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Simple, trace level derivatization and liquid-liquid extraction method of a genotoxic impurity 2,4-dichlorobutanoic acid in antiepileptic drug substance: Levetiracetam by GC-MS technique.

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ABSTRACT

The objective of this work was to develop and validate a simple derivatization and liquid–liquid extraction (LLE) procedure for GC-MS analysis of 2,4-Dichlorobutanoic acidor 2,4-Dichlorobutyric acidin Levetiracetam drug substance. The analyte was treated with methanol sulfuric acid mixture and extracted by water and n-Hexane, the supernatant allowed to separate into two phases, the derivative of analyte separated into the upper layer and detected by gas chromatography-mass spectrometry by using a short mid-polar capillary GC column at a high column-head pressure. Total run time of the analysis was15 min. Chromatographic conditions were achieved on DB-624 (6% cyanopropylphenyl and 94% dimethylpolysiloxane copolymer) capillary column (30 m long, 0.32 mm internal diameter, 1.8 µm diameter) using helium as a carrier gas at a flow rate of 1.5 mL/min.The process was optimized by a design of experiments.

Method validation parameters achieved as per regulatory requirements. The method was validated showing that the detection limit and quantitation limit of this impurity were 0.05 µg/g and 0.14 µg/g respectively. A good linearity over the 0.14-0.72 µg/g concentration range with correlation coefficient > 0.999, providing satisfactory results in terms of intra-day and inter-day precision as well as an optimal accuracy.

Thus, proving that thedescribed GC-MS method could be employed for fast and simple analysis ofgenotoxic impurity 2,4-Dichlorobutanoic acid in Levetiracetam drug substance.

Keywords: Levetiracetam, Genotoxic impurity, GC-MS,Development, Liquid–Liquid extraction, Method validation.

INTRODUCTION

Levetiracetam is an antiepileptic drug substance medicinally effective in generalized, partial epilepsy syndromes and has been recommended as the treatment of epilepsy either monotherapyin the case of partial seizures or as an adjunctive therapy (for partial myoclonic and tonic-clonic seizures) [1-3].The chemical name of Levetiracetam is(S)-ethyl-2-oxo-1-pyrrolidine acetamide. This antiepileptic drug that is chemically unrelated to the traditional antiepileptic drugs in current use [4, 6]. Levetiracetam is also useful as alone or in combination with other drugs, for the treatment of mania, migraine and bispolar disorders [5]. Levetiracetam is dose dependent and daily therapeutic dosage of drug ranged from 1000 to 3000 mg [7], with serum concentration ranges from about 10-50µg/mL [4,5].

2,4-Dichlorobutanoic acid is a genotoxic impurity formed as by-product in the synthetic process of 4-Chloro butyryl chloride, which is starting material of Levetiracetam drug substance [8, 9]. The schematic representation of drug synthesis and impurity carryover to final drug is shown in Figure 1.According to the European Medicines Agency (EMEA) and from US Food and Drug Administration (USFDA) the projected use of a threshold of toxicological concern (TTC), it is established that genotoxic impurities will be limited to a daily dose of 1.0–1.5 µg/day [10, 11].As a result it is necessary for process chemists to avoid such genotoxic impurities in the manufacturing process [12], though it would be difficult or not viable to eliminate PGIs completely from the synthetic scheme. Therefore it is a great face up to analyst to develop a right analytical method to quantify the impurity precisely and control their levels in drug substances.

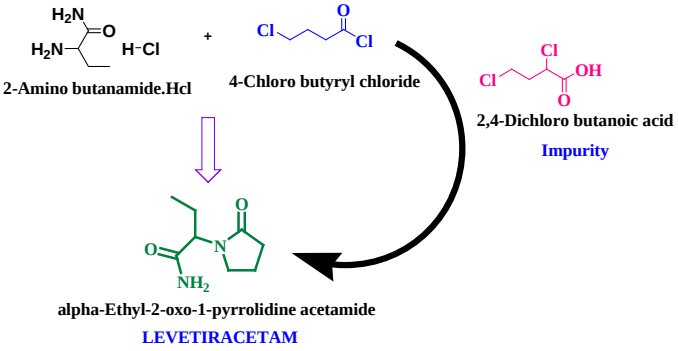


Figure 1: Schematic representation of levetiracetam synthesis and 2,4-Dichlorobutanoic acid impurity carry over through the route synthesis of starting material 4-Chloro butyryl chloride.

The regulatory bodies may be anticipated to control the levels of 2, 4-Dichlorobutanoic acid to 0.5 ppm in the drug substance (assuming TTC concept for maximum daily dose 3000 mg of Levetiracetam). Development of an analytical method capable of such a lower level of detection is a great challenge to analyst for controlling of this impurity. Conservative analytical instruments in pharmaceutical industries such as HPLC and GC detection must be employed as the impurity standards in first attempt for genotoxic impurities[14,15], but these have some drawbacks, because in HPLC and GC techniques,responses of impurities at trace levels not always possible and not to be characterized on line, if that is new impurity. Hence accurate determinations at trace levels with above techniques are not enough. Spectrometric techniques are needful for the trace level quantification. The literature survey exposed that some spectrometric methods and HPLC methods were developed for the determinations of Levetiracetam in-human plasma, Levetiracetam it's toxic impurity, levetiracetam and lamotrigine determinations in whole blood, levetiracetam its Carboxylic Metabolite in human plasma, using a Nitrogen-Phosphorus Detector for Levetiracetam by GC and determination of Levetiracetam in plasma studies [16-23].

Ever since no method has been in the past period reported for the quantification of 2,4-Dichlorobutanoic acidin Levetiracetam, a challenge was made to develop a highly specific, selective and accurate GC-MS method for the quantification which gives superior trace level analysis. The developed method is extremely reproducible, necessary lesser time for analysis and validated as per International conference of Harmonization (ICH) guidelines [24].

EXPERIMENTAL

Chemicals and reagents

Solvents were used in experiments obtained from analytical grade. Methanol (99.99 %), Sulfuric acid (98.12 %), n-Hexane (99.53 %) and water were attained in their best quality by Merck (Mumbai,India). 2,4-Dichlorobutanoic acid (93.1 %) and Levetiracetam drug substance (99.6 %) were utilized from APL research centre-II (a division of Aurobindo pharma limited, Hyderabad, India).

Preparations of stock, standard solutions and sample solutions

Stock standard solution (0.05 µg/mL) was prepared by dissolving 2,4-Dichlorobutanoic acid in derivative solution i.e. methanol-sulfuric acid (90:10, v/v).Finally desired concentration 0.5 µg/g of standard solution (with respect to sample concentration 40 mg/mL)was prepared by transferring 2.0 mL of stock standard solution to a glass vial and capped. Heated the vial at 80°C for 15 min. Then cooled this solution to room temperature. To this solution, added 1.0 mLof water and 2.0 mLof n-Hexane then vortex the solution for 1.0 min. Allowed the two phases to separate for 1.0 min., collected the upper layer (n-Hexane layer) and transferred into a 2 mL auto sampler vial for injection.

Test sample of Levetiracetam drug substance was prepared by diluting to 40 mg/mL by adding 2.0 mL of derivative solution, 1.0 mL of water, 2.0 mL of n-Hexane then derivatizedthe solution and collected the upper organic layer like as above. The standard solutions and test samples were optimized to achieve a desired signal-to-noise ratio (S/N) and desired peak shape.

Instrumentation

GC-MS analysis was carried out with a 7890B coupled with 5977A quadrupole mass detector (MSD) and the mass Spectrometer equipped with an Electron Ionization (EI) source, GC sampler 80 (Auto sampling unit) (Make: Agilent Technologies, Santa clara, CA, USA).InDB-624 (6% cyanopropylphenyl and 94% dimethyl poly siloxan) capillary column (30 m long, 0.32 mm internal diameter, 1.8 µm diameter) injections (2.0 µL) were performed in 1:2 split mode, capillary injector temperature at 200°C and a helium flow of 1.5 mL/min. The GC oven temperature was initially set at 80°C for 2 min, raised at a rate of 20°C/min up to 240 °C, where it was maintained for 5 min, for an overall runtime of 15 min. Temperatures were set at 250 °C for the ionization source and at 240 °C for the interface. Ms on time, Ms off time were set at between 6.0-10.0 min respectively and Dwell time 100 ms. GC-MS spectra of methyl ester of 2,4-Dichlorobutyric acid was initially acquired in full-scan mode (mass range 29–300 m/z, source energy 70 eV) and subsequently in acquisition mode of Selective Ion Monitoring (SIM). Data handling system was MASS HUNTER version 0704 to monitor the output signals for pre-processing the analytical raw data. Electron ionization (EI) mode was used for the quantification of impurity derivative at m/z 108 for quantifier, m/z 75 for qualifier.

Validation study

The analytical method was fully validated in accordance with the recommendations of following US FDA and ICH guidelines. The following parameters were investigated: specificity, limit of detection (LOD), limit of quantitation (LOQ), linearity, accuracy, range, intra-day and inter-day precision i.e. method precision, and intermediate precision.

Specificity

The specificity of this method gave the unbiased assessment of 2,4-Dichlorobutanoic acid in the presence of other constituents (Levetiracetam and its related substances). It has been done by analyzing the Levetiracetam and impurity solutions. The solutions of Levetiracetam test sample and Levetiracetam spiked with 2,4-Dichlorobutanoic acid at specification level prepared and injected for analysis. Further test sample of Levetiracetam and Levetiracetam spiked with impurity at specification level injected in MS SI Mmode was tested for the interference. From the results we concluded that no interference observed at the retention time of Methyl ester of 2,4-Dichlorobutanoic acid. The corresponding chromatogram is shown in Figure 2. Hencethis method is selective and specific for the determination of 2,4-Dichlorobutanoic acid content in Levetiracetam drug substance.

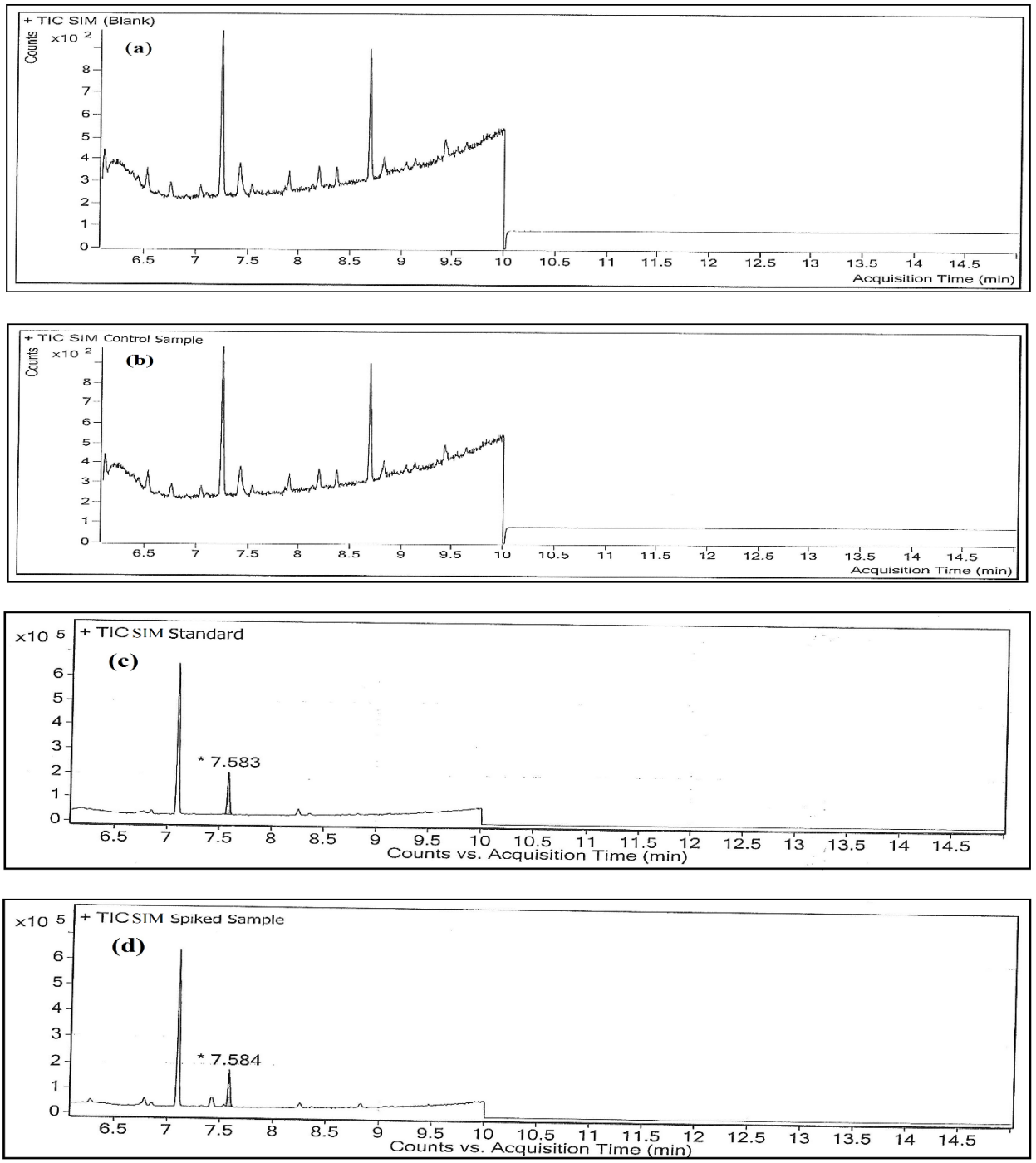


Figure 2: Chromatograms of (a) Blank (b) Control sample of Levetiracetam (c) Standard (d) Control sample spiked with 2,4-Dichlorobutanoic acid at 0.5 µg/g level

Linearity

The linearity of this analytical method was confirmed with a six-point calibration plot of LOQ to 150% of analyte concentrations (LOQ, 50, 80, 100, 120 and 150%) shown in Figure 3. A graph between the peak areas versus the concentrations within the range of 0.14-0.72 µg/g was linear. This data consists of slope, intercept, and correlation coefficient values. The correlation coefficient value for methyl ester of 2,4-Dichlorobutanoic acid peak was 0.9996. An excellent correlation result from this linearity experiment proved that the responses of mass spectrometer were

proportional to the concentrations of 2,4-Dichlorobutanoic acid was shown in Table 1.

Precision

The repeatability and intermediate precision experiments were performed to assess the precision of this developed method. For repeatability, six sample solutions were prepared individually using single batch of Levetiracetam drug substance spiked with 2,4-Dichlorobutanoic acid at 0.50 µg/g level. Same procedure was followed on different day, different analyst, and different column lot to evaluate the intermediate precision. Over all RSD observed 2.4% for the results of repeatability and intermediate precision experiments, which indicate this test method is precise and rugged were shown in Table 1.

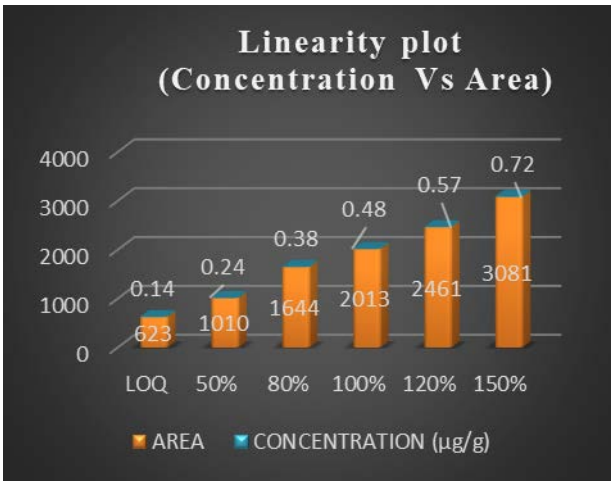


Figure 3: Linearity graph for methyl ester of 2,4-Dichlorobutanoic acid from LOQ to 150% level

Table 1: Intra-day and Inter-day precision at 0.5 µg/g concentration and linearity data of the impurity in the concentration range of 0.14 - 0.72 µg/g level.

Injection ID	Impurity Content (µg/g)		Linearity		
	Intra-day	Inter-day	Points	Concentration (µg/g)	Area of Methyl ester of 2,4-Dichlorobutanoic acid
1	0.41	0.43	1	0.14	623
2	0.42	0.43	2	0.24	1010
3	0.42	0.44	3	0.38	1644
4	0.42	0.44	4	0.48	2013
5	0.41	0.43	5	0.57	2461
6	0.41	0.43	6	0.72	3081
Mean	0.42	0.43	**Statistical analysis**		
Standard deviation	0.01	0.01			
%RSD	2.4	2.3	Slope	4263.666	
Over all %RSD	2.4		Intercept	7.487	
			Correlation coefficient	0.9996	

Limit of Detection (LOD) and Limitof Quantification (LOQ)

Determination of signal to noise ratio is the sign for calculating LOD and LOQ values in method validation. The LOD and LOQ values of 2,4-Dichlorobutanoic acid (as Methyl ester of 2,4-Dichlorobutanoic acid) wereobtained by injecting the standard stock solution in lower levels with respect to the drug substance concentration of 40 mg/ml and determining their S/N ratios. LOD and LOQ values were evaluated by their concentrations were lowered sequentially such that they yield S/N ratios >3.0 and >10.0 respectively were depicted in Figure 4. The predicted concentrations were verified for precision by injectingeach solution six times for analysis. The LOD and LOQ values calculated from S/N ratio were shown to be 0.05µg/g and 0.14 µg/g respectively. LOD value is showing almost 3 times less than LOQ which was shown in Table 2.

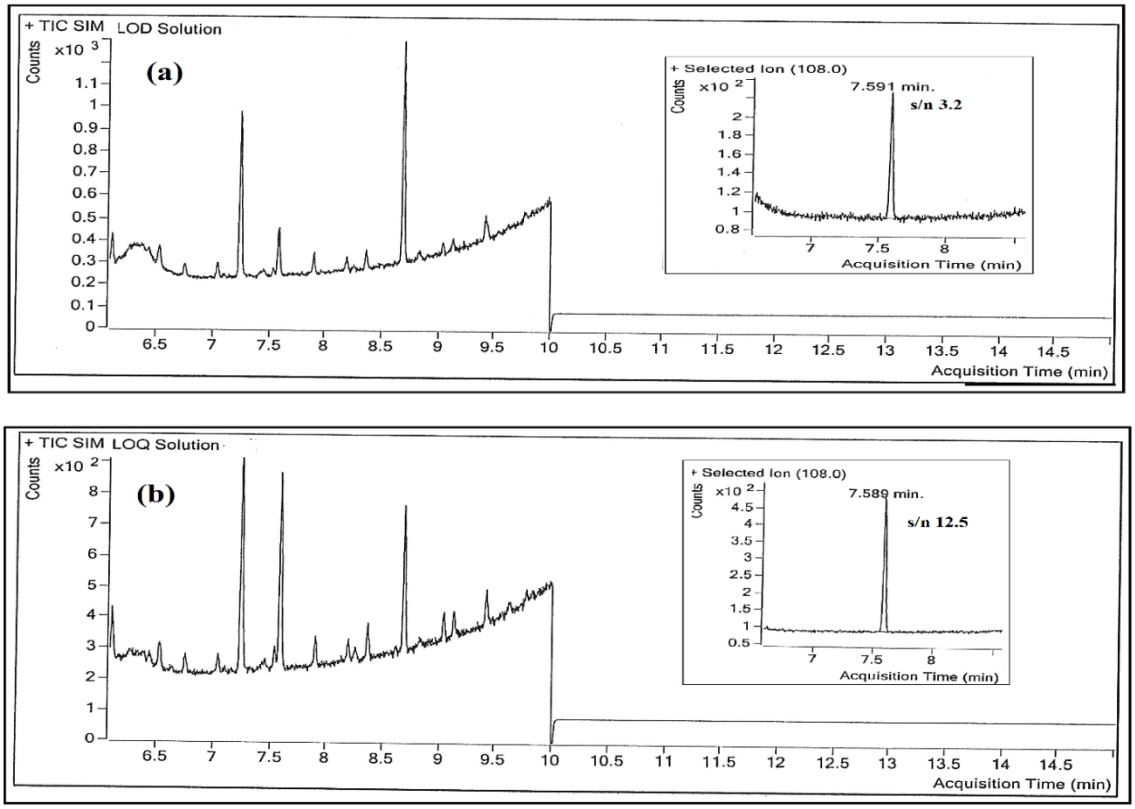


Figure 4: (a) Signal to noise of LOD solution 3.2 (b) Signal to noise of LOQ solution 12.5.

Table 2: Analysis in five different batches of Levetiracetam drug substance and LOQ and LOD concentrations.

No. of Samples	LOQ *	LOD #	Impurity
	Concentration (µg/g)	Concentration (µg/g)	Result (µg/g)
Sample-1	0.14	0.05	Not detected
Sample-2	0.14	0.05	Not detected
Sample-3	0.14	0.05	Not detected
Sample-4	0.14	0.05	Not detected
Sample-5	0.14	0.05	Not detected
* Limit of quantification # Limit of detection			

Recovery

Accuracy of the method was determined by recovery studies. Levetiracetam drug substance sample solutions were prepared, each in triplicate, as control sample (unspiked sample) and as spiked sample by spiking with 2,4-Dichlorobutanoic acid at LOQ level, 50%, 100% and 150% of specification level and analyzed each solution in GCMS. The accuracy studies were carried out and the percentage recovery and percentage relative standard deviation of the recoveries were calculated shown in the Table 3. These results indicate that the method has an acceptable level of accuracy from LOQ to 150% of specification level.

Table 3: Evaluation of recovery at four different concentrations of the impurity

Recovery Level	Amount added concentration (µg/g)	Amount found concentration (µg/g)	% Recovery
LOQ(0.14µg/g) Level	0.14	0.12	85.7
	0.14	0.12	85.7
	0.14	0.12	85.7
50% (0.24 µg/g) level	0.24	0.21	87.5
	0.24	0.21	87.5
	0.24	0.22	91.7
100% (0.48 µg/g) level	0.48	0.41	85.4
	0.48	0.42	87.5
	0.48	0.42	87.5
150% (0.72 µg/g) level	0.72	0.63	87.5
	0.72	0.63	87.5
	0.72	0.64	88.9

Stability study

The stability experiments were performed thoroughly to evaluate the stability of standard solution and sample solution. Standard solution and sample solution spiked with 2,4-Dichlorobutanoic acid at 0.5 µg/g level were prepared as per test method and analyzed initially and different time intervals by keeping the solutions at room temperature. The percentage difference between the peak areas of methyl ester of 2, 4-Dichlorobutanoic acid in the standard and spiked sample solutions after 24 hours were 0.7% and 0.1% respectively. There was no change in the amount of impurity during this stability study. It concludes that standard and spiked sample solutions are stable for 24 hours at room temperature. Stability studies calculations shown in the Table 4.

Table 4: Solution stability data of impurity in diluents

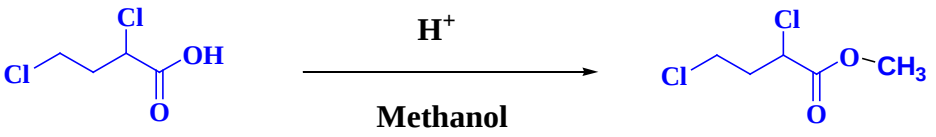
	Temperature about 25˚C± 2˚C / after 24 hours			Temperature about 25˚C± 2˚C / after 24 hours		
Name	**Standard area (Initial)**	**Standard area (after 24 hours)**	% Difference	**Spiked sample area (Initial)**	**Spiked sample area (after 24 hours)**	% Difference
Impurity	2232	2247	0.7	2040	2037	0.1

RESULTS AND DISCUSSIONS

Method development

Optimization of mass spectrometer conditions

A gas chromatography/mass spectrometric method was developed for the analysis of 2,4-Dichlorobutanoic acid in Levetiracetam drug substance. The main objective of this method development was to achieve trace level, simple, efficient and rapid determination of 2,4-Dichloro butanoic acid in Levetiracetam drug substance and to facilitate the method for routine use in quality control. Due to the unique impurity structure and elimination of back ground noise for impurity; single ion monitoring (SIM) mode was selected for detection of Impurity. This mode permits considerable enhancement of selectivity and sensitivity for screening of impurity quantification. Optimization of mass spectrometric conditions of 2,4-Dichlorobutanoic acid stocksolution (0.05µg/mL) was prepared in derivative solution(Prepared methanol and sulfuric acid in the ratio of 90:10 v/v) and allowed to generate derivative spectra in scan mode Figure 5.Stable and intense m/z fragment ions were selected for quantitation of methyl ester derivative. Stock solution (0.05 µg/mL) was diluted to get a final concentration of 0.05 µg/g.This solution was used to generate the derivative quantitative signal out puts for fragments at m/z 108 for quantifier, m/z 75 for qualifier. The ion source, interface temperatures, and dwell time parameters were optimized to get proper impurity optimum response.



2,4-Dichloro butanoic acid **Methyl 2,4-Dichloro butanoate**

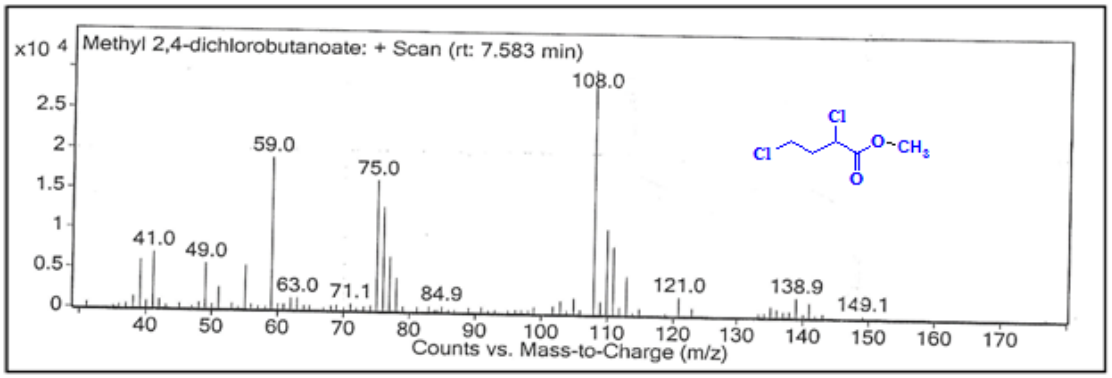


Figure 5: Mass spectra of Methyl ester of 2,4-Dichlorobutanoic acid

Choice and separation of column

The primary goal of this development was separate and quantifythe2,4-Dichlorobutanoic acid with excellent peak shape. Various attempts were performed with different columns i.e.DB-1701 (14% cyanopropylphenyl and 86% methylpolysiloxane copolymer) capillary column (30mlong, 0.32 mm internal diameter, 1.8 µm diameter) and DB-1 (100% dimethylpolysiloxane copolymer) capillary column (30 m long, 0.32 mm internal diameter, 1.8 µm diameter)by using column flow of 1.5 ml/min but, in all the above conditions the separation of the related substances of Levetiracetam drug substance with analyte impurity was not satisfactory and also lower recoveries found. Later, DB-624 (6% cyanopropylphenyl and 94% dimethylpolysiloxane copolymer) capillary column (30 m long, 0.32 mm internal diameter, 1.8 µm diameter) was used for better response of analyte and related substance peaks were well resolved from analyte peak.

Optimization of chromatographic conditions

Operating conditions of this GC-MS method were optimized by using analytical column namely, DB-1701 (14% cyanopropylphenyl and 86% methylpolysiloxane copolymer) capillary column of 30 m long, 0.32 mm internal diameter, 1.8 µm diameter with column flow rate of 1.5 mL/min with Helium as carrier gas and head space GC injection volume was 1.0 mL. Column oven temperature was 70˚C for 5 min. then raised to 220°C with a ramp of 15°C/min. Standard solution of 0.5 µg/g(w.r.to sample concentration of40 mg /mL) was injected to optimize tuning the GC-MS system. The response of impurity peak observed very low and peak shape was not good.

To overcome this challenge,DB-624 (6% cyanopropylphenyl and 94% dimethylpolysiloxane copolymer) capillary column of 30 m long, 0.32 mm internal diameter, 1.8 µm diameter with column flow rate 1.5 mL/min with Helium as carrier gas and injection volume 2.0 µL. Column oven temperature was 80˚C for 2 min. then raised to 240°C with a ramp of 20°C/min. Derivatized standard solution (0.5 µg/g w.r.to sample concentration of 40 mg /mL)injected to optimized tuning the GC-MS system. This injection provided neat peak shapes with good responses.

Optimization and preparation of sample

In the pharmaceutical impurity analysis, one of the most important tasks is sample preparation because the influence of matrix in trace levels also leads to loss of analyte precision, sensitivity, and recovery. Distinct diluents in different ratios *viz*, methanol, methanol in water, methanol-sulfuric acid (90:10, v/v) were checked for solubility of the drug, impurity as well as chromatographic performance. Solubility of both impurity and drug substance were good in methanol-sulfuric acid (90:10, v/v). Stock solution (0.05 µg/mL) was prepared by dissolving 2,4-Dichlorobutanoic acid in derivative solution i.e. methanol-sulfuric acid (90:10, v/v). Finally, desired concentration of 0.5 µg/g of standard solution (with respect to sample concentration 40 mg/mL) was prepared by transferring 2.0 mL of stock standard solution into a glass vial and heated the vial at 80°C for 15 min. Then cooled the solution to room temperature. To this solution added 1.0 mL of water and 2.0 mL of n-Hexane then vortex the solution for 1.0 min for mixing. Allowed the two phases to separate for 1.0 min. Collected the upper layer (n-Hexane layer) and transferred into a 2 mL vial for injection.

Test sample of Levetiracetam drug substance was prepared by diluting to 40 mg/mL by adding derivative solution and transferring 2.0 mL of this solution to a glass vial and heated the vial at 80°C for 15 min. Then cooled the solution to room temperature. 1.0 mL of water and 2.0 mL of n-hexane then vortex the solution for 1.0 min for mixing. Allowed the two phases to separate for 1.0 min. Collected the upper layer (n-Hexane layer) and transferred into a 2 mL vial for injection.

An excellent peak shapes, good responses, and fine recoveries were found with these sample preparations. 2,4-Dichlorobutanoic acid converts into Methyl ester of 2,4-Dichlorobutanoic acid compound in the presence of Methanol and sulfuric acid. Therefore, in this method 2,4-Dichlorobutanoic acid is quantified as Methyl ester of 2,4-Dichlorobutanoic acid.

CONCLUSION

The main object of this proposed work is to establish an exceptionally more selective and accurate method for the estimation of 2,4-Dichlorobutanoic acid impurity in the Levetiracetam drug substance by using GC-MS in an EI ionization mode. This established method was validated to provide specificity, precision, linearity, and good accuracy. The quite low LOD and LOQ values (i.e.0.05 ppm and 0.14 ppm) were attained as per international standards. The stability of method indicates that it could be utilized for routine analysis of samples in production stages. This method is very useful for the trace level quantification of 2,4-Dichlorobutanoic acid impurity in the Levetiracetam drug substance.

DECLARATIONS OF INTEREST

The authors declare no conflict of interest.

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HIGHLIGHTS

1) First developed trace level analysis of 2,4-Dichlorobutanoic acid PG impurity in the Levetiracetam drug substance.
2) This developed GC-MS method is very sensitive and validated.
3) The quantification and detection levels are found very low

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