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METHOD DEVELOPMENT AND VALIDATION FOR QUANTIFICATION OF BENZENE AND ETHYLENE DICHLORIDE IN BACLOFEN DRUG SUBSTANCE BY GC-HS USING FID DETECTOR

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Abstract:
A proficient GC-HS method was developed for quantification of Benzene and Ethylene Dichloride in Baclofen drug substance with flame ionization detector (FID) using column DB-1, (30m length X 0.32mm diameter) 5µm film thickness, Part No.: 123-1035, Make: Agilent. Nitrogen is used as Carrier gas at with flow rate of 1.0 ml/minute. The proposed method was validated for System suitability, Specificity, Linearity, LOD and LOQ determination, Recovery, Precision, Range and Robustness. All the parameters were found within the acceptable limits. Linearity of Benzene and Ethylene Dichloride is in the range of LOQ to 150%. The established methodology was commercially useful, specific, accurate, precise and suitable for the analysis of Benzene and Ethylene Dichloride in Baclofen drug substance.

***Keywords**: Gas Chromatography with Head space (GC-HS), Guideline for Residual Solvents Q3C(R8) and Method Validation, Baclofen drug substance, Benzene and Ethylene Dichloride*

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INTRODUCTION:

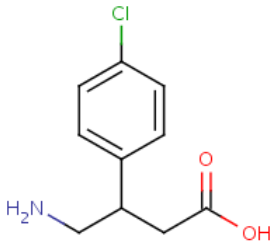
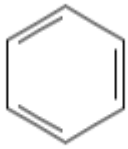
Baclofen is used to treat muscle spasticity; it is FDA-approved for managing reversible spasticity, particularly for the relief of flexor spasms, clonus, and concomitant pain, common sequelae of spinal cord lesions, and multiple sclerosis. Baclofen also has several off-label uses. Baclofen (beta-[4-chlorophenyl]-GABA) is an agonist at the beta subunit of gamma-aminobutyric acid on mono and polysynaptic neurons at the spinal cord level and brain. The thinking is that baclofen reduces the release of excitatory neurotransmitters in the pre-synaptic neurons and stimulates inhibitory neuronal signals in the post-synaptic neurons with resultant relief of spasticity.

Benzene and Ethylene Dichloride are organic solvents used in the synthesis of 3-(4-Chloro phenyl) Glutaric acid, that is key starting material of Baclofen. Both Benzene and Ethylene Dichloride are falls under Class 1 solvent list of Guideline for Residual Solvents Q3C(R8), so need to control at 2ppm and 5ppm respectively. And these organic solvents cannot be removed completely during the synthesis and even purification. Thus, monitoring of these residual organic solvent impurities in the drug substance is mandatory according to regulatory requirements to ensure human safety.

Generally, in the pharmacopoeias like USP, BP, EP, IP etc. monographs, specific methods for residual solvents have not reported for drug substance and drug products. To determine and quantitate at such lower level of Benzene and Ethylene Dichloride in Baclofen drug substance, highly sensitive, selective and accurate analytical methods is required. A rugged GC-HS method has been developed for Benzene and Ethylene Dichloride in Baclofen. In this work, over seven other residual solvents (Methanol, Ethanol, Isopropyl alcohol, Ethyl acetate Tetrahydrofuran, trimethylamine and Toluene) used in manufacturing of Baclofen were separated to prove the specificity for Benzene and Ethylene Dichloride in Baclofen.

The objective of this work was to develop a simple and rapid GC-HS method which would be accurate and robust. The method was validated according to ICH guidelines.

Compound name, Chemical formula, Molar mass and Structure formula are mentioned below:

Sr. No.	Compounds Name	Chemical formula	Molar mass (g/mol)	Structure formula
1	Baclofen	$C_{10}H_{12}ClNO_2$	213.661	
2.	Benzene	C_6H_6	78.11	
3	Ethylene dichloride	$C_2H_4Cl_2$	98.96	

METHODOLOGY DEVELELOPED AND MATERIALS USED:

Chemicals and Reagents

Methanol (GC-HS grade, Make: Sigma Aldrich), Ethanol (GC-HS grade, Make: Sigma Aldrich), Isopropyl alcohol (GC-HS grade, Make: Sigma Aldrich), Ethyl acetate (GC-HS grade, Make: Sigma Aldrich), Tetrahydrofuran (GC-HS grade, Make: Sigma Aldrich), Toluene (GC-HS grade, Make: Sigma Aldrich), Dimethyl sulfoxide (GC-HS grade, Make: Sigma Aldrich), Benzene (GC-HS grade, Make: Sigma Aldrich), Ethylene dichloride (GC-HS grade, Make: Sigma Aldrich)

INSTRUMENTATION

Gas chromatography, model no:2010 with head space, Model no.: HS-20 Make: Shimadzu was utilized for this work.

Chromatographic conditions: The column DB-1, (30m length X 0.32mm diameter) 5µm film thickness, Part No.: 123-1035, Make: Agilent. Column oven temperature; Initial temperature (50 ºC) for 5 min; increased to 100 ºC @ 5 ºC/min, hold for 0 min; then increased to 220 ºC @ 12 ºC/min, hold for 12 min; detector temperature: 300 ºC; Carrier gas: Nitrogen employed as a carrier gas with an invariable pressure of 6 psi. Make-up gas for FID: nitrogen gas with 40 mL/min flow rate was used; Fuel gases: Used hydrogen gas and zero air with flow rate of 40 and 400 mL/min. correspondingly. Split ratio: 1:1. Total run time of chromatography: 35 min.

Head space conditions: Oven temperature: 80 ºC; Transfer line temperature: 100 ºC; Loop temperature: 90 ºC; Vial equilibration duration: 30 min; Vial pressurization duration: 1.0 min; Loop equilibration time: 0.05 duration; Loop fill time: 0.1 min; Inject duration: 1.0 min; Vial pressure: 20 psi.

Preparation of blank, standard and sample solution: The diluent used was N, N-Dimethylformamide same was used as Blank solution. Residual solvents standard solution was prepared by using Benzene and Ethylene dichloride reference standards to attain a concentration of about 0.0006 mg/mL of Benzene and 0.0015 mg/mL of Ethylene dichloride. Further transfer 1 mL of standard solution into 20 mL headspace vial, add 1 mL water into it. Crimp the vial with an aluminum crimp cap containing a PTFE/Silicon septum. For sample solution preparation accurately weigh and transfer about 300.0 mg of test sample into a 20 mL headspace vial. Add 1.0 mL of diluent and 1 mL water to same vial. Crimp the vial with an aluminum crimp cap containing a PTFE/Silicon septum [This standard solution concentration is equal to about 2 ppm of Benzene and 5 ppm of Ethylene dichloride, against for 75 mg/mL of test concentration as per the ICH specification limits].

Acceptance criteria for System Suitability:

%RSD: The Relative Standard Deviation of peak areas of six replicate injections of standard solution and bracketing standard should not be more than 10.0.

Calculation: Calculate the residual solvents (in ppm) by using the following formula.

$$\textbf{Conc. in. ppm} = \frac{(AT - AB) \times WS \times DT}{(AS - AB) \times DS \times WT} \times P \times 10000$$

AB = Average peak area of respective analyte in the chromatogram obtained from blank ,AT = Peak area counts of respective analyte in the chromatogram obtained from the sample solution, AS = Average peak area counts of respective analyte standard in the chromatogram of the standard solution, WS = Weight of respective analyte standard, WT= Weight of sample solution, DT= Dilution factor of sample solution, DS= Dilution factor of respective analyte standard solution, P= Purity of respective analyte standard used.

Observation: In the above method, Benzene and Ethylene Dichloride are separated well with good resolution to other Baclofen residual solvents impurities with good symmetrical factor. Hence this method is suitable for Validation.

VALIDATION RESULTS AND DISCUSSION

Specificity:

The specificity is defined as the ability to assess unequivocally the analyte in the presence of components that may be expected to be present such as residual, degradation product and matrix components. In HPLC method, it is assured/proved by complete separation of peak of analyte from other peaks that are of other impurities that might be present in sample or blank.

Inject the Blank (as Diluent), Standard solution and spiked solution. Check the interference at the retention time of analyte. There should not be any interference in blank (as Diluent) and spiked sample at the retention time of analyte. If any peak is present at the retention time of analyte its response should

not be more than 20% of the response at the quantification limit (LOQ).

There is no interference observed between the responses of blank (as diluent) and spiked solution at RT of Benzene and Ethylene Dichloride. Hence, the method is very selective and specific for the estimation Benzene and Ethylene Dichloride residues in presence of seven other residual solvents (Methanol, Ethanol, Isopropyl alcohol, Ethyl acetate Tetrahydrofuran, trimethylamine and Toluene) used in manufacturing in Baclofen.

GC-HS Chromatograms of study

Fig No. 1: Blank Chromatogram

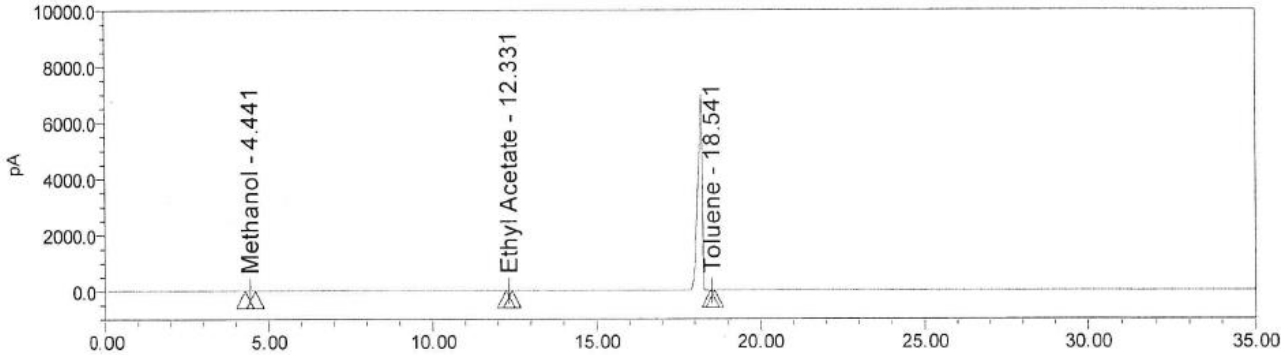


Fig No.2: Standard Chromatogram

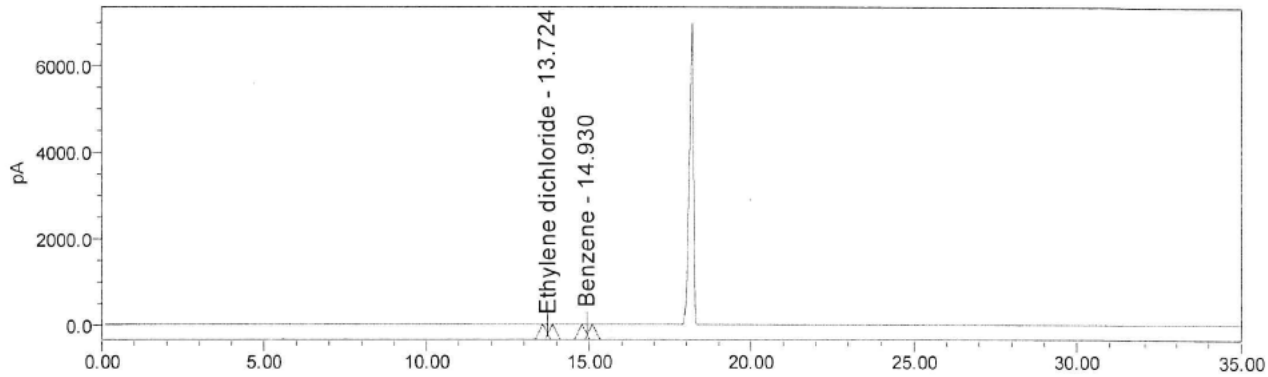


Fig No 3.: Spiked Chromatogram

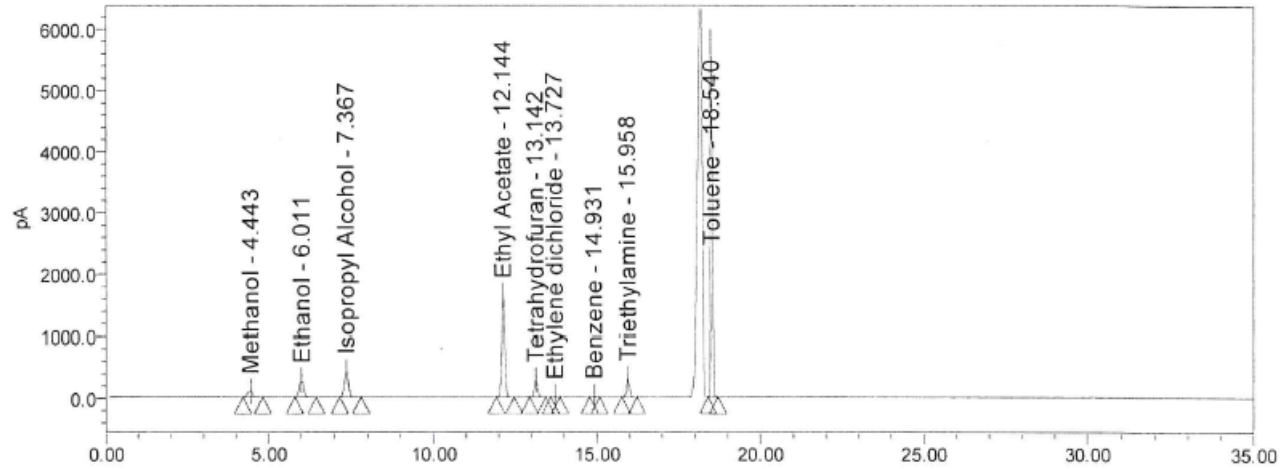


Table 1: Specificity Verification: Interference study

Name of Solvent	Retention time (Minutes)
Methanol	4.4
Ethanol	6.0
Isopropyl alcohol	7.3
Ethyl acetate	12.1
Tetrahydrofuran	13.1
Ethylene dichloride	13.7
Benzene	14.9
Triethylamine	15.9
Toluene	18.5

Linearity:

A linear relationship should be evaluated across the range of the analytical procedure. It may be demonstrated directly on the analyte by dilution of a standard stock solution using the proposed procedure. Linearity should be evaluated by visual inspection of a plot of signals as a function of analyte concentration or content. If there is a linear relationship, test results should be evaluated by appropriate statistical methods by calculation of a regression line. The correlation coefficient, y-intercept, slope of the regression line should be calculated.

The test method linearity was established from, six levels of concentration over the range LOQ to 150% of, ICH limit for each residual solvent impurity. A linear correlation, and regression were determined among the concentrations, and peak area responses of each residual solvent. The correlation coefficient (r) and regression coefficient (R2) values, for both residual solvent impurities found to be higher than, 0.990. The statistical characteristics like slope, y-intercept and, % y-intercept were interpreted and found within the acceptable, limit for both solvent impurities. The data tabulated in Table-2 demonstrate the linearity of procedure.

Table 2: Linearity for Benzene and Ethylene Dichloride

	Benzene			Ethylene dichloride		
Preparation	**Concentration**	**Concentration**	**Average Area response**	**Concentration**	**Concentration**	**Average Area response**
	(ppm)	**(mg/mL)**		**(ppm)**	**(mg/mL)**	
Standard at LOQ Level	0.6	0.00018	7.203	1.5	0.00045	3.003
Standard at 50% Level	1	0.0003	12.693	2.5	0.00075	5.005
Standard at 75% Level	1.5	0.00045	18.732	3.8	0.00113	7.396
Standard at 100% Level	2	0.0006	25.352	5	0.0015	9.86
Standard at 120% Level	2.4	0.00072	28.79	6	0.0018	11.295
Standard at 150% Level	3	0.0009	33.945	7.5	0.00225	13.437
Correlation Coefficient (R)	0.995285			0.9971		
Squared Correlation Coefficient (R^2)	0.990592			0.994209		
Slope	37500			5850		
Equation	Y = 3.75e+004X + 1.41e+000			Y = 5.85e+003X + 6.46e-001		

Limit of detection (LOD):

It is the smallest amount or concentration of an analyte that can be estimated with acceptable reliability. The detection limit is determined by the analysis of standard with known concentrations of analyte and by establishing the minimum level at which the analyte can be reliably detected.

The limit of detection is determined by establishing the signal to noise ratio. Inject the blank and standard solutions at lower concentration and calculate the signal to noise ratio.

A signal-to-noise ratio between 3:1 estimating the detection limit.

The detection limit for Benzene and Ethylene Dichloride in Baclofen is found 0.2ppm and 0.5ppm respectively. For details, refer Table 3.

Table 3: LOD Precision and s/n Ratio

Analyte	Conc. ~(ppm)	Mean S/N ratio
Benzene	0.2	11
Ethylene Dichloride	0.5	5

Limit of quantitation (LOQ):

The Quantitation limit is generally determined by the analysis of samples with known concentrations of analyte and by establishing the minimum level at which the analyte can be quantified with acceptable accuracy and precision.

The limit of quantification is determined by establishing the signal to noise ratio. Inject the blank sample and the spiked sample at LOQ level in six replicates and calculate signal to noise ratio and the % RSD at LOQ level.

A signal-to-noise ratio between 10:1 estimating the quantification limit.

The quantification limit for Benzene and Ethylene Dichloride in Baclofen is found 0.6ppm and 1.5ppm respectively. For details, refer Table 4.

Table 4: LOQ Precision and s/n Ratio

Analyte	Conc. ~ (ppm)	Mean S/N ratio	%RSD
Benzene	0.6	27	4.26
Ethylene Dichloride	1.5	11	4.19

Recovery:

Recovery means the percentage of the true concentration of a substance recovered during the analytical procedure.

Recovery assessed using a minimum of 6 determinations over a minimum of 3 concentration levels.

Acceptable limits for a recovery result during validation should be within the range of 70% - 120%.

The percentage of average recovery for Benzene and Ethylene Dichloride in Baclofen found >95% at 2.0ppm, and 5.0ppm respectively. For details, refer Table 5.

Table 5: Recovery results

Solvent	Accuracy at LOQ	Accuracy at 100%	Accuracy at 150%
Benzene	111.53	102.48	104.89
Ethylene Dichloride	115.35	107.48	109.24

Precision: (Method Precision)

The precision determined under equal conditions with same homogeneous spiked sample (six different sample preparation) as per recommended test method and % RSD of the results obtained shall be calculated.

The repeatability is established by estimating the six replicates of spiked sample and calculates the % RSD of the results obtained.

The %RSD of results for the analysis of spiked sample should not be more than 10%.

The % RSD of six different sample preparation found <10% refer Table-6.

Table 6: Method Precision results

Analyte	%RSD for spiked samples (6 Test Preparations)
Benzene	1.55
Ethylene Dichloride	1.23

Precision: (Intermediate Precision)

Intermediate Precision means the susceptibility of an analytical method to changes in experimental conditions which can be expressed as different columns, different analyst and different days.

Intermediate Precision of the method is established by estimating the six replicates of spiked sample by different analysts, on different days and on different columns. Calculate the % RSD of the results obtained.

The %RSD of results for the analysis of spiked sample should not be more than 10%.

The % RSD of six different sample preparation found 10% refer table-7

Table 7: Intermediate Precision results

Analyte	%RSD for spiked sample (6 Test Preparations)
Benzene	3.58
Ethylene Dichloride	2.43

Robustness:

The robustness of an analytical procedure is measure of its capacity to remain unaffected by small, but deliberate variations in method parameters and provides an indication of its reliability during normal usage.

The robustness of the GC-HS method is established by estimating minimum six replicates of standard solution. Calculate the accuracy and % RSD of the results.

The Relative Standard Deviation (%RSD) of peak areas of six replicate injections of standard solution for each solvent peak should not be more than 10.0.

The Relative Standard Deviation (%RSD) of peak areas of six replicate injections of standard solution for Benzene and Ethylene dichloride peak was found below 10.0 with all the robust conditions refer Table – 8

Table-8: Conditions for Robustness and their observations

S. No.	Conditions for Robustness	%RSD Benzene	%RSD Ethylene dichloride
1	Column oven temp. Low (-2°C)	3.2	1.6
2	Column oven temp. High (+2°C)	6.1	3.6
3	Linear velocity Low (-2 cm/sec)	3.3	1.5
4	Linear velocity High (+2 cm/sec)	4.7	2.8
5	Column oven ramp rate Low (-2°C)	4.8	3
6	Column oven ramp rate High (+2°C)	6.5	4.1
7	HS injection time low (-0.10 min)	1.5	0.6
8	HS Injection time high (+0.10 min)	1.6	1.2
9	HS oven temperature low (-5°C)	3.3	1.9
10	HS oven temperature high (-5°C)	5.1	3.5

DISCUSSION:

A chromatographic method involves demonstrating specificity, which is the ability of the method to accurately measure the analyte response in the presence of all potential sample components. The chromatographic parameters were fixed and GC-HS with FID detector system was studied for suitability of residual solvents analysis. The developed method was performed for linearity, precision, Accuracy, specificity, LOD LOQ determination and Robustness.

CONCLUSION:

A simple and sensitive method for the quantification of Benzene and Ethylene Dichloride in Baclofen drug substance by using GC-HS with FID detector was developed, validated in accordance to ICH validation guidelines and applied for the analysis of Baclofen samples. The method was validated to ensure the feasibility of the method for its application in routine analysis. The LOQs achieved through this method were very low. The methodology established was specific, robust, accurate, sensitive and linear in the range LOQ to 150% specification limit as per ICH.

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