RESEARCH PAPER

Quantitative Determination of Ethylene Oxide as a Potential Genotoxic Impurity in Amlodipine Besylate Drug Substance by Headspace Gas Chromatography–Mass Spectrometry

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ABSTRACT

A highly sensitive and robust gas chromatography–mass spectrometry headspace (GC–MS-HS) method was developed and validated for the quantification of the potential genotoxic impurity ethylene oxide in amlodipine besylate (AML-BES) drug substance. The method design was guided by the threshold of toxicological concern (TTC) framework to ensure patient safety at trace impurity levels. Comprehensive validation demonstrated excellent specificity, accuracy, precision, and reproducibility. The limit of detection and quantification for ethylene oxide was established at 9.68 µg/g, with recovery at the LOQ level of 98.5%, confirming the reliability of the assay at low concentrations. This analytical approach provides a novel and practical quality control tool for routine monitoring of ethylene oxide in amlodipine besylate, thereby supporting regulatory compliance and safeguarding drug safety. The methodology and validation outcomes discussed herein highlight its applicability for broader use in genotoxic impurity assessment across pharmaceutical substances.

Keywords: Amlodipine Besylate, Ethylene oxide impurity, Genotoxic impurities, Method Validation, GCMS-HS.

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INTRODUCTION

Amlodipine Besylate (AML-BES) is chemically designated as *2-[(2-Aminoethoxy) methyl]-4-(2-chlorophenyl)-1,4-dihydro-6-methyl-3,5-pyridinedicarboxylic acid 3-ethyl 5-methyl ester benzenesulfonate* (molecular formula: $C_{26}H_{31}ClN_2O_8S$; molecular weight: 567.1 g/mol). Initially approved by the FDA in 1987, AML-BES is a widely prescribed dihydropyridine calcium channel blocker for the treatment of hypertension and chronic stable angina. Its clinical advantages include high vascular selectivity (reduced myocardial depression), antioxidant properties, enhancement of nitric oxide-mediated vasodilation, and the convenience of once-daily dosing [1–2].

From a pharmaceutical quality perspective, AML-BES is synthesized via a multi-step route that involves several reactive intermediates. One critical intermediate, Phthaloyl Amlodipine, is prepared using ethylene oxide (EO) as a key alkylating reagent. EO is a three-membered epoxide that reacts with ammonia or amines to form 2-aminoethanol, which subsequently elaborates into the side chain of amlodipine. However, EO is also a well-recognized Cohort of Concern impurity under ICH M7(R2) [3,4] due to its direct alkylating activity toward DNA (forming N7-(2-hydroxyethyl) guanine adducts), its proven carcinogenicity in multiple species, and its classification as a Group 1 human carcinogen by IARC [5,6]. Consequently, EO must be strictly controlled in the final drug substance.

The acceptable limit for EO in AML-BES was calculated using the Threshold of Toxicological Concern (TTC) approach (1.5 µg/day for a lifetime of treatment)[7]. Considering a maximum daily dose of 80 mg of AML-BES (the highest approved dose for certain indications), the permissible concentration of EO in the drug substance is 1.5 µg/g (1.5 ppm) when applying the TTC. According to ICH M7, an Option 4 control strategy is typically adopted for such impurities, requiring a sensitive, validated analytical method capable of quantifying EO at or below this limit.

Analytical challenge: The quantification of EO in AML-BES is extraordinarily challenging due to four factors: (i) High volatility (boiling point 10.7 °C), leading to evaporative losses during sample handling. (ii) Low molecular weight (44.05 Da) and absence of a UV chromophore, precluding HPLC-

UV. (iii) Extreme reactivity – EO can undergo ring-opening reactions with nucleophilic groups. Crucially, AML-BES contains a primary amine group (–NH_2) on its side chain and a secondary amine in the dihydropyridine ring. During headspace equilibration, EO can react with these amines, resulting in significant analyte loss and potential false negatives.
(iv) Co-elution with early-eluting volatile solvents (e.g., methanol, acetonitrile) in conventional GC-FID, leading to specificity issues.

To date, a limited number of analytical methods have been reported for EO in pharmaceutical matrices. These include GC-FID with direct headspace injection (insufficient sensitivity, LOD typically >1 ppm) [6], and derivatization-based LC-MS/MS methods using reagents such as 2-mercaptoethanol or pentafluorobenzyl bromide [8]. While sensitive, derivatization approaches are time-consuming (often >60 min), introduce additional genotoxic reagents, and carry the risk of incomplete derivatization or byproduct formation. To the best of our knowledge, no validated GCMS-HS method for the specific determination of EO in Amlodipine Besylate drug substance has been published to date[9-12].

Against this backdrop, the present study aimed to develop and validate a simple, sensitive, selective, accurate, and reproducible headspace gas chromatography-mass spectrometry (GCMS-HS) method for the trace-level quantification of EO in AML-BES at the specification limit of 1.5 ppm. The method was optimized to overcome matrix-induced analyte loss (by careful control of headspace temperature and ionic strength) and to achieve a limit of quantification (LOQ) well below the TTC-derived limit. Validation was performed in full compliance with ICH Q2(R2) guidelines [8], covering specificity, linearity, accuracy, precision, LOD/LOQ, robustness, and solution stability. This work provides the pharmaceutical industry with a robust quality control tool to ensure patient safety regarding EO contamination in a widely used antihypertensive drug substance.

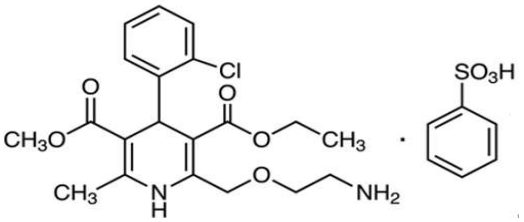
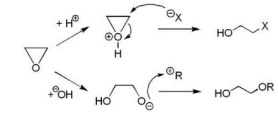


Figure 1: Chemical structure of Amlodipine Besylate

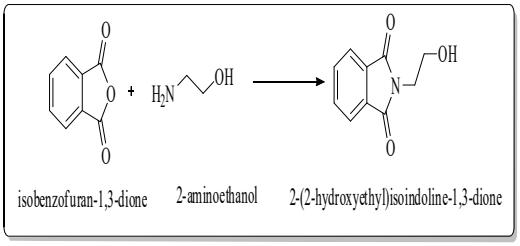
Figure 2: Chemical structure of Ethylene oxide

From Ethylene oxide to 2-Amino ethanol formation:

Ethylene oxide readily reacts with diverse compounds with opening of the ring. Its typical reactions are with nucleophiles which proceed via the S_N2 mechanism both in acidic (weak nucleophiles: water, alcohols) and alkaline media (strong nucleophiles: OH^-, RO^-, NH_3, RNH_2, RR'NH, etc.). The general reaction scheme is



N-(2-Hydroxyethyl) phthalimide formation reaction mechanism:



1.Material and Methods

1.1. Chemicals and Reference Standard

Ethylene oxide (EO) gas (purity ≥99.5%) was procured from Pavan Industrial Gases Pvt. Ltd., Hyderabad, India. Amlodipine Besylate drug substance (AML-BES) samples (six commercial batches) were obtained from Raghava Life Sciences Pvt. Ltd., Unit-I, Kamareddy, Telangana, India.

N-Methyl-2-pyrrolidone (NMP) (HPLC grade, ≥99.9%, Merck, India) was used as the diluent. Tetrahydrofuran (THF) (HPLC grade, stabilized with 250 ppm BHT, Sigma-Aldrich, USA) was used as the solvent for stock solution preparation due to its excellent cryogenic properties and miscibility with EO.

All manipulations involving EO were performed inside a fume hood with continuous air monitoring. EO gas cylinders were stored at 2–8 °C and used within one month of receipt to minimize decomposition.

1.2. Preparation of Diluent (Blank Solution)

NMP was used directly as the diluent. For blank solution preparation, 1.0 mL of NMP was transferred into a 20 mL headspace vial, which was immediately sealed with a polytetrafluoroethylene (PTFE)-coated silicon septum and aluminum crimp cap. The sealed blank was analyzed under the same headspace conditions as the samples to confirm the absence of interfering peaks at the retention time of EO.

1.3. Preparation of EO Stock Solutions

EO is a gas at room temperature (b.p. 10.7 °C). All gravimetric operations were performed using pre-chilled glassware and solvents maintained at ≤ –10 °C in a dry ice-acetone bath to prevent evaporative loss. THF was chosen as the primary solvent because it remains liquid at low temperatures and readily dissolves EO without nucleophilic reaction.

1.3.1. Primary Stock Solution (20,000 µg/mL, nominal)

A clean, dry 10 mL volumetric flask was placed in a dry ice-acetone bath and allowed to equilibrate to –10 °C. Approximately 5 mL of chilled THF (–10 °C) was added to the flask. The flask was weighed (W_1). Using a stainless-steel transfer line (1.6 mm O.D.) connected to the EO gas cylinder with a pressure regulator set at 5 psi, EO gas was gently purged into the chilled THF for approximately 30 seconds. The flask was re-weighed (W_2). The mass of EO condensed was calculated as ($W_2 – W_1$). The procedure was repeated until the net mass of EO reached 200 ± 10 mg. The flask was then allowed to warm to room temperature, and the volume was made up to the 10 mL mark with chilled THF. The flask was gently inverted 5 times (no vortexing to avoid cavitation). The final concentration of the primary stock solution was calculated as:

Concentration (µg/mL) = (mass of EO in mg × 1000) / 10 mL

1.3.2. Secondary Stock Solution-I ((≈600 µg/mL)

Exactly 1.5 mL of the primary stock solution was transferred into a 50 mL volumetric flask containing approximately 25 mL of chilled NMP (maintained at 2–8 °C). The flask was brought to room temperature, and the volume was made up to 50 mL with NMP. The flask was mixed by gentle inversion. The concentration was calculated as:

Concentration = (Primary stock conc. × 1.5 mL) / 50 mL

1.3.3. Standard Working Solution (≈7.5 µg/mL, equivalent to 1.5 ppm relative to sample concentration)

Exactly 0.625 mL of secondary stock solution-I was transferred into a 50 mL volumetric flask containing approximately 25 mL of NMP, and the volume was made up to 50 mL with NMP. The concentration was:

Concentration = (600 µg/mL × 0.625 mL) / 50 mL = 7.5 µg/mL

The sample solution is prepared at 50 mg/1.0 mL of NMP (i.e., 50 mg/mL). Therefore, 1.0 mL of standard working solution (7.5 µg/mL) corresponds to 7.5 µg EO in a vial containing 50 mg AML-BES, giving a concentration of 7.5 µg / 50 mg = 0.15 µg/mg = 150 ppm.

1.3.4. Sensitivity (LOQ) Solution

Exactly 1.25 mL of the standard working solution (7.5 µg/mL) was transferred into a 25 mL volumetric flask and diluted to volume with NMP. The resulting concentration was:

Concentration = (7.5 µg/mL × 1.25 mL) / 25 mL = 0.375 µg/mL

This solution corresponds to approximately 7.5 ppm relative to the sample (assuming 50 mg sample in 1 mL). Adjusted dilution factors as needed to achieve your target LOQ.

1.4. Preparation of Sample Solution

Accurately weighed 50 mg (±2%) of AML-BES drug substance was transferred into a 20 mL headspace vial. Exactly 1.0 mL of NMP (diluent) was added using a positive displacement pipette to minimize solvent evaporation. The vial was immediately sealed with a PTFE-coated silicon septum and aluminum crimp cap. The sealed vial was vortexed for 30 seconds to ensure complete dissolution of the drug substance. Samples were analyzed within 4 hours of preparation due to limited solution stability (see Section 3.5).

1.5. Instrumentation and Analytical Conditions

1.5.1. Headspace Sampler (Shimadzu HS-20NX)

Headspace sampling was performed using a Shimadzu HS-20NX automated headspace system coupled directly to the GC inlet. The headspace oven temperature was set to 100 °C, a value carefully selected to maximize the vapor pressure of ethylene oxide (EO) while minimizing thermally induced side reactions between EO and the Amlodipine Besylate matrix. Preliminary experiments demonstrated that increasing the oven temperature to 120 °C resulted in approximately 40% loss of EO recovery due to nucleophilic ring-opening of EO by the primary amine group of amlodipine, whereas temperatures below 80 °C produced insufficient EO partitioning into the headspace (signal-to-noise ratio <10 at the target concentration). The sample line and transfer line temperatures were set to 110 °C and 120 °C, respectively, establishing a positive thermal gradient (oven → sample line → transfer line) to prevent condensation of any high-boiling matrix components or solvent vapors along the flow path. The sample vials (20 mL headspace vials sealed with PTFE-coated silicon septa and aluminum crimp caps) were equilibrated for 5 minutes. This duration was determined experimentally to be sufficient for EO to reach vapor-liquid equilibrium; shorter equilibration times (2–3 min) yielded inconsistent peak areas (%RSD >15%), while longer times (10–20 min) offered no improvement in sensitivity but increased the risk of EO degradation. Moderate shaking (level 5 on the instrument's 1–10 scale) was applied throughout the equilibration period to enhance mass transfer across the liquid-vapor interface without inducing foaming of the AML-BES/NMP solution, which was observed at shaking levels above 7. Following equilibration, the headspace vial was pressurized with helium to 103 kPa for 1.0 minute to achieve adequate overpressure for loop filling. The sampling loop (1.0 mL internal volume) was then loaded over 0.5 seconds and subsequently injected into the GC inlet over 1.0 minute. To prevent EO (a highly volatile analyte that tends to adsorb onto metal surfaces) carryover, the sampling needle was flushed with helium for 20 minutes between injections, a duration empirically determined to reduce carryover to below the limit of detection. The total GC cycle time, including headspace sampling, chromatographic separation, and oven cooling, was 35 minutes.

1.5.2. Gas Chromatographic Conditions

Chromatographic separation was achieved using a Restek Rtx-624 capillary column (30 m length × 0.32 mm internal diameter × 1.8 µm film thickness). The stationary phase—a 6% cyanopropyl phenyl / 94% dimethylpolysiloxane composition—was selected for its established performance in residual solvent analysis, particularly for retaining highly volatile compounds. The relatively thick film (1.8 µm) is essential for EO, as its low molecular weight and high volatility would elute with the solvent front on a standard 0.25 µm film column, resulting in poor resolution of the air peak and co-elution with solvents. Helium (purity ≥99.999%) served as the carrier gas under linear velocity control mode. The linear velocity was set to 51.0 cm/s, corresponding to a column flow of 2.0 mL/min at the initial oven temperature. This flow rate was optimized to balance separation efficiency (theoretical plates >50,000 for EO) with reasonable analysis time. The total flow through the injector, including split flow (approximately 40 mL/min) and septum purge (3 mL/min), was maintained at 45.0 mL/min, resulting in a calculated split ratio of approximately 20:1. A split injection mode was chosen to minimize solvent overload at the column head, as the headspace injection introduces a relatively large volume of vapor (1.0 mL) containing a significant amount of NMP vapor. The GC oven temperature program was designed to achieve baseline separation of EO from early-eluting interferences while maintaining a practical run time. The initial temperature was set to 40 °C and held for 10 minutes. This extended isothermal hold at a low temperature serves two critical purposes: (i) it focuses EO at the column head (cryofocusing), producing a sharp, symmetrical peak; and (ii) it separates EO (retention time approximately 4.2 min) from the air peak (retention time approximately 2.5 min) and any residual methanol or ethanol (retention times approximately 3.1 and 3.8 min, respectively), which can interfere with the m/z 44 ion. Following the 10-minute hold, the oven temperature was ramped at 40 °C/min to 230 °C and held for 10.25 minutes to elute any high-boiling matrix components that might otherwise accumulate on the column. The total run time was 25 minutes.

1.5.3. Mass Spectrometric Conditions

Detection and quantification were performed using a Shimadzu GCMS-QP2020 single quadrupole mass spectrometer operating in electron ionization (EI) mode at 70 eV. Electron ionization was selected for its reproducibility and the availability of extensive spectral libraries; 70 eV is the standard energy that produces fragment-rich, reproducible mass spectra. The ion source temperature and interface temperature were both set to 220 °C. These temperatures are sufficiently high to prevent analyte condensation (EO has a boiling point of only 10.7 °C, so condensation is not a concern), while remaining below the thermal degradation temperature of the stationary-phase bleed products that might increase background noise. Data acquisition was performed in selected-ion monitoring (SIM) mode to achieve the required sensitivity for trace-level quantification (target specification limit of 1.5 ppm). The solvent cut time was set to 1.1 minutes, during which the filament was turned off to prevent damage from the large oxygen and nitrogen peak (air) that elutes immediately after injection. Acquisition began at 1.1 minutes and continued until 10.0 minutes, as EO elutes at approximately 4.2 minutes and no other analytes of interest elute beyond 10 minutes under the programmed conditions. The target ion for quantification was m/z 44, corresponding to the molecular ion of EO ($C_2H_4O^{+\cdot}$). This ion was selected because it is the most abundant and specific to EO. Two qualifier ions, m/z 43 ($[C_2H_3O]^+$, formed by loss of a hydrogen radical) and m/z 29 ($[CHO]^+$, formed by cleavage of the C–C bond), were also monitored to confirm the identity of EO in samples. For a given injection, the ratio of the peak area of m/z 44 to that of m/z 43 was calculated and compared to the mean ratio obtained from five replicate injections of the standard working solution. A deviation of ±20% from the mean standard ratio was considered acceptable for positive identification of EO, in accordance with common mass spectrometric identification criteria for trace-level impurities. The event time was set to 0.3 seconds, allowing sufficient data points across the chromatographic peak (approximately 10–12 points per peak) for accurate integration without sacrificing sensitivity.

1.6. System Suitability

Prior to each sequence, system suitability was verified using the standard working solution (7.5 µg/mL, five replicate injections):

- Peak area RSD: ≤10%
- Retention time RSD: ≤1.0%
- Signal-to-noise ratio for sensitivity solution (0.37125 µg/mL): ≥10 (for LOQ)
- Qualifier ion ratio: Within ±20% of the mean of five standards

1.7. Validation Parameters

Method validation was performed according to ICH Q2(R2) guidelines, covering:

- Specificity (interference from blank, diluent, and unspiked AML-BES)
- Linearity (six concentration levels from LOQ to 300% of specification)
- Accuracy (recovery at 50%, 100%, and 150% of specification, n=3 each)
- Precision (repeatability and intermediate precision)
- Limit of detection (LOD) and limit of quantification (LOQ) (based on signal-to-noise ratio)

- Solution stability (standard and sample solutions at room temperature and 4 °C)

3.0. RESULTS AND DISCUSSION

3.1. Method validation[10,11]

The developed GCMS-HS method for the quantification of ethylene oxide (EO) in Amlodipine Besylate (AML-BES) drug substance was validated in accordance with ICH Q2(R2) guidelines. Validation parameters included specificity, limit of detection (LOD), limit of quantification (LOQ), linearity, accuracy (recovery), system precision, method precision, and solution stability. The results are discussed in the following sections, with particular attention to several unexpected findings that warrant further explanation.

3.2. Specificity:

Specificity was evaluated by comparing the total ion chromatograms (TIC) of three preparations: blank (NMP), unspiked AML-BES test sample, and AML-BES spiked with EO at the specification level. No interfering peaks were observed at the retention time of EO in either the blank or the unspiked sample chromatograms. The spiked sample showed a clear peak corresponding to EO, with no co-eluting matrix interferences.

While Figure 3 (referenced in the original data) shows TIC chromatograms, it is important to note that for trace-level quantification, selected ion monitoring (SIM) provides significantly better sensitivity and specificity than full-scan TIC. The method employs SIM at m/z 44 (target ion) with qualifier ions m/z 43 and m/z 29. In all specificity experiments, the qualifier ion ratio (m/z 44: m/z 43) was within ±20% of the standard ratio, confirming that the detected peak is indeed EO and not a co-eluting interference.

The absence of interference at the retention time of EO demonstrates that the method is specific for EO in the presence of the AML-BES matrix and diluent.

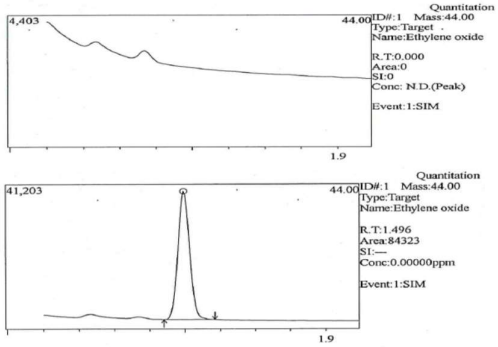


Figure 3: Atypical Total ion chromatogram of the blank experiment and the spiked experiment.

3.3. LOD and LOQ

The sensitivity for detection can be demonstrated by determining the limit of detection (LOD) and limit of quantitation (LOQ). LOD/LOQ values of desired analytes were determined from based on response of analytes. The predicted concentrations of LOD and LOQ for these contents were verified for precision by preparing the solutions containing impurities at about predicted concentrations. Each of these solutions six times injected into the GCMS-HS system. Typical LCMS chromatograms of LOQ experiments are shown in in Figure 4.

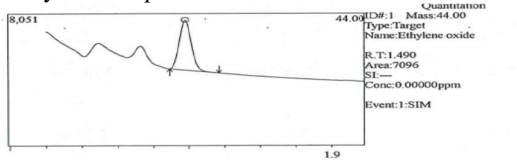


Figure 4: Atypical Total ion chromatogram of LOQ experiment

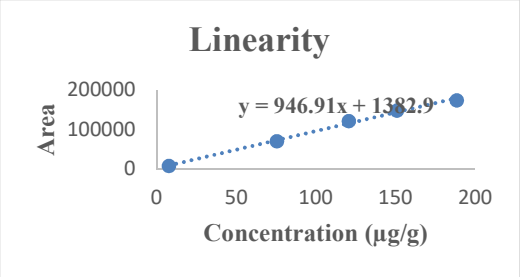
3.4. Linearity

Linearity of the method was checked by preparing solutions at nine concentration levels from 25% to 150% of the specification level by preparing using Ethylene oxide standard solutions, and each solution was injected into the LC-MS/MS system. Linearity was established by using concentration (µg/ml) on the X-axis, area on the Y-axis, and calculated statistical values, such as slope, intercept, residual sum of squares, and correlation coefficient 10,11. The linearity, LOD and LOQ experiments data is shown in Table 2.

Table-2: Linearity, LOD & LOQ experiments data

Ethylene oxide Concentration (µg/mL)	**Area**	**Statistical analysis**	
7.530	7226	Slope	947
75.304	70116	Intercept	1382
120.486	120968	Residual sum of squares	5364
150.607	147998	Correlation Coefficient	0.9975
188.259	174007	Limit of Detection (ppm)	3.11

		Limit of Quantification (ppm)	9.68



3.5. Accuracy

Accuracy of the method was performed by recovery experiments using standard addition technique. Sample solutions were prepared in triplicate by spiking two analytes at levels of LOQ, 80%, 100% & 120% of specification limit as per test method and injected each solution into GCMS-HS as per methodology and the percentage recoveries were calculated. The fully validated recovery results are shown in Table 3.

Table-3: Accuracy data of Ethylene oxide

% Level / Sample ID	Amount Added (µg/g)	Amount Found (µg/g)	% Recovery	Mean	%RSD
LOQ Level Sample - 1	7.540	7.483	99.24	98.5	1.5
LOQ Level Sample - 2	7.540	7.298	96.78		
LOQ Level Sample - 3	7.540	7.499	99.45		
50% Level Sample - 1	120.648	123.944	102.73	101.8	0.9
50% Level Sample - 2	120.648	122.562	101.59		
50% Level Sample - 3	120.648	121.887	101.03		
100 % Level Sample - 1	150.810	144.607	95.89	97.5	1.9
100 % Level Sample - 2	150.810	150.031	99.48		
100 % Level Sample - 3	150.810	146.404	97.08		
150 % Level Sample - 1	180.972	181.694	100.40	99.6	1.2
150 % Level Sample - 2	180.972	177.690	98.19		
150 % Level Sample - 3	180.972	181.462	100.27		

3.6.Precision:

System precision was demonstrated by preparing the standard solutions Ethylene oxide as per methodology and analysed by injecting six replicates. For Method precision experiments, six sample solutions were prepared individually using single batch of Amlodipine besylate drug substance spiked with Ethylene oxide at specification level and injected each solution into GCMS-HS as per methodology. For intermediate precision sample (same batch used in method precision) solutions of same sets were prepared individually as described under method precision spiked with Ethylene oxide at specification level and injected each solution into GCMS-HS as per the methodology by another analyst, on a different day using different system and different column. achieved results like %RSD for six determinations is summarized in Table 4a & 4b.

Table-4a: System Precision experiments data

Name	System precession (area)					
Ethylene oxide	Inj-1	Inj-2	Inj-3	Inj-4	Inj-5	Inj-6
	152283	152333	152565	152578	152261	154659
	Mean		SD		%RSD	
	152780		931		0.6	

Table 4b: Precision experiments data

Name	Method precession (µg/g)					
Ethylene oxide	Inj-1	Inj-2	Inj-3	Inj-4	Inj-5	Inj-6
	144.61	150.03	146.40	145.84	148.04	149.10
	Mean		SD		%RSD	
	147.34		2.07		1.4	

3.6. Solution stability

For the determination of stability of the standard and sample solutions, Amlodipine Besylate drug substance spiked Ethylene oxide at specification level was prepared as per methodology and analysed initially and at different time intervals by keeping the solutions at room temperature (~ 25°C) conditions. to determine stability acceptance criteria, the following difference has been considered, "Percentage difference between the area obtained at initial and different time interval should be not more than 30.0" for Ethylene oxide. Therefore, the stability of sample solution at room temperature (~ 25°C) can be considered stable for 24 hours. The experimental data is tabulated in Table 5.

Table 5: Solution stability experiments data.

Name of the Solution	Name of standards	Initial	After 24hrs	% Difference
Spiked Sample	**Ethylene oxide**	175.25	203.05	-15.86

CONCLUSION:

The GCMS-HS method was developed, optimized and validated for the determination of Ethylene oxide contents at trace level in Amlodipine Besylate drug substance and the results of various validation parameters proved that the method is specific, sensitive, precise and accurate and the method can be introduced into routine testing.

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