidance. Therefore, it was discovered that the validated ALS-GC-MS approach was linear, specific, sensitive, accurate, and precise making it appropriate for regular quality control monitoring of genotoxic impurities in Aripiprazole monohydrate drug substance.

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Author contributions

Naga Hyndavi Pothamsetti: Conceptualization, methodology, formal analysis, investigation, visualization, drafting, editing; Bala Krishna Kolli: Project administration, review, and supervision.

Conflict of interest

The authors declare that they have no conflict of interests.

Disclosure statement

We authorize to publish the article without any conflict.

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