

# The development of a GC-FID method for indirect quantification of chloroacetyl chloride, a potential genotoxic impurity, in chlordiazepoxide hydrochloride drug substance

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## ABSTRACT

This study presents an innovative gas chromatographic-flame ionization detection (GC-FID) method, developed and validated in accordance with The International Council for Harmonisation (ICH) guidelines, for the quantitative analysis of chloroacetyl chloride, a potentially genotoxic impurity, in chlordiazepoxide hydrochloride active pharmaceutical ingredient. Due to the reactive and genotoxic nature of chloroacetyl chloride, precise quantification is imperative in the active pharmaceutical ingredient. The proposed method involves the conversion of chloroacetyl chloride into methyl 2-chloroacetate (MCA), enabling indirect quantification via a GC-FID approach employing a DB wax column. The validated GC-FID method displays exceptional features, such as remarkable linearity, ranging from 0.38 to 1.8 ppm with a correlation coefficient of 0.9998, as well as low detection and quantification limits of 0.19 and 0.38 ppm, and the method is specific without interference. The precision of the method expressed as the % RSD was 0.53%. The sample recovery ranging from 97.3% to 101.5%, confirms the method's accuracy. Furthermore, three different batches of chlordiazepoxide hydrochloride underwent evaluation using this method. In conclusion, this method offers a highly sensitive approach for the precise quantification of chloroacetyl chloride in chlordiazepoxide hydrochloride drug substance, thereby ensuring compliance with the stringent safety standards of the pharmaceutical industry.

## INTRODUCTION

Chloroacetyl chloride, often abbreviated as chloroacetyl chloride (CAC), is a well-established raw material essential in both the pharmaceutical and chemical industries. Its primary purpose lies in the synthesis of critical intermediates and active pharmaceutical ingredients. This compound, known for its highly reactive and corrosive nature, poses significant hazards when it comes into contact with the skin, leading to severe chemical burns. In addition, chloroacetyl chloride can readily permeate the skin and affect the respiratory system [1]. Notably, chloroacetyl chloride exhibits favorable solubility in both water and methanol [2,3]. Its molecular structure

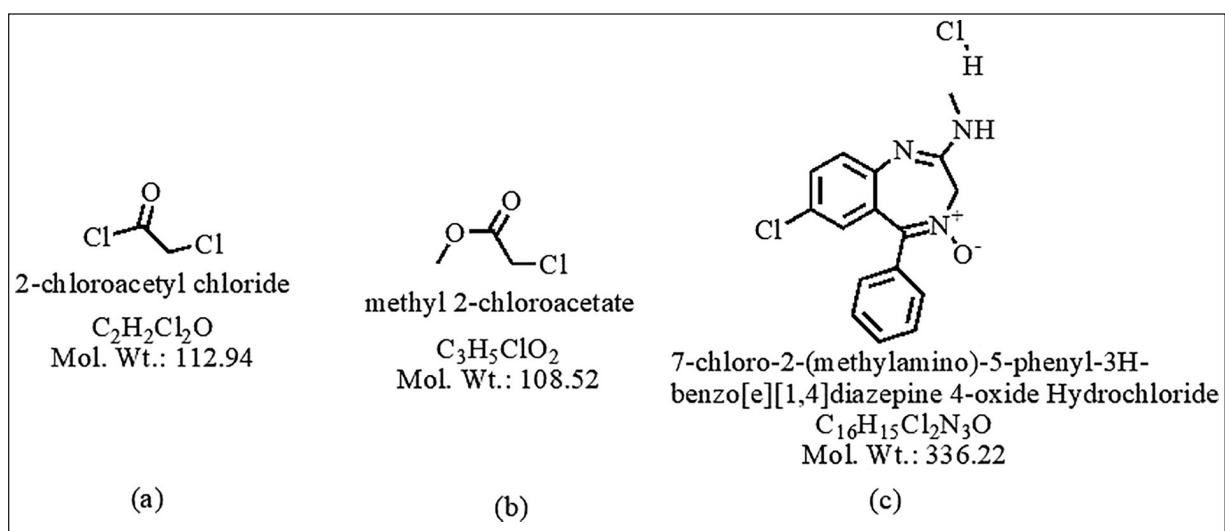
[4], includes a reactive “acyl-chloride” functional group (COCl), rendering it highly reactive toward nucleophiles. This characteristic makes it particularly suitable for acylation reactions [5], wherein it reacts with nucleophiles to generate acyl derivatives. However, it is crucial to acknowledge its extreme toxicity and corrosiveness, as the U.S. Environmental Protection Agency classifies it as an exceedingly hazardous substance. Comparatively, MCA, another chemical compound is considered “toxic” but may possess a lower degree of hazard when compared to chloroacetyl chloride. MCA (CH<sub>3</sub>O<sub>2</sub>CCl) contains an ester functional group, which exhibits reduced reactivity in comparison to acyl chlorides. Esters can undergo nucleophilic substitution reactions, but their reactivity is generally lower due to the presence of an electron-withdrawing chlorine atom. Interestingly, there is a paucity of information regarding the genotoxicity of chloroacetyl chloride. Hence, we base the risk assessment on the principles of the threshold of toxicological concern [6–9] and the structure-activity

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relationship [10–20]. Chloroacetyl chloride is categorized as a class 3 substance. Considering its potential to react with DNA and its potential genotoxicity [21–23]. An evaluation in accordance with the International Conference of Harmonization (ICH) M7 guidelines is imperative [24–27]. Several analytical methods have been employed to analyze chloroacetyl chloride. For instance, Khan *et al.* [28] utilized a capillary zone electrophoretic method, Morissette *et al.* [29] employed a derivatization GC method with a limit of quantification (LOQ) of 0.03%w/w. Langvardt *et al.* [30] established a derivatization procedure using electron capture gas chromatography (GC). Ajit Anerao *et al.* published a GC technique for measuring the levels of chloroacetyl chloride and thionyl chloride in tadalafil [31]. Kennedy [32] studied quantitative analysis of acid chlorides using an automatic cold on-column injection method. Klein *et al.* [33] developed an high performance liquid chromatography (HPLC) method for determining chloroacetyl chloride in the air. McCullough *et al.* [34] documented their study involving the collection of chloroacetyl chloride from the air using solid support, with its quantification performed via ion chromatography. Zhou *et al.* [35] developed a chemical derivatization HPLC method for the determination of chloroacetyl chloride and chloroacetic acid in raw material of Azintamide. Furthermore, Langhorst [36] developed a resin-coated solid sorbent tube for monitoring air bon reactive chemicals including chloroacetyl chloride, acetic anhydride, and isocyanatoethyl methacrylate, derivatized using a reagent 1-(2-pyridyl) piperazine, and analyzed using an high-performance thin layer chromatography chromatographic technique. To the best of my knowledge, researchers have not reported any methods for determining trace levels of chloroacetyl chloride in chlordiazepoxide hydrochloride.

A notable feature of chloroacetyl chloride, when used as a pharmaceutical raw material, is that it often remains in trace amounts in the final Active Pharmaceutical Ingredients, in the present study Figure 1 shows the structures of chloroacetyl chloride, MCA, and chlordiazepoxide hydrochloride

(APIs). This retention occurs due to the incomplete consumption of reagents in chemical reactions (as illustrated in Figure 1. In light of this, Liu *et al.* [37] emphasize the importance of sensitive analytical techniques for pharmaceutical genotoxic impurities (GTIs), especially when dealing with trace levels at ppm (parts per million) concentrations. The identification, determination, and management of GTIs in pharmaceutical substances have become a significant concern, particularly in the context of their potential association with human cancer [38–42]. In summary, derivatization procedures are widely used for CAC estimation, which enhances the sensitivity and selectivity of analytical techniques but comes with several drawbacks. They involve additional steps, leading to increased sample handling and the potential for errors and contamination. The derivatization process is time-consuming, adding to the overall analysis time and reducing sample throughput. Some analytes may be sensitive to the reagents and conditions, leading to degradation or decomposition [43,44]. Derivatization can increase the cost of analysis due to the need for specialized reagents and equipment, and it requires a high level of skill and expertise. Contamination risks are higher, and chemical waste is generated, adding to environmental concerns. Interference from the reagents and limitations on the applicability of derivatization to all compounds further compound these drawbacks, making it important to carefully consider its utility in specific analytical applications also this literature review underscores the need for rigorous assessment and control of traceable reagents in pharmaceutical production. These impurities, including chloroacetyl chloride, may pose unintended risks such as genotoxicity or carcinogenicity. Consequently, their concentrations must be minimized to levels deemed negligible in terms of human safety, even when complete elimination is unattainable. Chloroacetyl chloride, due to its high reactivity, can be converted to a more stable form, MCA, through a nucleophilic acylation reaction with methanol, as depicted



**Figure 1.** The structure of (a) chloroacetyl chloride, (b) MCA, and (c) chlordiazepoxide hydrochloride.

in Figure 2. This transformation enables the subsequent determination of MCA using GC with a flame ionization detector method, offering a straightforward and sensitive means of indirectly estimating chloroacetyl chloride. This process has been verified through analytical techniques such as mass spectrometry, and gas chromatographic-flame ionization detection (GC-FID), with comparisons to standard MCA and CAC compounds.

In this study, chloroacetyl chloride underwent complete conversion to MCA in the presence of methanol, as demonstrated by GC. Notably, Figures 8 and 9 display matching retention times and peak areas for MCA and chloroacetyl chloride standards 1.5 ppm spiked in chlordiazepoxide hydrochloride, providing clear evidence of the successful transformation and validating the complete conversion. In addition, liquid chromatography-mass spectrometry (LC-MS) analysis reinforced these findings, with the mass spectrum aligning with the expected profile of MCA. In the presence of Dichloromethane and Ethylene Dichloride, chloroacetyl chloride exhibits stability, as depicted in Figures 13 and 14. However, when subjected to methanol, it undergoes conversion to MCA, as evidenced by the LC-MS mass spectrum provided in the supplementary material. These results collectively offer robust scientific evidence for the efficient conversion of chloroacetyl chloride to MCA in the specified experimental conditions.

## EXPERIMENTAL STUDY

### Materials and methods

#### Chemicals and standards

We procured HPLC-grade methanol ( $\geq 99\%$ ) from standard reagents. Loba Chemie India supplied AR-grade chloroacetyl chloride ( $\geq 99\%$ ). We used MCA (98%), manufactured by AVRA. Flowchem Pvt. Ltd. generously provided chlordiazepoxide hydrochloride API-free samples, with the purity of the APIs exceeding 99.5%.

#### Instrumentation

We used a Shimadzu GC model The GC-2010 pro instrument featured a flame ionization detector and AOC20i is the auto-sampler model. The GC capillary column, DB Wax, with dimensions of 15 m length, 0.530 mm diameter, and a 1.0  $\mu\text{m}$  film thickness, was sourced from Agilent Technologies. We employed a Shimadzu make weighing balance model

AP225WD, with 0.1 mg accuracy. We used standard volumetric flasks and pipettes for standard and sample preparations.

### Chromatographic conditions

The final chromatographic conditions for the optimized method were established as follows: We utilized a DB Wax column with dimensions of 15 m in length and 0.53 mm in diameter, featuring a particle size of 1.0  $\mu\text{m}$ . Initially, the oven temperature was set at 40°C for 5 minutes. Subsequently, it was ramped up at a rate of 10°C per minute until reaching 200°C, where it was maintained for 5 minutes. The injector temperature was set to 150°C, and the detector temperature was maintained at 230°C. The column flow rate was kept at 5 ml/minutes with a 2:1 split ratio. A 2  $\mu\text{l}$  injection volume was introduced, using methanol as the diluent.

### Preparing standard and sample solutions

To prepare the standard stock solution, we precisely weighed around 10 mg of “chloroacetyl chloride” and added it to a 100 ml standard volumetric flask. Then, we dissolved and diluted it with methanol until it reached the 100 ml mark, resulting in a solution with a concentration of 100  $\mu\text{g/ml}$  for chloroacetyl chloride in the form of MCA.

To prepare the standard solution, we precisely transferred 1 mL of the standard stock solution into a 100 ml standard flask and subsequently diluted it to the specified volume with methanol. A subsequent dilution of 0.75 ml of this solution with methanol in a 20 ml volumetric flask yielded a concentration of 37.5 ng/ml.

For the preparation of the test sample, we transferred 50 mg of chlordiazepoxide hydrochloride sample, dissolving it in 2 ml of methanol, thereby achieving a concentration of 25 mg/ml.

During the system suitability testing, we injected the 1.5 ppm standard solution six times in replicates, to ensure that the relative standard deviation (RSD) for the area response of the standard solution remained below 10.0%.

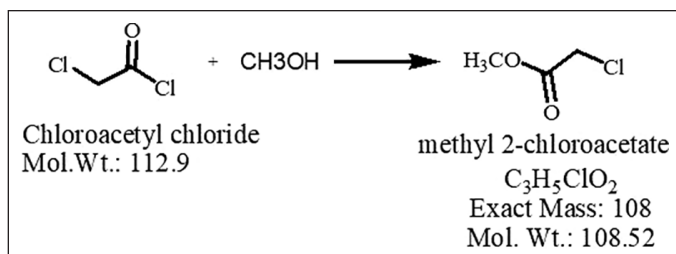
The LOD solution was prepared based on a signal-to-noise (S/N) ratio, yielding an observed lowest detectable concentration of 0.19 ppm, with an S/N ratio of 3.35.

To determine the LOQ solution, we applied a S/N ratio of 10.07, yielding the lowest quantifiable concentration of 0.38 ppm.

To assess linearity, we generated a series of solutions utilizing the standard stock solution, spanning a concentration range from 12.5% to 120%.

### Method development

We aimed to quantify chloroacetyl chloride as Methyl 2-chloro acetate in “chlordiazepoxide hydrochloride” active drug substances during method development. Initially, the research was followed by a flow chart as mentioned in Figure 3, we made several attempts using different GC stationary phases, including DB-5, DB-624, and Rtx-225. Ultimately, the DB-Wax GC column yielded a suitable response from the analyte. We evaluated various diluents, such as methylene dichloride, ethylene dichloride, ethanol, dimethyl formamide, dimethyl Acetamide, and dimethyl sulfoxide. Methanol,



**Figure 2.** Nucleophilic acylation reaction, chloroacetyl chloride reacts with methanol to produce MCA.

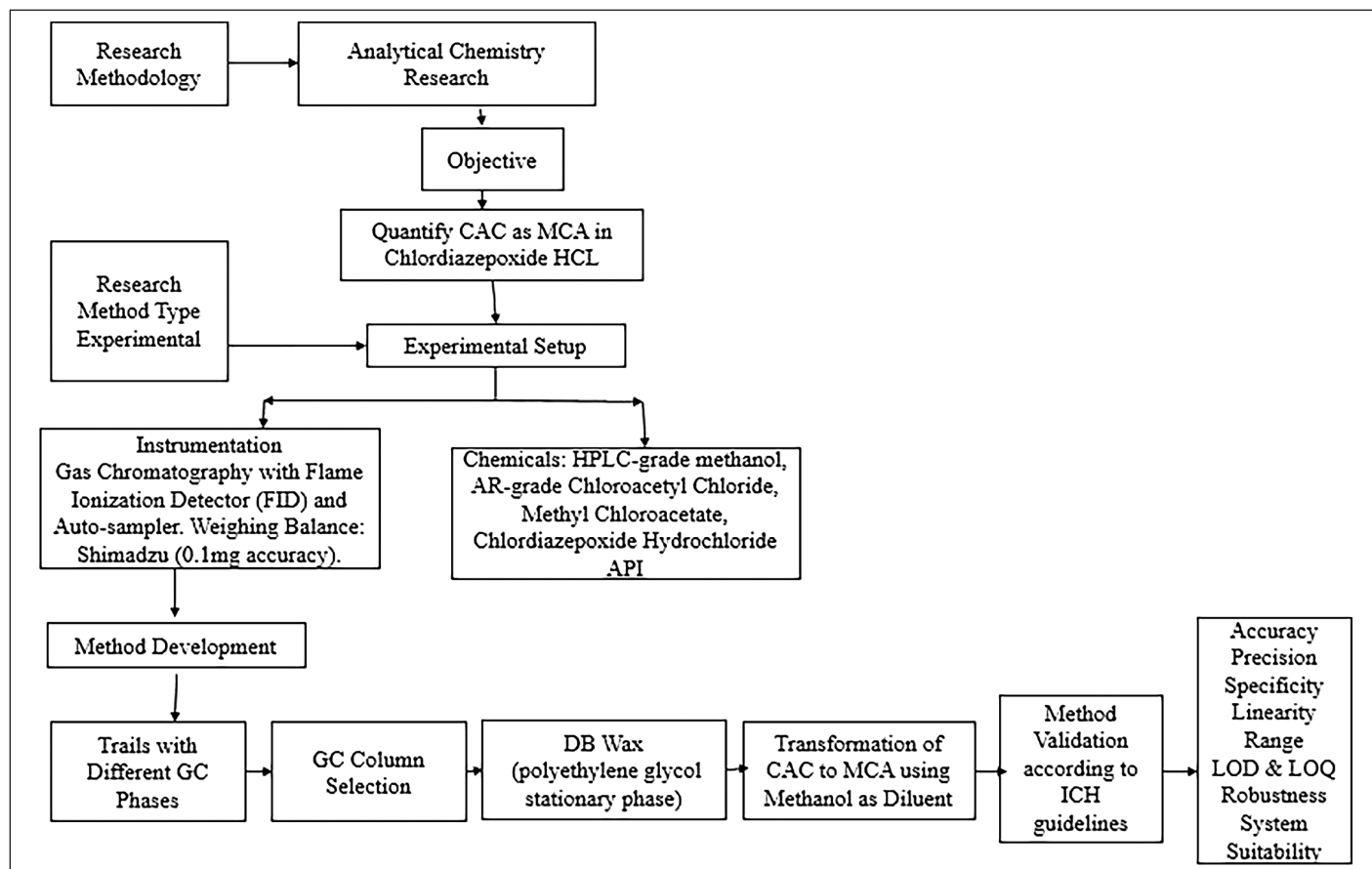


Figure 3. Flow chart of experimental work.

chosen for its ability to convert chloroacetyl chloride to MCA, was the selected solvent. Initially, we used the GC column DB-5 containing 5%-phenyl-methyl polysiloxane stationary phase, and prepared a 1.5-ppm standard solution for injection into the chromatographic system. Although we detected 1.5 ppm of chloroacetyl chloride, the peak shape was asymmetric, and the area response was low. Subsequently, we tried the GC column DB-624 a mid-polar column containing “6% cyanopropyl/phenyl, and 94% polydimethylsiloxane” stationary phase, and again, 1.5 ppm of chloroacetyl chloride was detected, but with a low area response and an improper baseline. We then attempted the Rtx-225 column containing a “cross-bonded 50% cyanopropyl methyl/50% phenyl methyl” stationary phase, and similar challenges were encountered with a low area response and an improper baseline. Finally, we selected the GC column DB-Wax column containing “polyethylene glycol” stationary phase, and used methanol as the diluent to convert chloroacetyl chloride to MCA, resulting in a well-detected 1.5 ppm of MCA in methanol using the DB Wax GC column. The method development was further validated with the DB Wax capillary column and methanol as the diluent, providing superior peak shape and baseline separation. The retention time of MCA was observed at approximately 10.80 minutes.

## RESULTS AND DISCUSSIONS

### Analytical method validation

We conducted the validation process, following the ICH-Q2 (R1) guidelines [45]. To affirm the established GC method’s dependability for the indirect determination of chloroacetyl chloride content in chlordiazepoxide active drug compounds. Table 1 displays the percent RSD (percentage RSD) of MCA (1.5 ppm) from six replicates, demonstrating a percentage RSD of 1.386% when utilizing the DB Wax GC column. The validation, in alignment with ICH recommendations, encompassed the assessment of the following parameters: system suitability and specificity, accuracy, limits of quantification and detection, range and linearity, precision, and solution stability.

#### Specificity and system suitability

In the presence of potential interfering chemicals, specificity ensures precise measurement and differentiation of the target analyte, while system suitability validates the consistent and dependable performance of the analytical system. To confirm the proper functioning of the system and data generation, we prepared a 1.5-ppm methyl 2-chloro acetate standard solution in methanol and introduced it into the system



six times. The percentage RSD for the six replicates of the standard solution was 1.386, significantly below the maximum acceptable threshold of 15.0%, thus confirming the system's suitability.

In the assessment of method specificity, we also introduced various listed solvents; however, no interference was observed at the retention time of the analyte peak. Further testing revealed the absence of interference from the blank at the 10.8-minute retention time, corresponding to the methyl 2-chloro acetate standard peak. Consequently, we concluded that the MCA peak remained unaffected by the presence of diluent (blank) peaks.

### Precision

The method's precision demonstrates its ability to consistently generate reproducible and consistent results when analyzing the same sample under uniform conditions, indicating the degree of random error in measurements. In the system suitability results presented in Table 1, an RSD of 1.386 was obtained from six replicates of 1.5-ppm standard chloroacetyl chloride solution, demonstrating the accuracy of the method, to further assess precision; we prepared six homogeneous samples of chlordiazepoxide hydrochloride, calculating the percentage RSD for the content of chloroacetyl chloride in these samples. The chloroacetyl chloride content was found to be below the detection limit initially. Subsequently, we spiked known impurity chloroacetyl chloride up to the specification limit of 1.5 ppm to establish method precision. The percentage RSD for the content of chloroacetyl chloride in six preparations of spiked test samples was observed as 0.53%, which did not exceed the limit of 15.0%, as indicated in Table 2. Consequently, we have confirmed the method's precision.

### Detection and quantitation limits

We established the detection and quantitation limits through precise quantification of substances at the lowest levels within sample matrices. Utilizing the S/N ratio approach as presented in Table 3, we determined the limit of quantitation to be 0.38 ppm and the limit of detection to be 0.19 ppm and corresponding chromatograms as shown in Figures 4–6.

**Table 1.** The precision table contains results from six replicates of the chloroacetyl chloride standard solution (1.5 ppm).

Replicates	Standard solution (1.5 ppm) area responses	Retention time in minutes
1.	4,073	10.830
2.	4,027	10.832
3.	4,007	10.832
4.	4,070	10.834
5.	4,028	10.835
6.	4,056	10.838
Average	4,067.333	10.838
%RSD	1.386	0.006

**Table 2.** Spiked chloroacetyl chloride into chlordiazepoxide hydrochloride at a specification level of 1.5 ppm.

Sl.no	Standard 1.5 ppm area response	Retention time in minutes	Measuring the recovery of chloroacetyl chloroacetate spiked into chlordiazepoxide hydrochloride (ppm)
1	4,021	10.833	1.514
2	4,051	10.837	1.525
3	4,071	10.838	1.533
4	4,045	10.837	1.523
5	4,017	10.834	1.513
6	4,022	10.852	1.515
Avg	4,037.833	10.839	1.520
SD	21.414	0.007	0.008
%RSD	0.530	0.064	0.520

**Table 3.** Detection limit and quantification limit results.

Test	Concentration (ppm)	Area response	S/N ratio
Limit of detection 12.5%	0.19	453	3.4
Limit of quantitation 25%	0.38	907.3	10.1

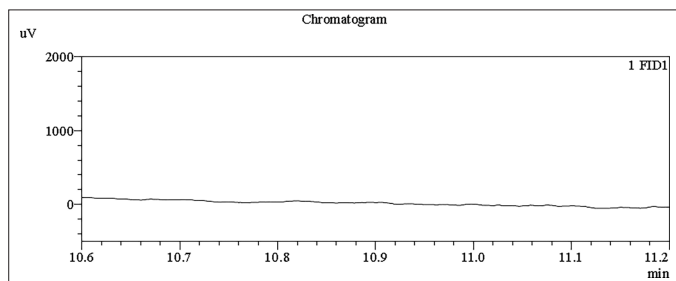
### Linearity and range

In the process of method validation, we assess the method's ability to generate accurate and proportionate analytical responses within a defined concentration range, thereby ensuring its suitability for quantitative analysis. We meticulously prepared standard solutions of methyl 2-chloro acetate at diverse concentrations spanning from the limit of detection to 120% (12.5%, 25%, 50%, 80%, 100%, and 120%) of the working level (1.5 ppm). Subsequently, we conducted chromatographic analyses on these solutions, as outlined in Table 4 and Figure 7, to ascertain the linearity and range of the method.

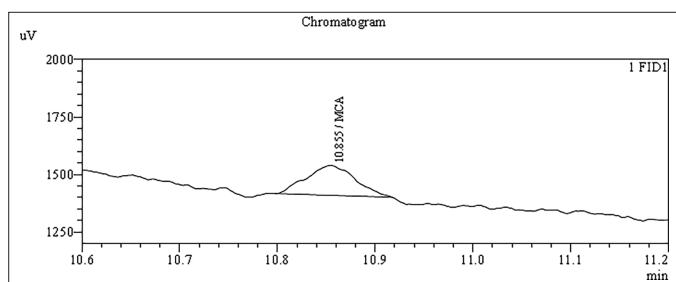
We calculated the correlation coefficient between the mean area response and the standard solution concentration. The correlation coefficient obtained from the linearity graph plotting the mean area response against concentration in ppm was 0.9996. The linearity extended from the LOQ (0.38 ppm) to the higher level (1.8 ppm) and the regression statistics for linearity were displayed in Table 5.

### Accuracy (recovery)

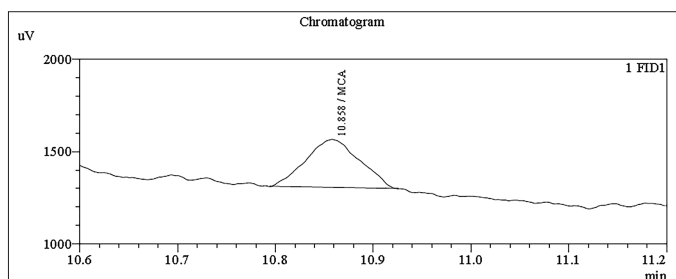
We evaluate the method's capability to accurately quantify and recover known analyte quantities, thereby demonstrating its precision and reliability in real-world sample analysis. For each level, we prepared three sample solutions by spiking a homogeneous chlordiazepoxide hydrochloride sample at concentrations of 25%, 50%, 100%, and 120%. We subsequently analyzed these solutions using the prescribed method and calculated the recovery rates to assess the



**Figure 4.** GC-FID chromatogram diluent blank chromatogram, no interference observed around main peak retention time 10.8 minutes.



**Figure 5.** GC-FID chromatogram: MCA spiked sample (0.19 ppm LOD)



**Figure 6.** GC-FID chromatogram: MCA spiked sample (0.38 ppm LOQ).

procedure's accuracy. The recovery rates ranged from 97.3% to 101.5% for solutions within 25% to 120% concentration range, confirming the method's accuracy, as outlined in Table 6.

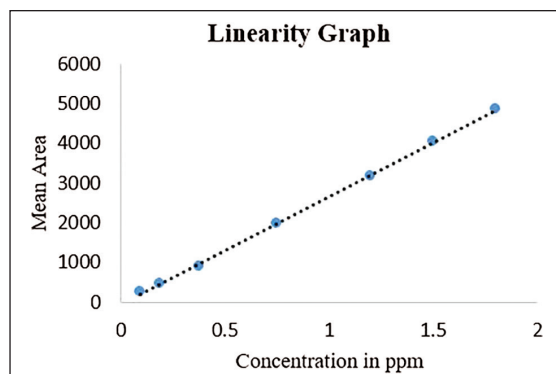
Finally, we conducted an indirect assessment of chloroacetyl chloride content by employing the developed and validated method on the chlordiazepoxide hydrochloride test samples. Chloroacetyl chloride was not detected in three batches of API samples as shown in Figures 10–12 and assay Table 13.

### Robustness

We assessed the method's robustness by evaluating its ability to consistently yield reliable results despite minor variations in experimental conditions, demonstrating its resilience and reliability in practical applications. Through a series of experiments, we demonstrated that this method maintains its robustness even when subjected to slight variations in gas chromatographic conditions. Throughout the

**Table 4.** Linearity and the range of the method.

S.no.	Percent with respect to the specification limit 1.5 ppm	ppm	Average area response	Correlation coefficient
1	12.5	0.19	463.6	0.9996
2	25	0.38	907.3	
3	50	0.75	1,981.0	
4	80	1.2	3,156.0	
5	100	1.5	4,039.0	
6	120	1.8	4,837.3	



**Figure 7.** Linearity graph mean area response versus concentration in ppm.

**Table 5.** Regression statistics for linearity.

Multiple R	0.999845092
R square	0.999690208
Adjusted R square	0.99961276
Standard error	0.012561618
Observations	6

experimental process, systematic alterations were implemented to optimize various instrumental parameters. Noteworthy adjustments included fluctuations in the carrier gas flow rate, ranging from 4.5 to 5.5 ml/min, with corresponding results meticulously documented in Table 7. In addition, subtle modifications were made to the detector temperature, demonstrating a range of variation from 220°C to 240°C, as systematically illustrated in Table 8. The injector temperature experienced adjustments within the interval of 135°C to 165°C, meticulously detailed in Table 9. Further refinements involved precise adjustments in the initial oven temperature, ranging from 36°C to 44°C, as systematically outlined in Table 10. Moreover, the temperature ramping rate underwent deliberate variation, oscillating between 8°C/min and 12°C/min, with methodical documentation provided in Table 11. These sequential and controlled changes were executed to assess their impact on the overall analytical performance and are fundamental to the refinement of the analytical methodology employed in this study. Under this diverse range of conditions, the tailing factor

**Table 6.** Accuracy of methyl-2- chloroacetate in chlordiazepoxide hydrochloride at different levels ( $n = 3$ ).

Level	Preparations	Amount measured (ppm)	Amount as such in run (ppm)	Amount added (ppm)	% Recovery	Average% recovery	% RSD of recovery
LOQ	01	0.370		0.38	97.49		
	02	0.365	Not detected	0.38	97.29	97.32	0.15
	03	0.365		0.38	97.19		
50%	01	0.762		0.75	101.54		
	02	0.762	Not detected	0.75	101.59	101.54	0.05
	03	0.761		0.75	101.49		
80%	01	1.200		1.2	100.00		
	02	1.202	Not detected	1.2	100.19	99.63	0.80
	03	1.185		1.2	98.71		
100%	01	1.521		1.5	101.43		
	02	1.529	Not detected	1.5	101.94	101.45	0.47
	03	1.515		1.5	100.98		
120%	01	1.811		1.8	100.59		
	02	1.815	Not detected	1.8	100.86	100.92	0.37
	03	1.824		1.8	101.33		

**Table 7.** Robustness study: carrier gas flow rate variation ( $n = 3$ ) versus area response of MCA, carrier gas flow rate (4.5 to 5.5 ml/minutes).

Retention time (minutes)	Area response for carrier gas flowrate 4.5 ml/minutes	Retention time (minutes)	Area response for carrier gas flowrate 5 ml/minutes	Retention time (minute)	Area response for carrier gas flowrate 5.5 ml/min
11.139	4,153	10.832	4,027	10.498	4,097
11.140	4,143	10.830	4,073	10.502	4,056
11.136	4,109	10.832	4,007	10.497	4,055
Avg	4,135.000	Avg	4,035.667	Avg	4,069.333
SD	23.065	SD	33.843	SD	23.965
%RSD	0.558	%RSD	0.839	%RSD	0.589
Tailing Factor	1.072	Tailing Factor	1.043	Tailing Factor	1.03
Theoretical Plate(USP)	182,548	Theoretical Plate(USP)	149,177	Theoretical Plate(USP)	140,289

**Table 8.** Robustness study: detector temperature variation ( $n = 3$ ) versus area response of MCA ( $^{\circ}\text{C}$  220–240).

Retention time (minutes)	Area response at detector temperature set 220 $^{\circ}\text{C}$	Retention time (minutes)	Area response at detector temperature set 230 $^{\circ}\text{C}$ )	Retention time (minutes)	Area response at detector temperature set 240 $^{\circ}\text{C}$
10.814	4,043	10.832	4,027	10.809	4,094
10.814	4,033	10.830	4,073	10.815	4,076
10.817	4,020	10.832	4,007	10.815	4,008
Avg	4,032.000	Avg	4,035.667	Avg	4,059.333
SD	11.533	SD	33.843	SD	45.358
%RSD	0.286	%RSD	0.839	%RSD	1.117
Tailing Factor	1.031	Tailing Factor	1.043	Tailing factor	1.016
Theoretical plate(USP)	162,428	Theoretical plate(USP)	149,177	Theoretical plate(USP)	157,286

**Table 9.** Robustness study: injector temperature variation ( $n = 3$ ) versus area response of MCA ( $^{\circ}\text{C}$  135–165).

Injector temperature manipulation 135 $^{\circ}\text{C}$ –165 $^{\circ}\text{C}$					
Retention time (minutes)	Area response at injector temperature set 135 $^{\circ}\text{C}$	Retention time (minutes)	Area response at injector temperature set 150 $^{\circ}\text{C}$	Retention time (minutes)	Area response at injector temperature set 165 $^{\circ}\text{C}$
10.780	4,006	10.832	4,027	10.762	4,056
10.785	4,019	10.830	4,073	10.772	4,020
10.797	4,016	10.832	4,007	10.784	4,086
Avg	4,013.667	Avg	4,035.667	Avg	4,054.000
SD	6.807	SD	33.843	SD	33.045
%RSD	0.170	%RSD	0.839	%RSD	0.815
Tailing Factor	0.962	Tailing Factor	1.043	Tailing Factor	1.157
Theoretical plate (USP)	145,024	Theoretical plate (USP)	149,177	Theoretical plate (USP)	161,028

**Table 10.** Robustness study: initial oven temperature variation ( $n = 3$ ) versus area response of MCA ( $^{\circ}\text{C}$  36–44).

Retention time (min)	Area response for initial oven temperature 36 $^{\circ}\text{C}$	Retention time (minutes)	Area response for initial oven temperature 40 $^{\circ}\text{C}$	Retention time (minutes)	Area response for initial oven temperature 44 $^{\circ}\text{C}$
11.285	4,025	10.832	4,027	11.312	4,040
11.286	4,065	10.830	4,073	11.316	4,086
11.285	4,059	10.832	4,007	11.316	4,038
Avg	4,049.667	Avg	4,035.667	Avg	4,054.667
SD	21.572	SD	33.843	SD	27.154
%RSD	0.533	%RSD	0.839	%RSD	0.670
Tailing Factor	1.072	Tailing Factor	1.043	Tailing Factor	1.034
Theoretical plate (USP)	182,548	Theoretical plate (USP)	149,177	Theoretical plate (USP)	179,942

**Table 11.** Robustness study: ramp rate ( $n = 3$ ) versus area response of MCA (8 $^{\circ}\text{C}$ /minutes to 12 $^{\circ}\text{C}$ /minutes).

Retention time (minutes)	Area response for ramp rate 8 $^{\circ}\text{C}$ /minutes	Retention time (minutes)	Area response for ramp rate 10 $^{\circ}\text{C}$ /min	Retention time (minutes)	Area Response for ramp rate 12 $^{\circ}\text{C}$ /min
11.553	4,024	10.832	4,027	10.230	4,089
11.553	4,013	10.830	4,073	10.231	4,045
11.556	4,068	10.832	4,007	10.245	4,085
Avg	4,035.000	Avg	4,035.667	Avg	4,073.000
SD	29.103	SD	33.843	SD	24.331
%RSD	0.721	%RSD	0.839	%RSD	0.597
Tailing factor	1.051	Tailing factor	1.043	Tailing factor	1.096
Theoretical plate (USP)	130,761	Theoretical plate (USP)	149,177	Theoretical plate (USP)	154,379

consistently stayed below 1.5, and the theoretical plate numbers consistently exceeded 100,000. In addition, we consistently observed peak area measurements with RSD below 5.0%.

#### Solution stability

A solution maintains its chemical and physical properties, including component concentration and integrity,

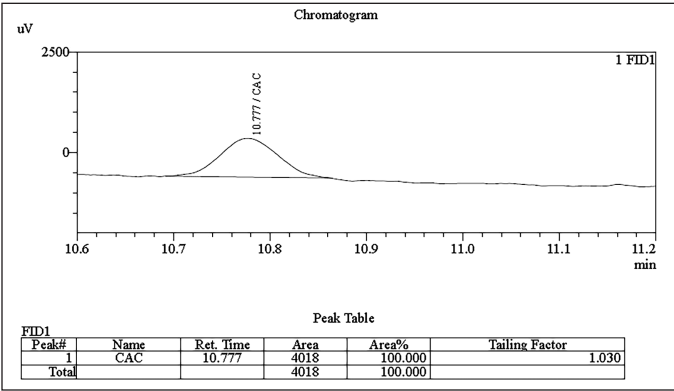


**Table 12.** Solution stability results.

S.no.	Stability interval	Peak response of 1.5 ppm of the standard solution
1	Initial preparation	4,071
2	After 24 hours	4,073
3	After 48 hours	4,038
4	After 72 hours	4,021
% RSD		0.63

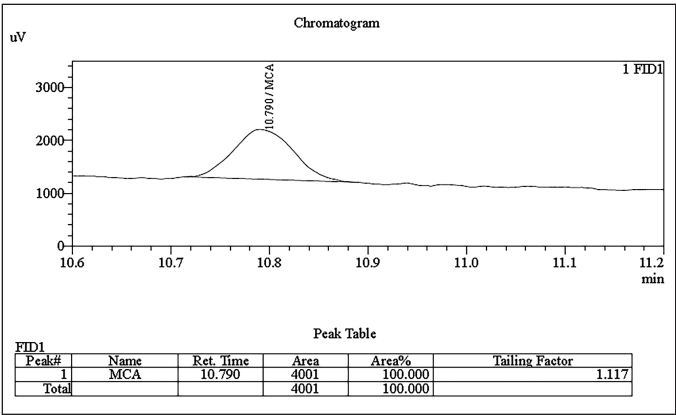
**Table 13.** Assay of chloroacetyl chloride into chlordiazepoxide hydrochloride.

S.no.	Standard 1.5 ppm area response	Retention time in minutes
1	4,018	10.777
2	4,004	10.778
3	4,016	10.790
4	4,001	10.790
5	4,047	10.799
6	4,060	10.800
Average	4,024	10.789
SD	23.89	0.01
% RSD	0.59	0.09
Chlordiazepoxide hydrochloride	Sample area response	Retention time in minutes
Blank-methanol	Not detected	10.70 to 10.80
Sample-1	Not detected	10.70 to 10.80
Sample-2	Not detected	10.70 to 10.80
Sample-3	Not detected	10.70 to 10.80

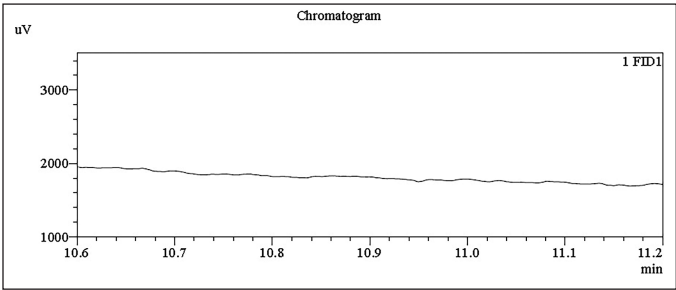


**Figure 8.** GC-FID chromatogram: chloroacetyl chloroacetate standard 1.5 ppm spiked in chlordiazepoxide hydrochloride sample.

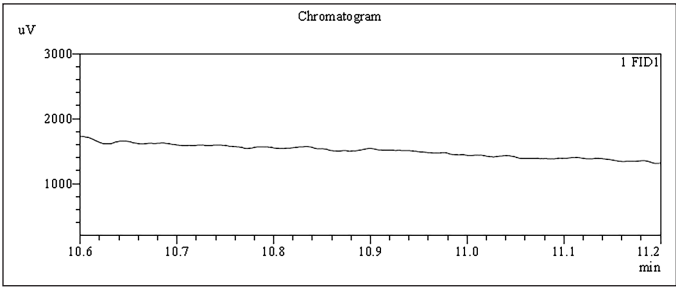
over a specified period under defined storage conditions, indicating its solution stability. To assess its stability over a 3-day period, we injected the standard solution of 1.5 ppm into the system, and the results in Table 12 demonstrate that the solution remained stable at room temperature (25°C) in clear glass throughout the entire duration.



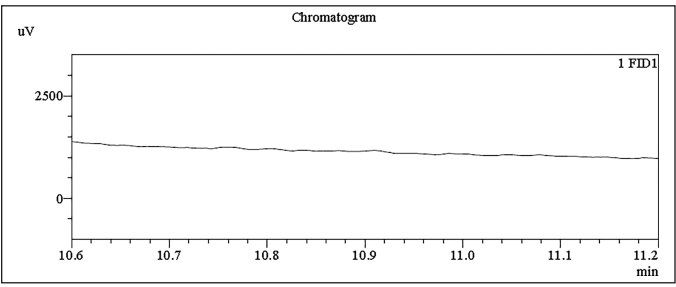
**Figure 9.** GC-FID Chromatogram: MCA Standard 1.5 ppm spiked in chlordiazepoxide hydrochloride sample.



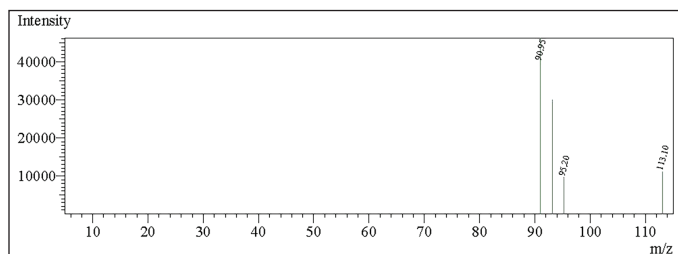
**Figure 10.** GC-FID chromatogram: chlordiazepoxide hydrochloride API sample-01 in diluent blank.



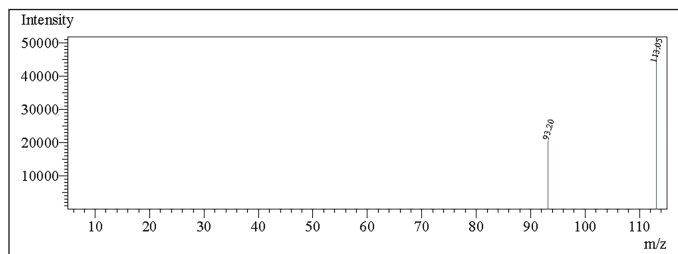
**Figure 11.** GC-FID chromatogram: chlordiazepoxide hydrochloride API sample-02 in diluent blank.



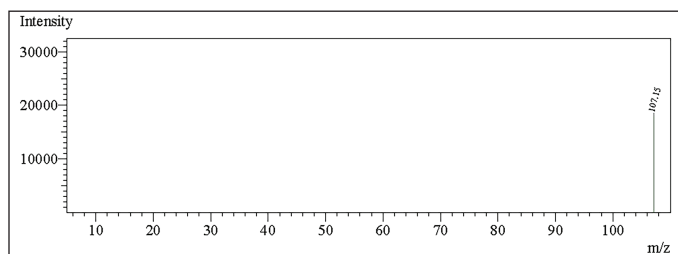
**Figure 12.** GC-FID chromatogram: chlordiazepoxide hydrochloride API sample-03 in diluent blank.



**Figure 13.** Mass spectrum of CAC in dichloromethane



**Figure 14.** Mass spectrum of CAC in ethylene dichloride.



**Figure 15.** Mass spectrum of CAC in methanol forming MCA at –ve polarity.

## CONCLUSION

In summary, we have successfully developed a simple, expeditious, and secure (GC-FID) method for the quantitative determination of chloroacetyl chloride in chlordiazepoxide hydrochloride drug substance. This method has demonstrated exceptional precision and accuracy throughout its development and validation stages. In full compliance with the rigorous guidelines outlined by the International Conference on Harmonisation (ICH), our method has excelled in numerous critical analytical parameters, including specificity, system suitability, precision, detection and quantification limits, linearity, accuracy (recovery), robustness, and solution stability for three days.

The successful application of this method in the analysis of three distinct batches of chlordiazepoxide hydrochloride samples, all of which did not exhibit any trace of MCA, underscores the method's reliability and suitability for use in the pharmaceutical industry. In upholding its commitment to ensuring patient safety, the pharmaceutical sector can confidently rely on this robust analytical approach to maintain the quality and integrity of the active drug substance.

## LIST OF ABBREVIATIONS

CAC, Chloroacetyl chloride; GC-FID, Gas chromatography with flame ionization detector; ICH, The International Conference of Harmonization; MCA, Methyl 2-chloroacetate

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## AUTHOR CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work. All the authors are eligible to be an author as per the International Committee of Medical Journal Editors (ICMJE) requirements/guidelines.

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## CONFLICTS OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

## ETHICAL APPROVALS

This study does not involve experiments on animals or human subjects.

## DATA AVAILABILITY

All data generated and analyzed are included in this research article.

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