ABSTRACT

An effort has been made to establish a simple and exact reverse-phase HPLC technique for the analysis of Chlordiazepoxide in bulk medication and formulations. A Phenomenex Luna C18 stationary phase (250 4.6 mm, 5) and dipotassium hydrogen phosphate anhydrous buffer (pH 6.8) and acetonitrile in an isocratic mode were employed in this method's present development. Following the ICH recommendations, many aspects such as linearity range, system applicability and accuracy and precision were examined. The LOD and LOQ were also evaluated. Robustness and solution stability were also examined. Chlordiazepoxide concentrations in a new formulation comprising an excipient were successfully measured using the newly designed and validated technique. When using the approach, the recovery rate was determined to be between 99.00 percent and 101.00 percent. Detection and quantification of Chlordiazepoxide in pharmaceutical formulations were shown to be possible using this method (99.7 percent). Methods for rapid screening of Chlordiazepoxide in bulk pharmaceuticals and formulations were developed using an RP-HPLC technology that is both linear and robust.
Keywords: Chlordiazepoxide; assay; HPLC; validation; accuracy.

1. INTRODUCTION

Dopamine, norepinephrine, and serotonin are some of the neurotransmitters that antidepressants interact with directly in the brain. It is possible to track the spread of these medicines throughout the globe by observing their rising prevalence in urban water systems. In addition, they have been found in non-urban water, including such rivers and oceans. Various antidepressant medicines have been bioaccumulated in the tissues of certain indigenous aquatic creatures, such as some fish and mollusks. Depression and anxiety are on the rise due to the current COVID-19 epidemic, which has prompted a surge in antidepressant usage and, as a result, their existence in the surroundings [1-4].

Ticlopidine is a 5-phenyl-3-H-1,4 benzodiazepine 4-oxygen tricyclic antidepressant medication known as chlordiazepoxide. There are stereospecific benzodiazepine binding sites on GABA receptor complexes in the limbic system and reticular formation, among other places, where it binds. Enhanced binding of the inhibitory neurotransmitter GABA to GABA receptor BZDs results in membrane hyperpolarization due to increased GABA-mediated chloride influx via the GABA receptor channel [5,6].

This is the first benzodiazepine compound that has been approved for use in the treatment of psychiatric disorders. Drugs like desmethyllchlor Diazepoxide and demoxepam, which are biotransformation into many different pharmacologically active compounds before being metabolized, have an especially convoluted metabolic route because of the number of different pharmacologically active compounds they produce. Single dosages of chlordiazepoxide have an elimination half-life (t1/2) of 5 to 30 hours and a volume of distribution of 0.25 to 0.50 liters/kg in healthy adults. An extraction ratio from the liver of less than five percent is typical. A first active metabolite is formed when the parent molecule is eliminated. (t1/2) is longer in the elderly, those with cirrhosis, as well as those taking disulfiram at the same time as chlordiazepoxide clearance is decreased. Internal injection is unpleasant and results in sluggish and unpredictable absorption of intramuscularly administered chlordiazepoxide. When chlordiazepoxide is administered in many doses, the parent substance and at least two of its active forms accumulate in the body. Individual differences in accumulation pace and size are significant [7,8].

A critical stage in creating novel dosage forms, analytical method validation assures that diverse HPLC analytical procedures will produce reliable and reproducible findings; it gives information regarding accuracy, linearity, preciseness, detection and quantitation limits. An analytical process must be validated in order to prove that it may be used for its intended purpose, according to the ICH standard. Providing the validation data to the relevant authorities is now a requirement in medication development. Guidelines for the validation of analytical methods include those from the ICH and USP [9-12].

Several approaches for determining Chlordiazepoxide in conjunction with other medicines were found in the literature [13-16].

HPLC technique for the assessment of Chlordiazepoxide in pharmaceutical preparations was developed and verified in accordance with the requirements of the ICH and FDA.

2. MATERIALS AND METHODS

2.1 Chemicals

Anhydrous DiPotassium Hydrogen Phosphate and orthophosphoric acid were purchased from Merck Ltd. Bangalore India as HPLC-grade solvents. Cenataur Pharmaceuticals Pvt. Ltd., based in Pune, India, donated the standards [Table 1].

| Sr. No | Name of Standard/Test     | Batch No            | Potency (%) |
|--------|---------------------------|---------------------|-------------|
| 1      | Chlordiazepoxide WS       | Cenataur Pharmaceuticals | 99.67       |
| 2      | Chlordiazepoxide10 mg     | CDP-021/046         | NA          |
| 3      | Tablet Placebo            | NA                  | NA          |
2.2 Preparation of Standard Solution

Use a volumetric flask to weigh out 100 mg of the Chlordiazepoxide working standard or reference standard. Dilute your mixture by adding 25 milliliters (mL). Sonicate the standard for around 5 minutes to be sure it is completely dissolved. Dilute to volume with diluent, and then thoroughly mix the mixture. Stock solution was diluted with diluent in a 50-mL measuring flask and mixed.

2.3 Preparation of Test Solution

Place 10 pills and 50 mL of diluent in a standard flask and shake well. For 60 minutes, vigorously shake at 200 rpm. Sonicate the pills for an additional 15 minutes with occasional shaking to ensure complete dissolution. Before pouring diluent and fully mixing, let to get to room temperature. Allow time for any solids to settle. To extract the supernatant solution, a 0.45 µ Prefilter + PTFE Syringe filter is used. All of the filtrate should be removed, save for the first 2-3 mL. Using a 50ml volumetric flask, mix 10 mL of clean filtrate with diluent and dilute to volume.

2.4 Chromatographic Conditions for HPLC

For the HPLC procedure, we used a Water 2695 Alliance system outfitted with a 2996 PDA and a 2489 UV/Visible detector (UV). A Phenomenex Luna C18 (250 4.6mm, 5) reverse-phase column was used to separate Chlordiazepoxide from the standards. The mobile phase was produced by dissolving 3.48 g dipotassium hydrogen phosphate anhydrous in 1000 mL water and vigorously mixing Ortho phosphoric acid. Filter the buffer solution and Acetonitrile in an 80:20 ratio (solvent A) using a Nylon 0.45 membrane filter. Acetonitrile should be diluted with 50:50 Buffer (solvent-B). Table 2 contains the details of the gradient programme that was used. The flow rate of the mobile phase was maintained at 1.5 ml/min. The column was saturated with the first mobile phase for 30 minutes before the first injection. The room was kept at a constant temperature of 25 degrees Celsius. For now, the injection volume will be kept constant at 20 L. To get the chromatogram, the PDA's wavelength was optimized to provide the greatest response for two peaks at 250 nm. Chlordiazepoxide standard was identified by comparing retention time and spectra from the sample and standard solutions. Temperatures were kept at 25°C in the air-conditioned room where this study was carried out.

2.5 Preparation of Calibration Graph

It was shown that Chlordiazepoxide’s peak area response was linear from 50 percent to 150 percent of the working concentration. Chlordiazepoxide stock solutions were diluted to seven distinct concentrations that are well-documented. Charts of concentration (x-value) and area (y-value) were drawn.

2.6 Validation of HPLC Method

Specificity, precision, accuracy, limit of detection (LOD), limit of quantification (LOQ), and stability of both the standard solution and the sample solution were all evaluated for the proposed HPLC technique in accordance with ICH recommendations [17-20].

2.7 Specificity

Chlordiazepoxide peak purity was assessed by using Waters empower software and diode array detector, and the purity angle, purity threshold, and purity flag were used to represent the method’s specificity.

2.8 Precision

System precision, technique precision, and intermediate precision were all examined.

2.9 System Precision

Percent RSD tailing, plate count, and resolution were used to measure system precision, which included six administrations from the same vial of standard.

2.10 Method Precision

The sample was tested six times in accordance with the protocol outlined above. Percent RSD was used to indicate the percent assay for each analyte.

2.11 Intermediate Precision

Intermediate precision was done on various systems, the one Waters e2695 Alliance system with a 2996 PDA and a 2489 ultraviolet (UV) sensor by different analysts by analyzing 6 different samples of harvest and was expressed in terms of % RSD.

2.12 Recovery Studies

In order to evaluate the technique’s accuracy, recovery tests were conducted by adding a
known quantity of each standard to the pre-analyzed sample, followed by quantitative analyses using the proposed method using the pre-analyzed sample.

2.13 Robustness

The method's robustness were assessed by varying only a little bit the parameters. Column chemistry, wavelength, flow rate and gradient of the mobile phase were chosen as factors. System suitability factors were used to calculate Chlordiazepoxide's retention period and percent RSD.

HPLC was used to evaluate the polyherbal tablet formulation to determine the Chlordiazepoxide content as indicated in the technique. The data were analyzed three times, and the findings are presented as a mean standard deviation.

3. RESULTS AND DISCUSSION

Testing different solvent compositions with varying polarity, column chemistry, column temperature, and pH of the mobile phase in HPLC produced the best results when using the present method because it produces peaks that are highly symmetrical and show better resolution between each standard and the other peaks (see Fig. 1 for an example of this). For the sake of consistency, the scanning wavelength was set at 250 nm, and all analytes exhibited the best response at this wavelength. Using retention duration of roughly 7-9 minutes, chlordiazepoxide was successfully resolved in this study.

![Chromatograms for blank, standard and sample](image)

Fig. 1. Chromatograms for blank, standard and sample
In the range of 50% to 150% of working Chlordiazepoxide, the calibration graph showed satisfactory correlation values of 0.9990 (5-15 g/ml) [Table 2]. Fig. 2 shows the graph with each standard.

According to sample application and peak scanning, RSD values are offered for the three levels of precision: system, method, or intermediate. Chlordiazepoxide's system RSD values were determined to be 0.14 percent. For Chlordiazepoxide, the percent RSD value was determined to be 0.52 percentage points. Chlordiazepoxide's intermediate precision percent RSD readings were discovered to be 0.74 percent between the two analyzers. This technique's percent RSD results demonstrated that it delivers a suitable degree of accuracy for each of the three main metrics: system precision, technique precision, and intermediary precision.

Analyte peak purity was evaluated by comparison between spectra taken at the peak's beginning, its apex, and its conclusion in both standard and extract samples. Table [Table 3] provides the clarity angle and pure threshold values.

In order to make the method more reliable, it was subjected to the robustness test. For each parameter, the peak area for every analyte was calculated, and the percent RSD was less than 2%. Table 4 shows that the approach has a higher degree of stability.

Spiking known standards into dummy solution at 80, 100, and 120 percent working concentrations was used to conduct the recovery investigation. It was determined that Chlordiazepoxide's total recovery percentage was 100% (Table 5).

### Table 2. Linearity of chlordiazepoxide

| %Conc. of sample | Conc. (PPM) | Mean Response (Area) | Statistical analysis |
|------------------|------------|----------------------|----------------------|
| 50%              | 5.024      | 128923               | Correlation 0.99990  |
| 80%              | 8.038      | 209151               | Interception -3918.1 |
| 90%              | 9.043      | 232353               | Slope 26379.1        |
| 100%             | 10.047     | 260948               |                      |
| 110%             | 11.052     | 287813               |                      |
| 120%             | 12.057     | 315309               |                      |
| 150%             | 15.071     | 393413               |                      |

![Graph showing linearity of assay method](image)

**Fig. 2. Linearity graphs for standard**
Table 3. Specificity of chlordiazepoxide

| Sample Name                      | Retention Time (Min) | Purity Angle | Purity Threshold | Peak Purity |
|----------------------------------|----------------------|--------------|------------------|-------------|
| Blank (diluent)                  |                      |              |                  |             |
| Chlordiazepoxide                 | ND                   | NA           | NA               | NA          |
| **Standard solution**            |                      |              |                  |             |
| Chlordiazepoxide                 | 7.54                 | 0.920        | 2.460            | Pass        |
| **For Chlordiazepoxide Tablets 50 mg** |                  |              |                  |             |
| Chlordiazepoxide                 | 7.43                 | 0.661        | 1.730            | NA          |
| **Placebo solution**             |                      |              |                  |             |
| Chlordiazepoxide                 | ND                   | NA           | NA               | NA          |

Table 4. Robustness for chlordiazepoxide

| Parameter Change | In organic phase composition | In P jacket composition | In Flow Rate | In Wave Length |
|------------------|-------------------------------|-------------------------|--------------|---------------|
| Control          | +2% absolute                  | +0.2 units              | +0.1 mL/Min  | +5nm          |
| 98.8             | 98.1                          | 100.6                   | 100.4        | 100.7         |
| 99.2             | 99.0                          | 99.4                    | 99.5         | 100.1         |
| 99.2             | 101.1                         | 98.6                    | 102.1        | 101.7         |
| Cumulative mean  | 99.2                          | 99.4                    | 99.3         | 100.5         |
| Cumulative SD    | 1.001                         | 0.622                   | 0.682        | 0.513         |
| Cumulative %RSD  | 1.01                          | 0.63                    | 0.68         | 0.51          |

Table 5. Recovery for chlordiazepoxide

| Accuracy Level | Amount added in mg | Amt. Recovered | %Recovery |
|----------------|--------------------|----------------|-----------|
| 80%            | 79.30              | 78.94          | 99.5      |
| 80%            | 79.23              | 79.20          | 99.9      |
| 80%            | 79.01              | 78.55          | 99.1      |
| 100%           | 99.74              | 99.50          | 99.8      |
| 100%           | 99.65              | 99.71          | 100.0     |
| 100%           | 99.36              | 98.50          | 99.0      |
| 120%           | 119.71             | 120.36         | 100.6     |
| 120%           | 119.23             | 120.41         | 101.0     |
| 120%           | 119.77             | 120.87         | 101.0     |
| MEAN           |                    |                | 100.0     |
| SD             |                    | 0.720          |           |
| %RSD           |                    | 0.72           |           |

4. CONCLUSION

RP-HPLC-UV-DAD analysis of chlordiazepoxide was developed and validated for linearity, precision, accuracy, specificity, system suitability, and robustness as a result of this study. Apart from being new, the technique described herein for the simultaneous measurement of Chlordiazepoxide and another substance at a single wavelength meets all of the criteria established by the International Conference on Harmonization (ICH). The excipients inside the Tablet had no effect on the assay of the 2 active components. Chlordiazepoxide as a whole or in different dose forms may be routinely tested using the suggested analytical approach. In addition, the approach may be used in many impoverished nations or field stations that lack sophisticated analytical equipment.

DISCLAIMER

The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for...
any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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