



RESEARCH ARTICLE



Received on: 05/03/14
Accepted on: 30/05/14
Published on: 15/06/14

Corresponding Author

R. Ramesh Raju

Department of Chemistry,
Acharya Nagarjuna University,
Nagarjuna Nagar-522 510,
Guntur, Andhra Pradesh, India



QR Code for Mobile users

Conflict of Interest: None Declared!

DOI: 10.15272/ajbps.v4i32.468

Method development and validation of chlordiazepoxide and amitriptyline hydrochloride in pharmaceutical formulations by RP-HPLC

D. Srikantha, R. Ramesh Raju*

Department of Chemistry, Acharya Nagarjuna University, Nagarjuna Nagar-522 510,
Guntur, Andhra Pradesh, India.

Abstract

A simple, precise and rapid, reversed phase-high performance liquid chromatography (RP-HPLC) method is developed for simultaneous determination of chlordiazepoxide and amitriptyline hydrochloride in tablet dosage forms. The HPLC conditions used are methanol: acetonitrile (75:25 v/v) mobile phase, Zodiac C18 column (250mm x 4.6mm x 5 μ m), pump pressure (9.5 MPa) and detection wavelength (221 nm). The measured elution times are 4.87 \pm 0.02 minutes for chlordiazepoxide and 7.38 \pm 0.02 minutes for amitriptyline hydrochloride, respectively. The method is validated for linearity range, recovery, robustness and sensitivity. Linearity is demonstrated for chlordiazepoxide and amitriptyline hydrochloride in the range 5-30 μ g/mL ($r_2 = 0.9998$) and 15-75 μ g/ml ($r_2 = 0.9997$). The mean recoveries ranged from 98.81-100.63% and 98.97-101.33% and the limit of detections (LOD) are 0.15 μ g/ml and 0.5 μ g/ml for chlordiazepoxide and amitriptyline hydrochloride, respectively. The proposed method proved to be specific, robust and accurate and has good signal to noise ratio with well resolved peaks for unambiguous determination of chlordiazepoxide and amitriptyline hydrochloride combination and in tablet dosage form.

Keywords: Chlordiazepoxide; Amitriptyline hydrochloride; RP-HPLC; Tablet dosage form; UV detection.

Cite this article as:

D. Srikantha, R. Ramesh Raju. Method development and validation of chlordiazepoxide and amitriptyline hydrochloride in pharmaceutical formulations by RP-HPLC. Asian Journal of Biomedical and Pharmaceutical Sciences; 04 (32); 2014. 8-14.

Introduction:

Benzodiazepines are being widely used for the treatment of anxiety and other related disorders. Sometimes these are also administered to patients along with tricyclic antidepressants (TCAs) as they both act on the central nervous system (CNS). Chlordiazepoxide, the first benzodiazepine developed in the 1950s is an anxiolytic benzodiazepine derivative, with anticonvulsant, sedative and amnesic properties^[1]. Its IUPAC name is 7-chloro-N-methyl-5-phenyl-3H-1, 4-benzodiazepin-2-amine-4-oxide. Its molecular formula is $C_{16}H_{14}ClN_3O$ and its molecular weight is 299.8 g/mol^[2-4]. It has also been used in the symptomatic treatment of alcohol withdrawal and for the management of anxiety disorders.

Depression, a disorder, is alarmingly on rise among the world population. For the treatment of depression, in general, tricyclic antidepressants (TCAs) are prescribed. These are one of the oldest classes of antidepressants that are still in extensive use. These antidepressants act on nerve cells in the CNS. Amitriptyline hydrochloride belongs to the class of tricyclic antidepressants and its IUPAC name is 3-(10, 11-Dihydro-5H-dibenzo [a,d] cyclohepten-5-ylidene)-N, N-dimethyl-1- propanamine. Its molecular formula is $C_{20}H_{23}N$, HCl and molecular weight is 313.9 g/mol^[2-4]. Its function is to prevent serotonin and noradrenaline from being reabsorbed back into the nerve cells in the CNS. Thus it aids to relieve depression. It is sometimes prescribed in combination with benzodiazepines in treating depression in people who are also anxious and agitated, or who are suffering from disturbances in sleep.

Literature survey reveals that various methods have been used for the determination chlordiazepoxide separately and in combination with other drugs. Chlordiazepoxide and its metabolites are determined by high performance liquid chromatography (HPLC) in human plasma^[5-7], human serum and in urine^[8] and along with its impurities^[9]. Methods like non-aqueous titration and potentiometry^[2-4] also have been demonstrated.

Similarly, amitriptyline hydrochloride has been studied as a single drug and in combination with other drugs by different techniques. It is measured in human whole blood along with its metabolites^[9], plasma^[10] and biofluids^[11]. To mention, non-aqueous titration, titrimetry and HPLC^[2-4,12], voltametry^[13], conductometry^[14], HPLC without organic solvent^[15], spectrophotometry^[19] and gas chromatography^[21] methods are demonstrated.

Furthermore, spectrophotometry^[16-19], first derivative spectrophotometry^[19,20], gas chromatography^[21], HPLC^[19,20] and high performance thin layer chromatography (HPTLC^[19]) are demonstrated for amitriptyline hydrochloride and chlordiazepoxide combination. So far, only two studies are reported for these drugs combination to the best of our knowledge^[19, 20]. This necessitates more detailed investigations for quantitative estimation of these two drugs.

In this paper, we report an improved simple and rapid method for simultaneous quantitative determination of chlordiazepoxide and amitriptyline hydrochloride in pharmaceutical formulations using RP-HPLC method. The developed method is validated following ICH guidelines^[22, 23] by checking the parameters like linearity, recovery, robustness and sensitivity. The proposed method enables quicker determination of antidepressants and benzodiazepines, which are used in combination.

Materials and methods:

The HPLC grade chemicals (methanol, water, acetonitrile) used in the present study are purchased from Merck Specialties private Limited, Mumbai, India. HPLC instrumentation

The instrument used to develop a high performance liquid chromatographic (HPLC) method for quantitative estimation of chlordiazepoxide and amitriptyline hydrochloride simultaneously is a PEAK HPLC system and it is operated in isocratic mode. The stationary phase is a Zodiac C_{18} column (250 mm x 4.6 mm, 5 μ m particles). It is equipped with a LC 20AT pump for delivery of solvent and a variable wavelength programmable LC – 7000 ultraviolet (UV)-detector. For injecting the samples a 20 μ L Rheodyne injection port is utilized. The experiments are performed at room temperature. The data is recorded using PEAK software and the obtained results are analyzed with Microsoft Excel. The maximum absorption wavelength of chlordiazepoxide and amitriptyline hydrochloride is determined by an ultraviolet (UV)-Visible Techcomp 230D6 spectrophotometer with HITACHI software. The degassing of the mobile phase is done by an ultrasonicator bath (Loba) and for weighing the materials a Denver (SI234) balance is used.

Preparation of standard solution

Chlordiazepoxide and amitriptyline hydrochloride drugs of 10 mg each are weighed and are dissolved in 10 mL of methanol separately in two different volumetric flasks. Then they are stirred in ultrasonicator for 2-5 minutes to dissolve completely.

The solutions are filtered through a 0.45 μm nylon membrane ultipore filter paper. A 1000 $\mu\text{g}/\text{mL}$ solution is prepared with proper dilution. From this 2 mL is further diluted to 20 mL to get a stock concentration of 100 $\mu\text{g}/\text{mL}$ solution. Thus two standard drug solutions with concentration 100 $\mu\text{g}/\text{mL}$ of chlordiazepoxide and 100 $\mu\text{g}/\text{mL}$ of amitriptyline hydrochloride are obtained. From this required concentrations are prepared by selective dilution.

Method development

The RP-HPLC method development started with preparation of suitable mobile phase and its composition, pH values, flow rate and detection wavelength. The pure drug of chlordiazepoxide and amitriptyline hydrochloride is injected into the HPLC system and run in different solvent systems. Different mobile phase solvents that are widely used like methanol, water and acetonitrile and their combinations are tried in order to find the best conditions for chlordiazepoxide and amitriptyline hydrochloride separation. It is observed that organic solvents methanol and acetonitrile gave satisfactory results compared to other combinations. The methanol and acetonitrile mobile phase system is tried with different proportions and with different flow rates to improve the peak shape, signal to noise ratio and to minimize the shift of the baseline.

Finally, the optimal condition of the mobile phase is chosen as methanol and acetonitrile in the ratio 75:25 v/v. This composition of the mobile phase resolved the two drugs very well. The mobile phase and samples are degassed by ultra-sonification for few minutes and then filtered with 0.45 μm ultipore filter paper. All the measurements are performed at constant temperature of the column. The pH value of the final mobile phase composition is 5.1 without the use of any buffer. The flow rate is set to 1.2 mL/minute after trying different flow rates. After several trials, elutes runtime is set to 12 minutes assuring no interferences as well as influence from the excipients.

Optimal Chromatographic conditions

The active pharmaceutical ingredient (API) concentrations used for the validation measurements for chlordiazepoxide is 15 $\mu\text{g}/\text{ml}$ and for amitriptyline hydrochloride is 37.5 $\mu\text{g}/\text{ml}$, respectively. These concentrations are chosen as the standard concentration because they are the optimum from the linearity measurements, Beer-Lambert's law and they also fall in the middle of the used concentration range. The remaining finally recommended optimized conditions are: pH - 5.1; detection wavelength - 221 nm; mobile phase-methanol: acetonitrile (75 : 25 v/v); Zodiac C₁₈ column (250 mm x 4.6 mm, 5 μm); elution isocratic mode, flow rate - 1.2 ml/min; runtime - 12 minutes and the pump pressure - 9.5 \pm 0.6 MPa.

Results and Discussion:

The absorption wavelength for chlordiazepoxide and amitriptyline hydrochloride is determined after several trials. The absorbance spectra of the diluted standard and working solutions of chlordiazepoxide and amitriptyline hydrochloride in methanol are recorded on a UV spectrophotometer. They are scanned in the wavelength 200 nm - 400 nm range using quartz cuvettes with 10 mm path length. The data is obtained as absorbance and it is shown in fig. 1 with wavelength (nm) as x-axis and absorbance (abs. units) as y-axis. The maximum absorption wavelength is observed at 221 nm for the two drugs. This is in good agreement with the reported wavelengths for these drugs combination within ten percent by^[20].

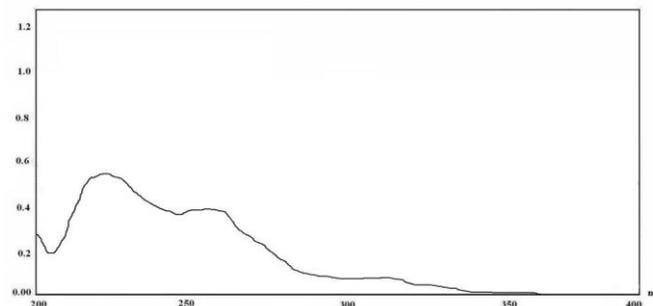


Figure 1: Absorption spectrum of chlordiazepoxide and amitriptyline hydrochloride. Maximum absorption wavelength is observed at 221 nm for the two drugs.

Method validation

After development of the proposed method, it is validated by measuring various parameters satisfying the ICH guidelines^[22]. The specificity of the developed RP-HPLC method is validated by complete separation of chlordiazepoxide and amitriptyline hydrochloride drugs by measuring the parameters retention time, tailing factor (t_r) and resolution. The measured peaks for both the drugs are sharper, have good signal to noise ratio and are well separated for a run time of 12 minutes. The chromatogram of the two drugs is shown in fig. 2 and the measured resolution for both drugs is 13.44. The retention times are 4.87 minutes for chlordiazepoxide and 7.38 minutes for amitriptyline hydrochloride, respectively. The retention times obtained are less than the reported values in^[20]. The tailing factor for chlordiazepoxide is 1.08 and for amitriptyline hydrochloride is 1.23, which fall in the range of the ICH guidelines (Table 1). The resolution reported here is better than the value reported using high performance thin layer chromatography (HPTLC) method^[20]. In addition, the specificity of the two drugs is also determined in commercial tablets where no interference and influence from other excipients is observed.

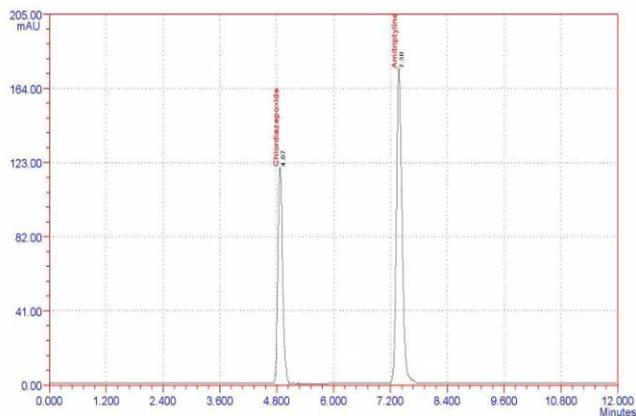


Figure 2: Chromatogram of chlordiazepoxide and amitriptyline hydrochloride standards. The retention times are 4.87 and 7.38 minutes, respectively.

Linearity

For chlordiazepoxide, the linearity is measured in the range 5-30 $\mu\text{g/ml}$ (in 5 $\mu\text{g/ml}$ steps). By plotting the peak areas as y-axis and the corresponding six concentrations ($\mu\text{g/ml}$) as x-axis the calibration graph is constructed. The peak areas are normalized to the 30 $\mu\text{g/ml}$ peak area for chlordiazepoxide and to the 75 $\mu\text{g/ml}$ peak area for amitriptyline hydrochloride, respectively. The plotted data is fitted using a linear regression of the form $y = mx + c$, where, 'm' slope of the curve and 'c' the intercept. The slope, $m = 0.0323 \pm 2.3 \times 10^{-4}$, intercept, $c = 0.0289 \pm 0.0044$ and the evaluated correlation coefficient for chlordiazepoxide is $r^2 = 0.9998 \pm 0.0047$. Similarly, the linearity of the peak areas is determined for six different concentrations of amitriptyline hydrochloride in the range 15-75 $\mu\text{g/mL}$ (15 $\mu\text{g/mL}$ steps). The data fitted to the linear function is found to be precise with a correlation, $r^2 = 0.9997 \pm 0.0066$, $m = 0.0135 \pm 1.26 \times 10^{-4}$ and $c = -0.0149 \pm 0.0061$. The linearity results for chlordiazepoxide and amitriptyline hydrochloride are given in Table 1 along with other validation parameters. The concentrations range studied for the two drugs showed no significant changes in the slope and intercept. The student t-test was performed for chlordiazepoxide and amitriptyline hydrochloride drugs. The respective t-values are 2.43 for amitriptyline hydrochloride and 6.54 for chlordiazepoxide at the 95% confidence interval.

Limit of Detection (LOD) and Quantification (LOQ)

The parameters limit of detection (LOD) and limit of quantification (LOQ) are calculated from the calibration equations from the limits based on the coefficient of variation and the slope. These parameters are checked in determining the sensitivity of the method developed for the analysis of the sample. These are calculated on the criteria $\text{LOQ} = 3.3\text{LOD}$, respectively. The detection limit, taken as the lowest absolute concentration of the analyzed in the sample. This can be detected but cannot be quantified under the stated experimental conditions.

The LOD and LOQ for chlordiazepoxide are 0.15 $\mu\text{g/ml}$ and 0.5 $\mu\text{g/ml}$, which are higher than the values reported in^[20]. Similarly, for amitriptyline hydrochloride, 0.5 $\mu\text{g/ml}$ is the LOD and 1.5 $\mu\text{g/ml}$ is the LOQ. These are also higher than the values reported in^[20].

Parameter	Chlordiazepoxide	Amitriptyline hydrochloride	Recommended values
Linearity range ($\mu\text{g/ml}$)	5-30	15-75	---
Correlation coefficient	0.9998 ± 0.00474	0.9997 ± 0.0066	>0.999
LOD ($\mu\text{g/ml}$)	0.15	0.5	---
LOQ ($\mu\text{g/ml}$)	0.5	1.5	---
Recovery (%)	98.97-101.33	98.81-100.63	80-110%
Robustness	Robust	Robust	Robust
Retention time (minutes)	7.38	4.87	---
Resolution		13.44	---
Theoretical Plates	20926	13351	>2500
Tailing factor (t_r)	1.08	1.23	$0.8 \leq t_r \leq 1.5$

Table 1: Summary of some validation parameters, system suitability parameters and ICH guide lines.

Precision

For chlordiazepoxide and amitriptyline hydrochloride drugs, precision of the method is validated through the intra-day and inter-day measurements. The repeatability of the two drugs for the application and for the measurement is studied with six samples each. Repeatability application is evaluated by measuring the peak area for each sample. For both intra-day and inter-day precision studies 37.5 $\mu\text{g/ml}$ of chlordiazepoxide and 15 $\mu\text{g/ml}$ of amitriptyline hydrochloride concentration is used. Intra-day precision is determined by analysis of standard solutions 3 times on the same day. For three consecutive days inter-day precision studies of the two drugs peak area variations are determined. The data obtained from intra-day and inter-day measurements are given in Table 2. The peak areas of six different samples ($n = 6$) for both the drugs are expressed in terms of mean of normalized area, standard deviation (SD) and their relative standard deviation/coefficient of variance (%RSD/CV). The peak areas are normalized with the peak area of one of the sample and then the mean is taken. The corresponding standard deviation (SD) and the relative standard deviation (RSD) are also given. The RSD of all the samples from intra-day (inter-day) measurements for chlordiazepoxide is 0.83(0.33) and for amitriptyline hydrochloride is 1.83(0.63),

which is less than 2%, respectively. These fulfill the ICH guidelines and hence the method can be said precise. The method is also validated for ruggedness for three samples each of chlordiazepoxide and amitriptyline hydrochloride drugs by two analysts. The results are presented in Table 2 with peak areas shown as mean of the normalized peak areas, their SD and the %RSD/CV. The less than 2% RSD obtained indicates the measurements satisfy the ICH guidelines for the drugs in combination.

Parameter	Chlordiazepoxide		Amitriptyline hydrochloride		Recommended values
	Normalized Area Mean SD (n=6)	%RSD	Normalized Area Mean SD (n=6)	%RSD	
Intra-day precision	0.99 ± 0.06	0.83	0.995 ± 0.003	± 1.83	<2%
Inter-day precision	1.08 ± 0.11	0.33	1.016 ± 0.01	0.63	<2%
Ruggedness	1.01 ± 0.01	1.0	0.99 ± 0.01	1.1	<2%

Table 2: Intra-day, inter-day precision and ruggedness results for chlordiazepoxide and amitriptyline hydrochloride.

Robustness

The robustness of the method is verified for chlordiazepoxide and amitriptyline hydrochloride drugs by deliberate changes made to parameters mobile phase volume ratio, pH of the solution and to the wavelength of detection. The results of robustness studies for the two drugs are given in Table 3 along with percentage change in peak areas with respect to the peak area of the standard solution. Concentration of chlordiazepoxide and amitriptyline hydrochloride solutions used for these studies is 15 µg/ml and 37.5 µg/ml, respectively. The effect of the changes in peak area is checked by consciously changing the mobile phase volume ratio (± 6.7% and ± 14.2%), pH value (± 2%) and wavelength (± 2.3%) from the optimized chromatographic conditions of these parameters. The percentage change in peak areas is calculated for each parameter and is found to be less than 2% for the two drugs and fulfill the ICH guidelines signifying the robustness of the method. The RSD values are also shown in Table 3.

The method is robust at less than 25% variation of the mobile phase volume ratio. However, the mobile phase composition variation is found to be less sensitive from the standard peak areas for chlordiazepoxide and sensitive for amitriptyline hydrochloride. In the case of pH, 2% increase resulted in decrease in peak area for chlordiazepoxide and increase in amitriptyline hydrochloride peak area. Similarly, for 2% increase of pH showed decrease in peak area of chlordiazepoxide

and increase in peak of amitriptyline hydrochloride. A 2.3% increase in wavelength showed increase in peak areas of both chlordiazepoxide and amitriptyline hydrochloride drugs. While, 2.3% decrease in wavelength resulted decrease in the chlordiazepoxide peak area and increase in peak area of amitriptyline hydrochloride.

Parameter	Value	Chlordiazepoxide ^a		Amitriptyline hydrochloride ^b	
		Area (mAU)	% Change	Area (mAU)	% Change
	Standard	62861	----	114122	----
Mobile phase	70:30 (v/v)	61658	1.91	116014	1.66
	80:20 (v/v)	62016	1.34	113228	0.78
Wavelength	226 nm	63963	1.75	114596	0.41
	216 nm	62488	0.59	115933	1.59
pH	5.0	62644	0.34	115512	1.22
	5.2	62732	0.20	115867	1.53

Table 3: Robustness data of chlordiazepoxide and amitriptyline hydrochloride. a) At 15 µg/mL b) At 37.5 µg/mL

Recovery

The accuracy of the method, in general, is verified by recovery experiments for the drugs under study. Generally used methods are: standard addition technique, internal standard method and/or external standard method. We employed the standard addition method for the recovery study of chlordiazepoxide and amitriptyline hydrochloride drugs. Three concentration levels (50%, 100% and 150%) are used to study the recovery. The analysis of each level is repeated three times (n = 3). Finally, the percentage recovery of chlordiazepoxide and amitriptyline hydrochloride drugs is compared with the actual amounts. The recovery results of the two drugs are presented in Table 4 in the form of mean recovery and their standard deviation, relative standard deviation and percentage error. The recovery ranged from 98.81-100.63% for chlordiazepoxide while it is 98.97-101.33% for amitriptyline hydrochloride, respectively. From the statistical parameters the results satisfy the ICH guidelines and suggest the proposed method is accurate.

Formulation assay

It is carried out on Amixide-H tablets (144 mg). The procedure is repeated 3 times, separately by weighing the tablet in powder form. Twenty tablets are powdered, weighed and the measured average weight is 5 mg for chlordiazepoxide and 12.5 mg for amitriptyline hydrochloride. The powder equal to 1 mg of the drug is taken and is dissolved in 10 ml of methanol. From 100 µg/ml solution, 15 µg/ml solution of chlordiazepoxide and 37.5 µg/ml of amitriptyline

hydrochloride are prepared. These concentrations are used for formulation assay studies. Assay results of the two drugs are expressed as percentage of label claims. The respective recoveries are 98.66% and 99.03% for chlordiazepoxide and amitriptyline hydrochloride, which is in good agreement within 90 to 100% of the label claim (Table 5). Only two dominant peaks in the

chromatogram are observed in the drug sample and almost no interference from excipients peaks that are normally present in the tablets (fig. 3). The demonstrated method is suitable for routine as well as bulk analysis of the compounds in pharmaceutical dosage form.

Analyte	Analyte added	Target (µg/ml)	Spiked (µg/ml)	Total (µg/ml)	Concen.found (µg/ml) Mean ± SD; RSD	Recovery% Mean±SD; RSD	%Error*
Chlordiazepoxide.	50%	10	5	15	15.10±0.20; 1.3	100.63±1.34; 1.33	0.769
	100%	10	10	20	19.76±0.13; 0.67	98.81±0.66; 0.67	0.387
	150%	10	15	25	25.09±0.35; 1.4	100.36±1.39; 1.4	0.809
Amitriptyline HCL	50%	25	12.5	37.5	37.21±0.24; 0.65	99.22±0.64; 0.65	0.376
	100%	25	25	50	49.48±0.41; 0.82	98.97±0.81; 0.81	0.468
	150%	25	37.5	62.5	65.87±0.34; 0.52	101.33±0.53; 0.52	0.301

Table 4: Recovery studies results of chlordiazepoxide and amitriptyline hydrochloride by standard addition method. *%Error = RSD/√n

Drug	Brand Name	Label claim	Amount prepared	Amount found	%Assay
Chlordiazepoxide	Amixide-H	5 mg	15 µg/ml	14.80 µg/ml	98.66
Amitriptyline hydrochloride		12.5 mg	37.5 µg/ml	37.14 µg/ml	99.03

Table 5: Results of formulation assay of commercial drug.

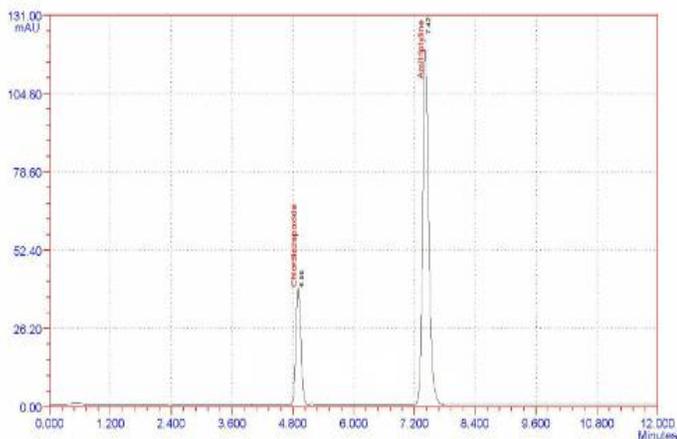


Figure 3: Chromatogram of Chlordiazepoxide and amitriptyline hydrochloride in Amixide- H tablets. The retention times are 4.90 and 7.42 minutes.

Conclusion

In conclusion, the proposed RP-HPLC method demonstrates the simultaneous estimation of two

drugs chlordiazepoxide and amitriptyline hydrochloride in tablet dosage preparations. Validation of the data of spiked samples by standard addition method and a commercial sample showed good recoveries and the precision measurements satisfy the ICH guidelines. The measured retention times indicate possibility for quick analysis of the samples in a shorter run time and the reported resolution shows well separated sharper chromatographic peaks. The mobile phase composition is relatively cheaper without the need for addition of a buffer. From the reported results, the proposed RP-HPLC method is superior to the earlier reported methods and could be used for determination of chlordiazepoxide and amitriptyline hydrochloride in tablet dosage form in bulk.

Acknowledgements

One of the authors (D. S.) wishes to thank the principal and the management of Loyola Institute of Technology and Management (LITAM), Sattenapalli, for their encouragement during the course of this work.

References

1. Sternbach LH. The story of benzodiazepines. *J Med Chem* 1979; 22(1): 1-7.
2. Indian Pharmacopoeia. Indian pharmacopoeia commission: Vol. 2. Ghaziabad, p 1054 and p 804.
3. British Pharmacopoeia. Her Majesty's stationary office, Vol.1. London, 2005, p 124 and p 438.
4. The United States Pharmacopoeia. United States Pharmacopoeial convention, 28th Revision: Rockville, MD, 2005, p 135 and p 435.
5. Ascalone V. Determination of chlordiazepoxide and its metabolites in human plasma by reversed-phase high-

performance liquid chromatography. J Chromatogr B 1980; 181 (1): 141-146.

6. Roberts SE, Delaney MF. Determination of chlordiazepoxide, its hydrochloride and related impurities in pharmaceutical formulations by reversed-phase high-performance liquid chromatography. J Chromatogr A 1984; 238: 265-272.

7. Soentjens-Werts, V, Dubois, JG, Atassi, G, Hanocq M. High-performance liquid chromatographic determination of chlordiazepoxide, its metabolites and oxaziridines generated after UV irradiation. J Chromatogr A 1994; 662 (2): 255-262.

8. Vree, TB, Baars, AM, Hekster, YA, van der Kleijn E. Simultaneous determination of chlordiazepoxide and its metabolites in human plasma and urine by means of reversed-phase high-performance liquid chromatography. J Chromatogr B 1981; 224 (3): 519-525.

9. French WN, Matsui FF, Smith SJ. Determination of major impurity in chlordiazepoxide formulations and drug substance. J Pharm Sci 2006; 64: 1545-7.

10. Gabrielle AS, Pierre S, Kathleen MG, Terrence FB. High-pressure liquid chromatographic determination of amitriptyline and its major metabolites in human whole blood. J Pharm Sci 1981; 71: 581-3.

11. Queiroz RHC, Lanchote VL, Bonato PS, de Carvalho D. Simultaneous HPLC analysis of tricyclic antidepressants and metabolites in plasma samples. Pharm. Acta Helvet 1995; 70 (2): 181-186.

12. Samanidou VF, Nika MK, Papadoyannis IN. Development of an HPLC method for the monitoring of tricyclic antidepressants in biofluids. J Separ Sci 2007; 30(15): 2391-2400.

13. Biryol I, Uslu B, Kucukyavuz Z. Voltametric determination of imipramine hydrochloride and amitriptyline hydrochloride using a polymer modified carbon paste electrode. J Pharm Biomed Anal 1996; 15: 371-381.

14. Ghani NTA, El-Nashar RM, Biome AA. Conductimetric determination of the antidepressants amitriptyline and dothiepin hydrochlorides and tranlycypromine hemisulphate in their pharmaceutical formulations. FABAD J Pharm Sci 2004; 29: 195-2001.

15. Fernández-Navarro JJ, Ruiz-Ángel MJ, García-Álvarez-Coque MC. Reversed-phase liquid chromatography without organic solvent for determination of tricyclic antidepressants. J Separat Sci 2012; 35: 10-11.

16. Deshmane GV, Kadam SS. Simultaneous spectrophotometric estimation of amitriptyline HCL and chlordiazepoxide. Indian drugs 1997; 34: 443.

17. Karpinska J, Suszynska J, Trace J. The spectrophotometric simultaneous determination of amitriptyline and chlorpromazine hydrochlorides in their binary mixtures. Microprobe Tech 2001; 19: 355-364.

18. Bakal RL, Manwar JV, Sahare AY, Bhajipale NS, Manikrao AM. Spectrophotometric estimation of amitriptyline HCL and chlordiazepoxide in pharmaceutical dosage form. Ind J Pharm Educ Res 2008; 42(1): 23-26.

19. Patel S, Patel NJ. Spectrophotometric and chromatographic simultaneous estimation of amitriptyline hydrochloride and chlordiazepoxide in tablet dosage forms. Indian J Pharm Sci 2009; 71(4): 472-476.

20. Mustafa AA, Mohamed E, Abdel-Hamid. Simultaneous high performance liquid chromatographic and first derivative spectrophotometric determination of amitriptyline hydrochloride and chlordiazepoxide in capsules. Anal Lett 1989; 22: 951-962.

21. Sane RT, Surve SR, Gangrade MG, Bapat VV, Chankar NL. Simultaneous gas chromatographic estimation of combined dosages. I-Amitriptyline hydrochloride with chlordiazepoxide,

II-Methocarbamol with ibuprofen, III-Paracetamol with diclofenac sodium, IV-Paracetamol with promethazine hydrochloride. Indian Drugs 1993; 30: 66-72.

22. ICH guidelines. ICH Harmonized Tripartite Guideline Q2 (R1), Validation of analytical procedures: text and methodology, Current Step 4 Version, 2005.

23. Ohannesian L, Streeter AJ. Hand book of Pharmaceutical analysis, Marcel Dekker Inc. NewYork 2002.