

# A Novel Validated Analytical Method Development for the Binary Mixture of Mebeverine and Chlordiazepoxide in Pharmaceutical Formulation and its Application to Stress Studies

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## Abstract

**Purpose:** A Stability indicating RP-HPLC method for the determination of Mebeverine and Chlordiazepoxide in bulk and pharmaceutical dosage form was developed and validated. Two methods are available for the determination of this combination and it is required to develop the novel method with cost effective, time consuming, with good sensitivity and less retention time and to know the interference of degradation products which can be performed in the routine analysis.

**Methods:** Stability indicating RP-HPLC method with chromatographic conditions include Agilent C18 column (250 mm×4.6 mm i.d., 5 μ particle size) and the mobile phase consists of 40:60v/v ratio of Methanol and Tri ethyl amine buffer pH 7.0 with OPA at a flow rate of 1.0ml/min at a ambient temperature and the injection volume was 20 μL.

**Results:** Quantification was achieved at 262 nm wavelength using UV detector and the retention times of Mebeverine and Chlordiazepoxide were found to be 3.40 and 7.45 mins. The developed method shows linearity in the range 27-216 μg/mL for Mebeverine and 1.8-7.4 μg/mL for Chlordiazepoxide. The LOD and LOQ values for Mebeverine were 2.2 μg/mL and 6.5 μg/mL, for Chlordiazepoxide 0.01 μg/ml and 0.03 μg/ml respectively. The regression coefficient for both drugs was found to be 0.999. The mean recoveries ranged from 99.99-100.004% and 99.97-100.01% for Chlordiazepoxide and Mebeverine respectively. The stability study results show that the method for the determination of both the drugs was stable. The percentage degradation for Mebeverine and Chlordiazepoxide were within the limit that is less than 30.

**Conclusion:** Both drugs were eluted with less retention times. Stability was performed which shows that the proposed method was stable. Degradation products were not interfered with the pure drugs of Mebeverine and Chlordiazepoxide. By the stress study the degradation pathways are studied. Hence the developed method was considered as the stability indicating method. The proposed method was performed based on the ICH guidelines.

**Keywords:** RP-HPLC; Mebeverine; Chlordiazepoxide; Forced degradation study; Methanol; Tri ethyl amine; ortho phosphoric acid

## Introduction

Mebeverine is chemically Benzoic acid, 3, 4-dimethoxy-, 4-[ethyl [2-(4-methoxyphenyl)-1-methylethyl] amino] butyl ester, with molecular formula  $C_{25}H_{35}NO_5$ . It is pale yellow crystalline powder which is freely soluble in water and methanol. Mebeverine is an antimuscarinic and belongs to a group of compounds called musculotropic antispasmodics. These compounds act directly on the gut muscles at the cellular level to relax them which relieves muscle spasm pains of gut. It is used for the treatment of irritable bowel syndrome as well as other conditions including chronic irritable colon, spastic constipation and mucous colitis.

Chlordiazepoxide HCl is 7-chloro-2-(methyl amino)-5-phenyl-3H-1, 4-benzodiazepine 4-oxide hydrochloride. Its molecular formula is  $C_{16}H_{14}ClN_3O.HCl$ . It is white or slightly yellow solid crystalline in nature and completely soluble in water, may be soluble or sparingly soluble in alcohol and practically insoluble in chloroform. Mechanism of action includes binding of the drug to stereo specific benzodiazepine binding sites on GABA receptors at several sites within the central nervous system which results in an increased binding of the inhibitory neurotransmitter GABA to the GABA (A) receptor. Hence benzodiazepines enhance GABA mediated chloride influx which results in membrane hyperpolarisation. The neuro inhibitory effect results in sedative, hypnotic, anxiolytic and muscle relaxant properties. By the muscle relaxant property it is used for treatment of irritable bowel syndrome along with Mebeverine.

From the literature survey it was concluded that only few RP-HPLC methods [1], have been reported for the determination of Mebeverine and Chlordiazepoxide in combination. Certain methods for the estimation of Mebeverine and Chlordiazepoxide in combined dosage forms include UV spectrophotometric method [2]. Mebeverine has been determined either alone or in other drug combination by various methods includes RP-HPLC [3-8] and Chlordiazepoxide also determined either alone or in other drug combination by various methods includes RP-HPLC [9-14], simultaneous spectrophotometric method.

Only few methods for the simultaneous estimation of Mebeverine and Chlordiazepoxide have been reported. So, the present work was done with aim to develop a new stability indicating RP-HPLC method and validated according to ICH guidelines.

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## Methodology

### Materials

**Instrumentation:** The separation was carried out on Waters 2695 HPLC system using Agilent C18 column (250×4.6 mm; 5 μm), isocratic HPLC pump and UV detector. Empower software was used for the data acquisition and quantification of peaks of Mebeverine and Chlordiazepoxide. Denver instrument- weighing balance, ultrasonicator bath model-UCA 701 was used.

**Chemicals and reagents:** Mebeverine and Chlordiazepoxide pure drug and capsules, HPLC grade Methanol (Merck), Acetonitrile of HPLC grade, ortho phosphoric acid, Triethylamine and HPLC grade analytical water were used.

**Chromatographic conditions:** The chromatographic column used was Agilent C18 250 mm x 4.6 mm, 5 μ particle size. The mobile phase consists of 40:60 ratio of Methanol and Tri ethyl amine buffer pH 7.0 (v/v) adjusted using ortho phosphoric acid. Flow rate was 1.0 mL/min at an ambient temperature and the chromatograms were monitored at a detector wavelength of 262 nm using UV-Detector. The injection volume was 20 μL.

### Preparation of standard solutions

**Solution A: Mebeverine:** Weigh accurately about 135 mg of Mebeverine pure drug into a 100 mL volumetric flask. 70 mL of mobile phase was added then sonicate to dissolve and dilute to volume with mobile phase

**Solution B: Chlordiazepoxide:** Weigh accurately about 5 mg of Chlordiazepoxide pure drug into a 100 mL volumetric flask. 70 mL of mobile phase was added then sonicate to dissolve and dilute to volume with mobile phase. Further dilute each 5mL of Solution-A and Solution-B to 50 mL with the mobile phase to get the concentrations of 135 μg/ml and 5 μg/ml, from which different concentrations are prepared according to the linearity range.

**Assay of sample solution:** 10 capsules were weighed and reweighed powder and tablets individually from capsules. Equivalent weight of 5 capsules of sample was taken into a 250 mL volumetric flask. Add 200 mL of mobile phase, sonicate to dissolve and dilute to volume with mobile phase. Filter through 0.45 μ Nylon syringe filter. Further dilute 5 mL to 100 mL with the mobile phase to get the concentrations of 135 μg/ml of Mebeverine and 5 μg/ml of Chlordiazepoxide respectively. Both Standard and sample solutions were injected into injection system and chromatograms were recorded.

### Method Validation

The validation of RP-HPLC method for the determination of Mebeverine and Chlordiazepoxide as per the protocol and to demonstrate that the method is appropriate for its intended use was studied for the following parameters. All the validation parameters were carried out according to ICH guide lines.

### Specificity

Specificity of an analytical method is ability to measure specifically the analyte of interest without interference from blank and known impurities. For this purpose blank chromatogram, standard chromatogram and sample chromatogram were recorded, at the retention times of drugs which confirm the response of drugs was specific. The specificity parameters were given.

### Accuracy

The accuracy parameter was carried out by the standard addition method at 80%, 100% and 120% levels of linearity and the recoveries obtained were given.

### Precision

The precision were checked by repeatedly injecting (n=6) solutions of 135 μg/mL Mebeverine and 5 μg/mL Chlordiazepoxide in combination.

### Intermediate precision (Reproducibility)

The precision study includes intraday and inter day of the proposed methods were determined by the corresponding responses three times on the same day and on three different days over a period of one week for three different concentration of 135 μg/mL Mebeverine and 5 μg/mL Chlordiazepoxide .

The % RSD values were low for Mebeverine and Chlordiazepoxide which reveal that the proposed method was precise.

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

The limit of detection (LOD) limit of quantification (LOQ) of the drug carry was calculated from the linearity curve using the following equation as per international conference harmonization (ICH) guidelines.

$$\text{LOD} = 3.3 \times \sigma / S$$

$$\text{LOQ} = 10 \times \sigma / S$$

$\sigma$  = standard deviation

S = slope

### Linearity and Range

Linearity of an analytical method is its ability to elicit the test results that are either directly or by a defined mathematical transformation which should be proportional to the analyte concentration in sample within a given range. Linear correlation was obtained between peak area vs. concentration of Mebeverine and Chlordiazepoxide were in the range of 27-216 μg/mL and 1.8-7.4 μg/mL .The linearity of the calibration curve was validated by the high value of correlation coefficient of regression equation and the results were given.

The analytical method shows the range which is the interval between the upper and lower levels of analyte (including these levels) that have been demonstrated with precision, accuracy and linearity.

### Robustness and Ruggedness

Robustness of the method was determined by carrying out the analysis at two different pH of mobile phase (i.e. 7.0 ± 0.5) and three different flow rates (i.e. 1 ± 0.2 mL/min)

The high % RSD values of robustness and for Mebeverine and Chlordiazepoxide with change in flow rate indicates that the method is not robust for change in flow rate. The low % RSD values of robustness for Mebeverine and Chlordiazepoxide with change in P<sup>H</sup> and Flow rate reveal that the proposed experimental method was robust.

Ruggedness of the method was determined by carrying out the analysis by two different analysts and the respective peak areas were noted. The result was indicated by % RSD (Figures 1-5).

## Stability Study

135 µg/ml Mebeverine and 5 µg/ml Chlordiazepoxide was prepared and the stability study was carried out for the standard drug solution at 0 hrs, 6 hrs, 12 hrs, 18 hrs and 24 hrs. The results reveal that the sample solutions are stable and accurate without interference.

## Forced degradation study

Forced degradation studies are also known as testing of stress, decomposition stress studies, forced decomposition studies, etc. Degradation conditions include hydrolytic conditions, oxidative conditions, photolytic conditions, thermal conditions and humidity. The results of degradation study were given in the table.

## Acid degradation

Powder equivalent to 135 mg of Mebeverine and 5 mg of Chlordiazepoxide were weighed accurately and transferred into two separate 100 mL round bottomed flasks, made up the mark with the solvent. Then 5 mL was transferred into 50 mL volumetric flask and diluted with the same solvent. From this 1ml was transfer into 10 mL volumetric flask and 1 mL of 0.1N HCl was added and reflux for 30 min at 60°C. Cooled to room temperature and neutralized with 1mL of 0.1N NaOH and made up the volume with HPLC grade solvent.

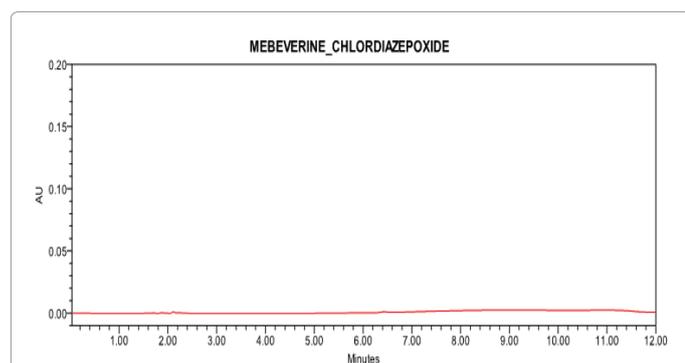


Figure 1: Blank Chromatogram of Mebeverine and Chlordiazepoxide at 262nm

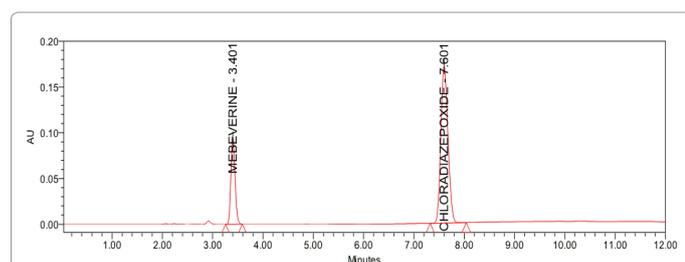


Figure 2: Chromatogram of Mebeverine and Chlordiazepoxide at 262nm in bulk analysis

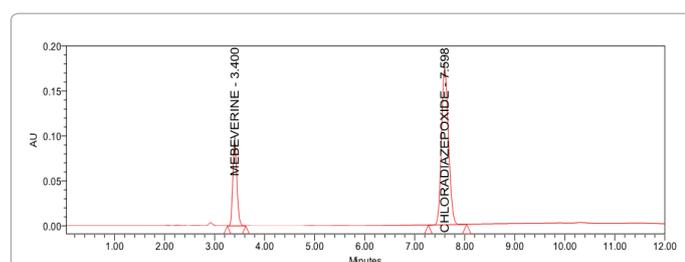


Figure 3: Chromatogram of Mebeverine and Chlordiazepoxide at 262nm in formulation analysis

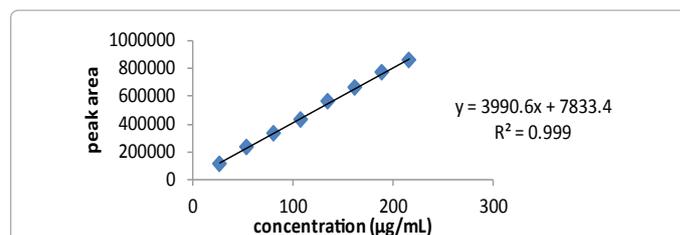


Figure 4: Linearity of Mebeverine: Calibration curve for Mebeverine at 262 nm

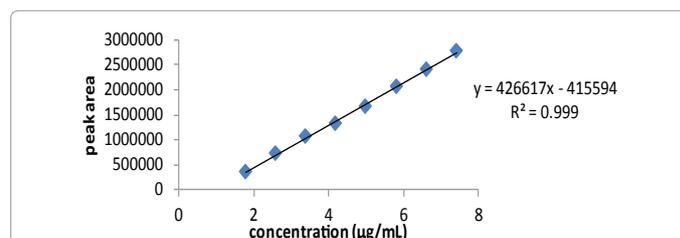


Figure 5: Linearity of Chlordiazepoxide: Calibration curve of Chlordiazepoxide at 262nm

## Base degradation

1ml (135 µg/mL and 5 µg/mL) of above solution of Mebeverine and Chlordiazepoxide were transferred into 10 mL volumetric flask and 1 ml of 0.1N NaOH was added and reflux for 30 min at 60°C. Cooled to room temperature and neutralized with 1mL of 0.1N HCl and made up the volume with HPLC grade water solvent.

## Peroxide

1ml (135 µg/mL and 5 µg/mL) of above solution of Mebeverine and Chlordiazepoxide were transferred to 10mL volumetric flask and 1mL of 3%v/v of H<sub>2</sub>O<sub>2</sub> was added and reflux for 30 min at 60°C. Cooled to room temperature and made up the volume with HPLC grade solvent.

## Thermal degradation

Weigh accurately 20 capsules and crush the tablets in it into fine powder and transfer capsule powder and tablet powder into two separate petridishes. Heat the samples in oven for about 6 hrs at 105°C. From this weigh accurately 100 mg of powdered sample into a 100 ml volumetric flask dissolve and dilute to volume with HPLC grade water. Transfer 1ml of above stock solution to 10 ml volumetric flask and filter the solution using 0.45 µ Nylon filter.

## Photolytic degradation

Photolytic degradation study was carried out by exposing the accurately weighed tablet and capsule powder to UV light in a photolytic chamber at 2600 lux for 24 hr, after 24 hrs weigh accurately 1522 mg of powdered sample into a 100 ml volumetric flask. Dissolve and dilute to volume with HPLC grade water. Transfer 1ml of above stock solution to 10 ml volumetric flask and filter the solution using 0.45 µ Nylon filter. Using the peak purity test, the purity of the drugs peaks were checked at every stage of above-mentioned studies (Tables 1-11).

## Discussion

The present study was carried out in order to develop a sensitive and accurate stability indicating RP-HPLC method for the simultaneous analysis of Mebeverine and Chlordiazepoxide in pharmaceutical dosage forms. The mobile phase consists of 40:60 ratio of methanol and Tri ethyl amine buffer pH 7.0 (v/v) on Agilent C18

Drug	Avgstd area	Avg sample area	Avgwt of tab. (mg)	Stdwt (mg)	Sample wt(mg)	Label amount (mg)	Std purity	Amount found (mg)	% assay
Chlordiazepoxide	1677568	1698788	327.5	5	5.05	5	100.3	5.05	100.3
Mebeverine	5436	541787	327.5	136.5	136.5	135	100.1	136.24	100.1

**Table 1:** Results of assay for formulation

S.No	Drug	Retention time(min)	Plate count	Tailing factor	Resolution
1	Mebeverine	3.400	7676	1.146	19.84
2	Chlordiazepoxide	7.598	13186	1.14	
Acceptance criteria			>2000	< 2	>1.5

**Table 2:** System suitability parameters for Mebeverine and Chlordiazepoxide

S. NO	% Level of Standard	Conc. Of working std. Added (µg/ml)	Peak area	Amount recovered	% recovery	Mean recovery	% R.S.D
1	80	40.5+67.5	427826	108.006	100.000	99.993	0.023
			427759				
			427965				
2	100	67.5+67.5	556811	134.94	99.97	99.993	0.023
			556620				
			556526				
3	150	94.5+67.5	662275	162.026	100.01	99.993	0.023
			662548				
			662324				

**Table 3:** Accuracy results of Mebeverine by RP-HPLC method

S. NO	% Level of Standard	Conc. Of working std. Added (µg/ml)	Peak area	Amount recovered	% recovery	Mean recovery	% R.S.D
1	80	1.35+2.25	1330215	3.6001	100.004	99.994	0.0072
			1330152				
			1330457				
2	100	2.25+2.25	1739493	4.49	99.99	99.994	0.0072
			1739265				
			1739350				
3	150	3.15+2.25	2065927	5.39	99.995	99.994	0.0072
			2065795				
			2065802				

**Table 4:** Accuracy results of Chlordiazepoxide by RP-HPLC method

S.No	Chlordiazepoxide		Mebeverine		
	Conc.(µg/ml)	Peak area	Conc.(µg/ml)	Peak area	
1	0.9	357120	27	113631	
2	1.8	719553	54	229332	
3	2.7	1058676	81	324241	
4	3.6	1330215	108	427826	
5	4.5	1739493	135	556811	
6	5.4	2065927	162	662275	
7	6.3	2404976	189	771203	
8	7.2	2768594	216	856249	
Regression equation		$y = 381324x + 10048$		$y = 4010x + 4873.7$	
Slope		381324		4010	
Intercept		10048		4873.7	
R <sup>2</sup>		0.999		0.999	

**Table 5:** Linearity for Mebeverine and Chlordiazepoxide at 262 nm

S.NO	TYPE	Chlordiazepoxide			Mebeverine		
		Mean area	Std. deviation	% RSD	Mean area	Std. deviation	% RSD
1	System precision	1677620	4429.59	0.264	543735	1894.68	0.348
2	Method precision	1673928	5814.12	0.347	541422	2780	0.513
3	Intermediate precision	1669300.6	8412.36	0.503	537365.8	2534.03	00047

**Table 6:** Precision studies by RP-HPLC method

s.no	Parameter	Mebeverine	Chlordiazepoxide	Limit
1	% RSD	0.348	0.264	NMR 2.0%

**Table 7:** Results of Ruggedness study by RP-HPLC

Variations	Chlordiazepoxide				Mebeverine			
	Retention time	Peak area	Plate count	% RSD	Retention time	Peak area	Plate count	% RSD
P <sup>H</sup> =6.5	7.165	1757140	17902	0.32	3.298	561797	8790	0.1
P <sup>H</sup> =7.5	9.24	2185148	16317	0.11	4.23	697128	9395	0.11
Flow rate 1.1mL/min	6.44	1446089	11260	0.21	2.84	465104	6662	0.26
Flow rate 0.9ml/min	9.24	218514	16317	0.23	4.23	697128	9395	0.2

**Table 8:** Results of Robustness study by RP-HPLC

Parameter	Chlordiazepoxide		Mebeverine	
	µg/mL	Area	µg/mL	Area
LOD	0.01	3968	2.2	9258
LOQ	0.03	11904	6.5	27355

**Table 9:** Sensitivity parameters (LOD & LOQ) by RP-HPLC

Time period (hours)	Chlordiazepoxide				Mebeverine				Resolution
	Retention time	Peak area	Tailing factor	Plate count	Retention time	Peak area	Tailing factor	Plate count	
6	7.595	1672263	1.16	12575	3.4	531947	1.12	7525	19.30
12	7.598	1662418	1.16	11832	3.399	534078	1.13	7203	18.82
18	7.606	1677732	1.13	13054	3.402	544222	1.14	7587	19.67
24	7.604	1674413	1.13	13500	3.402	541523	1.14	7881	19.98

**Table 10:** Results of stability study for Mebeverine and Chlordiazepoxide

Degradation	Sample	Chlordiazepoxide			Mebeverine		
		Mean area	% label claim	% degradation	Mean area	% label claim	% degradation
Control	1652.8	1693405	100	0	542695	100.3	-0.3
Acid	1849.2	1501905	79.3	20.7	483645	79.9	20.4
Alkali	1542.7	1158534	79.3	26.7	372786	73.8	26.5
Peroxide	1582.9	1167374	72	28	374187	72.2	28.1
Thermal	1510.9	1157940	74.8	25.2	373570	75.5	24.8
Photo	1522.3	1139725	73.1	26.9	366961	73.6	26.7

**Table 11:** Results of degradation study for Mebeverine and Chlordiazepoxide

column (240× 4.6 mm, 5 µm) analytical column was used to effect the separation of drugs and reference standard under isocratic conditions and to produce good resolution and free from tailing and fronting. Mebeverine and Chlordiazepoxide retention times were obtained at 3.40min and 7.59min. From the specificity chromatograms it was clarified that the peaks of pure drug and sample were not showing any interferences by comparing the blank chromatogram. In order to test the linearity of the method, dilutions of the working standard solutions of drugs were prepared in the range of 27-216 µg/mL for Mebeverine and 1.8-7.4 µg/mL for Chlordiazepoxide. A good linear relationship ( $r^2=0.99$ ) was observed between the concentrations of Mebeverine and Chlordiazepoxide and the corresponding peak areas. The method was duly validated by evaluation of the required parameters as per ICH guidelines. The system suitability parameters were within the limits as shown. The proposed method was found to be precise as the %RSD values for intra-day and inter-day were found to be less than 2%. The recoveries of Mebeverine and Chlordiazepoxide obtained from the pre-analyzed samples containing known amounts of added drug were shown which were within the acceptable range indicating the high accuracy of the proposed method. Robustness of the method was found out by testing the effect of small deliberate changes in the chromatographic conditions and the areas of corresponding peaks. The main factors selected in this method were the flow rate ( $\pm 0.2$ ) and the detection P<sup>H</sup> (0.5) and the results were recorded. LOD and LOQ of the method were calculated basing on standard deviation of the response and the

slope(s) of the calibration curve and the values for the proposed HPLC method were within the limits. The drug content in the formulation was quantified using the proposed method of analysis and the mean amount of Mebeverine and Chlordiazepoxide obtained in dosage form were in the range of 98%-102%. Stability study results and degradation results were recorded. The results shows that the drugs were degraded more by peroxide treatment and also reveals the degradation pathways by treating the drug with acid, alkali, peroxide, heat and light. All these results reveal that the method was stable without any interference, accurate, precise, less time consuming and economical.

## Conclusion

A RP HPLC method was developed and applied stress studies which explains that the method was stable, simple, accurate and sensitive for the determination of Mebeverine and Chlordiazepoxide in combined dosage form.

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