International Journal of Pharmacy
Journal Homepage: http://www.pharmascholars.com

Research Article **CODEN: IJPNL6**

A NEW VALIDATED STABILITY INDICATING HPLC METHOD FOR THE ESTIMATION OF RELATED SUBSTANCES OF LYSINE CLONIXINATE AND CYCLOBENZAPRINE HYDROCHLORIDE IN PHARMACEUTICAL DOSAGE FORMS

Morisetti. Nagendra Kumar*, V. Shanmukha Kumar. Jagarlapudi, P. Lohita Varma, Raja Sekhara Prasad . Kottapalli

Department of Chemistry, K.L.University, Vaddeswaram, Guntur District, Andhra Pradesh, India-522502.

***Corresponding author e-mail:** morisettinaga@gmail.com

ABSTRACT

A new gradient reverse phase high performance liquid chromatography method has been developed for quantitative determination of related substances of Lysine Clonixinate (LC) and Cyclobenzaprine Hydrochloride (CBP) in pharmaceutical dosage forms. Chromatographic separation achieved on a column L1, C18 250 mm x 4.6 mm; 5 µm with 0.025M Potassium dihydrogen phosphate pH 3.0 buffer as mobile phase-A and Acetonitrile as mobile phase–B at a flow rate of 1.7 mL min^{-1}. Diluent as water and acetonitrile of ratio 50:50 v/v with detection at 245 nm. The injection volume was 20 µL and a gradient program with run time of 60 minutes. The developed method validated according to ICH guideline with the parameters specificity, forced degradation (with stress conditions of acid, base, oxidative hydrolysis, thermal and photolytic degradation), limit of detection and limit of quantification, linearity, precision, intermediate precision, accuracy, robustness and ruggedness. From all validation parameters results the method proved as specific, stability indicating, precise, accurate, robust and rugged method.

Keywords: Lysine Clonixinate, Cyclobenzaprine Hydrochloride, Validation, HPLC.

INTRODUCTION

Cyclobenzaprine Hydrochloride (3-(5H-dibenzo[a,d] cyclohepten-5ylidene)-N, N-dimethyl-1-propanamine hydrochloride) has been considered structurally related to the first-generation antidepressants. Such tricyclics, including amitriptyline, act to inhibit the uptake of noradrenaline, resulting in increased transynaptic noradrenaline concentration. They have been shown to exert analgesic effects in chronic nerve and muscle pain, Cyclobenzaprine Hydrochloride may have a similar effect.

Cyclobenzaprine Hydrochloride is a muscle relaxant medication used to relieve skeletal muscle spasms and associated pain in acute musculoskeletal conditions. It is the best-studied drug for this application and it also has been used off-label for fibromyalgia[1] treatment. A new bedtime formulation of Cyclobenzaprine Hydrochloride is under development for the management of fibromyalgia syndrome. Cyclobenzaprine Hydrochloride (CBP) impurities[2] are namely 1) Dibenzocyclo heptenone, 2) Amitriptyline (10,11-Dihydro-N,N-dimethyl-5H-dibenzo[a,d]cycloheptene-propylamine) 3) Cyclobenzaprine related compound A (1-(5H-dibenzo[a,d][7]annulen-5-yl)-3-(dimethylamino) propan-1-ol), 4) Cyclobenzaprine N-Oxide (3-(5H-di benzo[a,d]cyclohepten-5-ylidene)-N,N-dimethyl-1-propanamine N-Oxide) and 5) Cyclobenzaprine related compound B (3-(5H-Dibenzo[a,d]cyclohepten-5-ylidene)-N-methyl-1-propanamine).

Lysine clonixinate (LC) 2-[(3-Chloro-2-methylphenyl)amino]nicotinic acid – L – lysine

(1:1))[3] is a NSAID (Non Steroidal anti Inflammatory Drug) derived from nicotinic acid that has proven to be effective in various pain syndromes such as renal colic and muscular pain[4]. Its therapeutic action is as Analgesic. The analgesic activity is mainly due to the inhibition of cyclooxygenase, the cyclooxygenase is the enzyme responsible for the production of prostaglandins [5].

Lysine Clonixinate is an analgesic drug with its effect on platelet Cyclooxygenase in man. LC is a cyclooxygenase inhibitor of moderate potency. It remains to be investigated whether mechanisms other than inhibition of cyclooxygenase contribute to the analgesic activity of lysine clonixinate. Lysine Clonixinate (LC) impurities are 1) 5-Chloro Clonixin (2-[(5-Chloro-2-methylphenyl)-amino]-nicotinic acid), 2) Chloramide (N-(3-Chloro-2-methylphenyl)-2-chloronicotinamide), 3) Dechloro Clonixin (2-[(2-methylphenyl)-amino]-3pyridinecarboxylic acid) and 4) Hydroxamide (N-(3-Chloro-2-methylphenyl)-2-hydroxynicotinamide)

Each tablet contains Lysine Clonixinate 125 mg and Cyclobenzaprine Hydrochloride 5 mg and tablets marketed as Dorixina Relax[6], Dorixina Flex. Its therapeutic action was Analgesic-Miorelaxant. The permitted maximum of tablets[6] was 6 tablets per day, it means 750 mg LC and 30 mg CBP per day allowed.

In the Literature very few individual methods available for Lysine clonixinate and Cyclobenzaprine hydrochloride. LC is in-house material, not in any monographs. CBP is available in USP and Ph.Eur as drug substance and drug product. Literature reveals that there is no single method for estimating the related substances of Lysine Clonixinate and Cyclobenzaprine Hydrochloride in a combined dosage forms. The main objective is to develop a single combination, simple, robust, rugged and reproducible HPLC method for the determination of related substances of Lysine Clonixinate and Cyclobenzaprine Hydrochloride in a single and combined dosage forms.

2. EXPERIMENTAL PART

2.1 Materials and Reagents: All standards, impurities, tablets of Lysine Clonixinate and Cyclobenzaprine Hydrochloride were supplied by Alphamed Formulations Pvt. Limited, Hyderabad, India. The HPLC grade Acetonitrile, Sodium hydroxide pellets, Hydrochloric acid, Orthophosphoric acid and Potassium dihydrogen orthophosphate were purchased from Merck. Hydrogen peroxide purchased from Rankem. Water was purified by Millipore Milli Q system.

***2.2 Equipments and Apparatus*:** HPLC system used namely Waters e2695 separation module with 2489 UV detector and with 2996 PDA detector. The column used was L1 with 25 cm x 4.6 mm; 5 µm (Inertsil ODS 3V) supplied by GL Sciences.

2.3 Chromatographic conditions: The separation between Lysine clonixinate, Cyclobenzaprine Hydrochloride and respective related substances was achieved using 0.025M Potassium dihydrogen orthophosphate buffer with pH adjusted to 3.0 using 1% ortho phosphoric acid solution as a mobile phase-A and Acetonitrile as mobile phase–B at a flow rate of 1.7 mL min^{-1} (gradient). Diluent was water and Acetonitrile in the ratio 50:50 v/v. Detection was at 245 nm, the injection volume was 20 µL and the run time 60 minutes. The Gradient program was given in Table 1.

2.4 Preparation of solutions

2.4.1 Diluted Standard solution preparation: Cyclobenzaprine hydrochloride of 0.001 mg mL^{-1} and Lysine Clonixinate of 0.025 mg mL^{-1} concentration solution prepared with diluent**.**

2.4.2 Sample preparation: The drug was extracted from tablet formulation of 125/5mg label claim using the diluent. Weighed and transferred 10 tablets into 100 mL volumetric flask, added 70 mL of diluent, kept on rotary shaker for 10 minutes at 200 rpm and sonicated for 30 minutes with intermediate shaking. Diluted to volume with diluent and mixed well. Centrifuged a portion of the sample solution at 3000 rpm for 10 mins. The concentration of LC in the sample was 12.5 mg mL^{-1} and CBP was 0.5 mg mL^{-1}.

2.4.3 Preparation of Spiked sample solution: (Refer Figure 2) Weighed and transferred 10 tablets into 100 mL volumetric flask, added 70 mL of diluent, kept on rotary shaker for 10 minutes at 200 RPM and sonicated for 30 minutes with intermediate shaking. Spiked all impurities of both actives at a concentration of 0.2 % to the respective test concentration. Diluted to volume with diluent and mixed well. Centrifuged a portion of the sample solution at 3000 rpm for 10 mins. In Spiked sample the concentration of LC impurities was about 0.025 mg mL^{-1} and CBP impurities was about 0.001 mg mL^{-1}.

3 EXPERIMENTAL METHODOLOGY

3.1 Development of Chromatographic Conditions: Several stationary phase columns C8, C18, CN and NH_2 were used for optimizing the chromatographic conditions. The experiments done for improvisation

of peak symmetry and resolution between all peaks. Different salt buffers such as phosphate (potassium), sodium per chlorate, acetate (ammonium) were evaluated for system suitability parameters and overall chromatographic performance. In the sequential trials potassium dihydrogen phosphate found to be suitable for separation of parent peak and impurities. The pH had an effect on the retention times of the LC, CBP and its related substances. Several trials at different pH (acidic side and basic side) tried. Resolutions and peak symmetry are found good at pH 3.0. To elute all impurities and highly non-polar impurities gradient programs tried. Gradient trial tried with mobile phase A as pH 3.0 buffer and mobile phase B as acetonitrile. Finally gradient program of 60 min with a injection volume of 20 µL and column temperature 40°C were selected for the estimation of related substances of Lysine clonixinate and Cyclobenzaprine hydrochloride in tablets. All impurities and actives were injected into the PDA detector and selected the suitable absorbance at wave length 245 nm. In Inertsil ODS 3V, 250 mm x 4.6 mm, 5 µm (From GL Sciences) all impurities were well separated from each other. This was a diluted standard method and established the RRF values, which were used in the calculation of % of impurities. Refer Table 2 for Retention times, Relative Retention time and Resolution values of all peaks.

The Optimized method: The optimized chromatographic conditions were Inertsil ODS 3V 250 mm x 4.6 mm, 5 µm with 0.025 mM Potassium dihydrogen phosphate buffer pH 3.0 as mobile phase A and acetonitrile as mobile phase B. The gradient program finalized for 60 min, injection volume of 20 µL and column temperature 40°C. The detection wave length as 245 nm.

4. VALIDATION OF METHOD AND RESULTS

4.1 System suitability, Specificity and forced degradation studies: The system suitability of the method evaluated by similarity factor method by injecting diluted standard solution twice and calculating the similarity factor and monitoring of tailing factor, theoretical plates for both actives from diluted standard. The similarity factor value found to be between 0.95 and 1.05, the tailing factor for LC and CBP from diluted standard solution was found to be less than 2.0 and theoretical plates of both actives found to be more than 2000 (Refer Table 3) .

The specificity of a method is its suitability for analysis of a substance in the presence of potential impurities. Stress testing of a drug product can help to identify likely degradation products, which can helps to establish degradation pathways and the intrinsic stability of the molecule. The specificity of the method for Lysine Clonixinate and Cyclobenzaprine Hydrochloride tablets has been determined in the presence of nine impurities and degradation products. The stress conditions and results mentioned in the following table (4). Peak purity has been checked for the active peaks by using PDA detector in stress samples.

There was no peak found at the retention time of Lysine Clonixinate, Cyclobenzaprine Hydrochloride and it's all nine impurities in blank and placebo blend chromatograms, proves no interference from blank and placebo. Peak-purity test results from the PDA detector confirmed that the peaks (Lysine Clonixinate, Cyclobenzaprine Hydrochloride) obtained from all the stress samples analyzed were homogeneous, pure, there were no co eluting peaks and mass balance found to be more than 95%. It confirming the Specificity and Stability indicating power of the method. Refer figure 3 for Chromatograms.

4.2 Limit of detection and quantification: The Limit of detection (LoD) and Limit of quantification (LoQ) for the nine impurities and two actives were established at the amounts for which the signal- to-noise ratio were 3:1 and 10:1 respectively by injecting a series of dilute solutions of predetermined known concentration. The precision at LoQ level also verified and found satisfactory. Refer Table 5 for results.

4.3 Linearity: The linearity solutions for the all substances (actives and impurities) were prepared by diluting the respective stock solutions to five different concentrations ranging from the LoQ to 125 % of the specification level (0.2% of test concentration). Each level solution injected thrice and considered the average value. The linearity graph plotted by using average area against concentration for all components.

The correlation coefficients of the calibration plots are reported. Calibration plots for all the related substances were linear over the ranges tested. The correlation coefficient (r) was >0.99 for all the components (Refer Table 5). The results shown there was an excellent correlation between the peak area and concentration for all components.

4.4 Precision: The precision of the method established by Repeatability and Intermediate Precision. Repeatability was verified by injecting six individual preparations of Lysine clonixinate 125 mg and Cyclobenzaprine hydrochloride 5 mg tablets sample solutions spiked with impurities at a

concentration of 0.2% of its respective test concentration. The intermediate precision of the method was also performed using different instrument (including column) and different analyst on different day. The intermediate precision was verified by injecting six individual preparations of Lysine clonixinate 125 mg and Cyclobenzaprine hydrochloride 5 mg tablets sample preparations spiked with all impurities at a concentration of 0.2% of test concentration.

The % RSD of peak area for each impurity calculated for precision, intermediate precision and found to be less than 10 %. The % RSD of peak area for each impurity was calculated from both combinable repeatability and intermediate precision and results found to be less than 10 %. These results proved the ruggedness of the method (For results refer Table 6).

4.5 Accuracy: The recovery of impurities was determined by spiking each impurity on test sample at three different levels starting from 50 % to 150 % of the 0.2 % of test concentration for all impurities. The diluted standard accuracy (which includes both actives) was also performed on the placebo for the unknown impurity accuracy. The recovery range for all impurities and actives was found to be between 90 % and 110 % (For results refer Table 6).

4.6 Robustness: To determine the robustness of the method the experimental conditions were deliberately varied and the resolution of all peaks (between actives and impurities) was evaluated. To study the effect of flow rate, flow rate was changed to 1.5 and 1.9 from 1.7 mL min^{-1}. The effect of mobile phase-A buffer pH was studied at pH 2.8 and 3.2. The effect of column oven temperature was studied at 35°C and 45°C. At each condition evaluated the system suitability and injected the spiked sample at all variable conditions.

In all the deliberate varied chromatographic conditions the selectivity as well as the performance of the method was unchanged on comparison of RRTs of all peaks and resolution between all peaks, it proves the robustness of the method.

In robustness effect of filter variability (effect of filtration) also verified by using the spiked test sample prepared and centrifuged a portion of the solution and filtered through 0.45 µm PVDF and Nylon filters. Injected the sample and calculated the difference of Maximum single impurity and total impurities between centrifuged and filtered samples.

The difference was less than 0.03% for maximum single impurity and less than 0.1 % for total impurities. It indicates that there is no effect of filtration on sample solution.

4.7 Solution and mobile phase stability: There is no significant changes in the amounts of the impurities were observed during solution stability and mobile phase stability experiments. The results from solution stability and mobile phase stability experiments confirmed that mobile phase, standard and sample solutions were stable for up to 48 hrs at bench top during determination of related substances.

5.0 CONCLUSION

The proposed method is highly selective, reproducible, specific, robust and rugged. This method can be used in the routine analysis of production samples. The information presented herein could be very useful for quality monitoring of lysine clonixinate and cyclobenzaprine hydrochloride in individual and combination dosage form samples and as well employed to check the quality during stability studies.

Figure 1: Chemical structures of Lysine Clonixinate and its impurities:

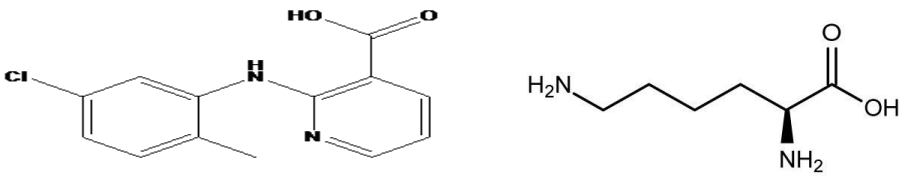

Fig 1.1 Lysine Clonixinate

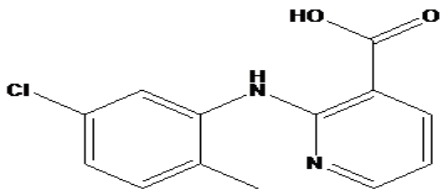

Fig 1.2 5-Chloro Clonixin

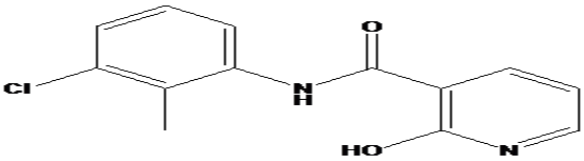

Fig 1.3 Chloramide

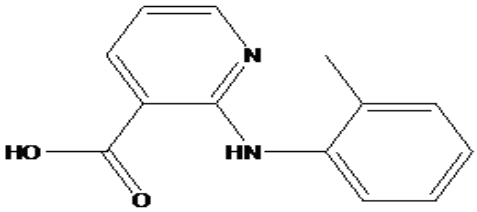

Fig 1.4 Dechloro Clonixin

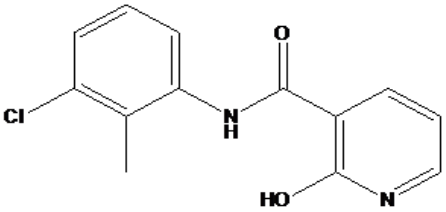

Fig 1.5 Hydroxamide

Chemical structures of Cyclobenzaprine Hydrochloride and its impurities:

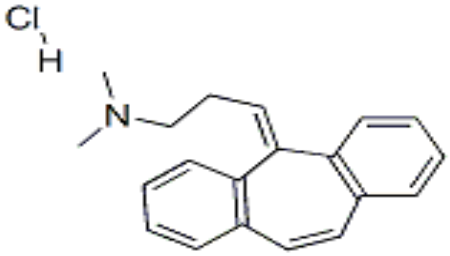

Fig 1.6 Cyclobenzaprine Hydrochloride

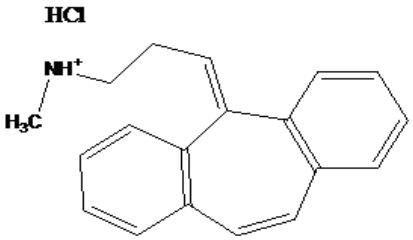

Fig 1.7 CBP Related Compound B

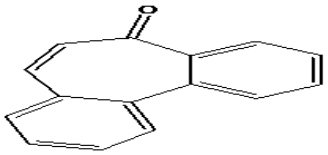

Fig 1.8 Dibenzocycloheptenone

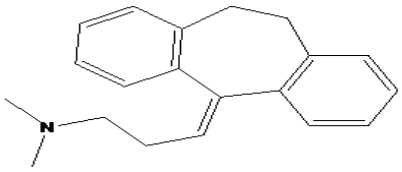

Fig 1.9 Amitryptiline

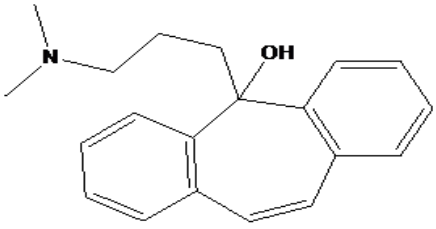

Fig 1.10 CBP Related Compound A

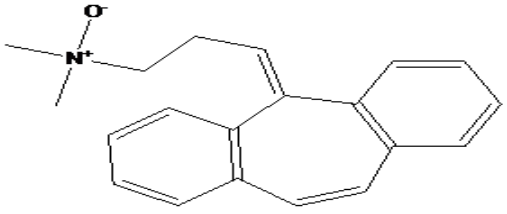

Fig 1.11 CBP N-oxide

Figure 2 Spiked Chromatogram

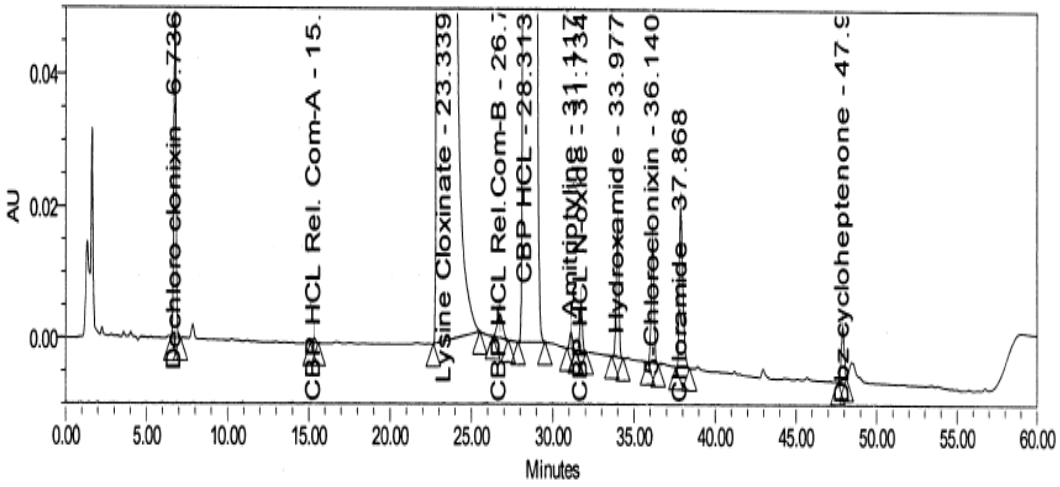

Figure 3 Forced Degradation Chromatograms:

Figure 3.1 Acid Degradation

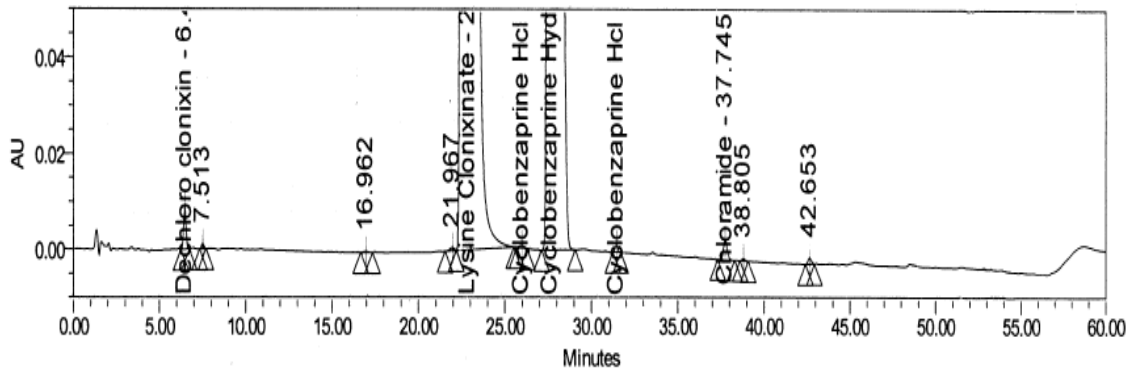

Figure 3.2 *Base* Degradation

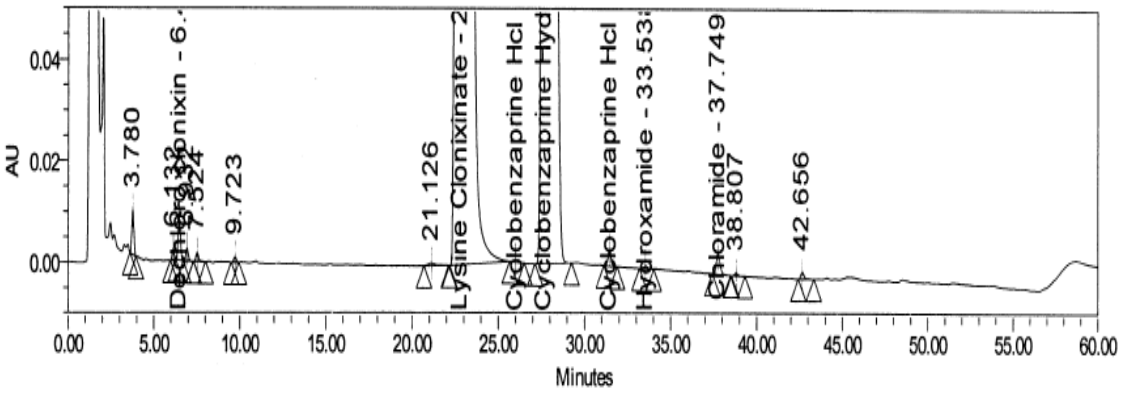

Figure 3.3 ***Peroxide*** **Degradation**

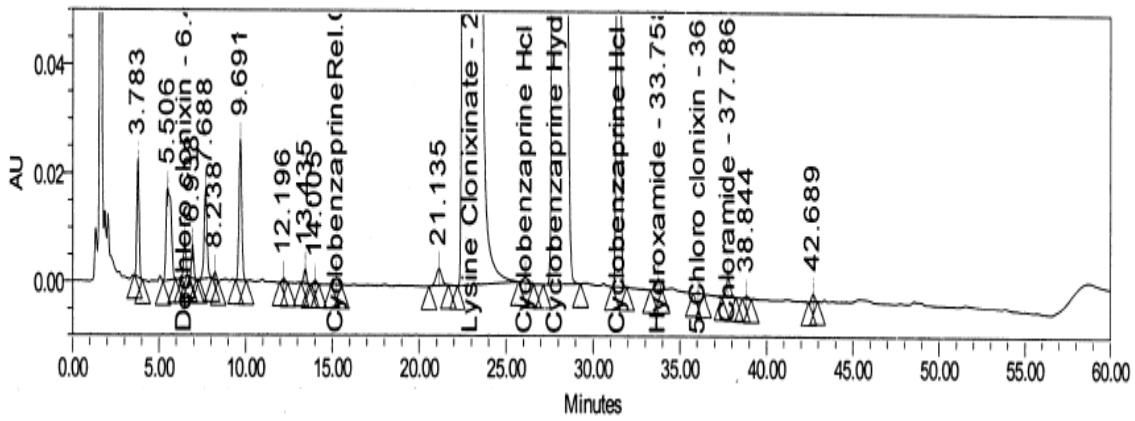

Figure 3.4 ***Thermal*** **Degradation**

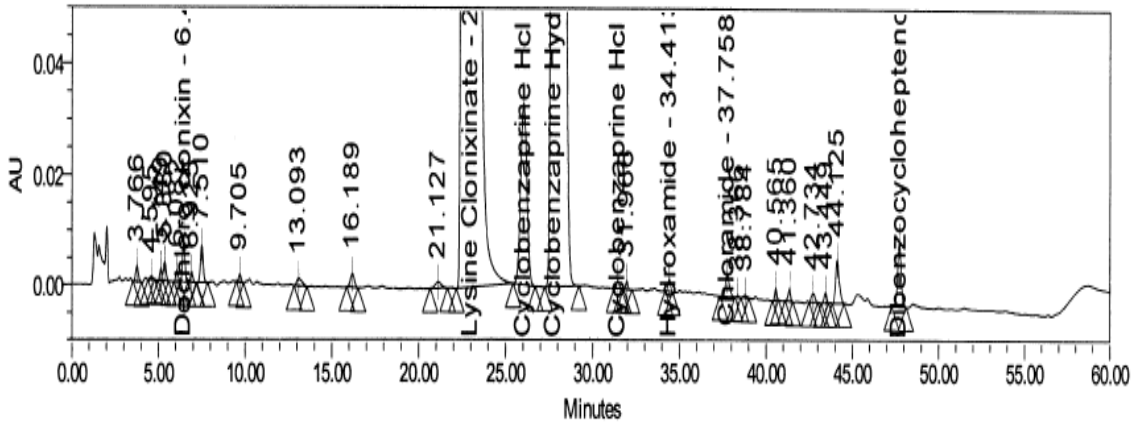

Figure 3.5 ***Photolytic*** **Degradation**

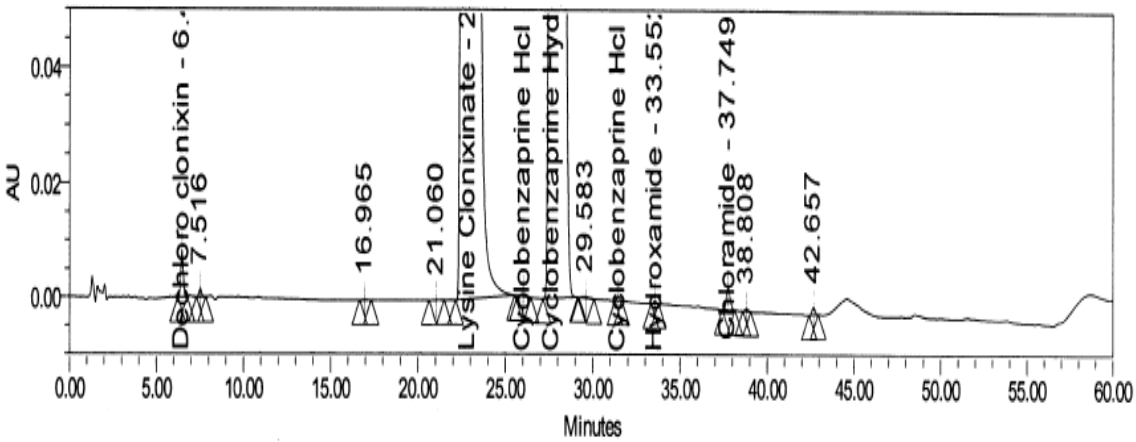

Table 1 Gradient Program

Time (in min.)	% Mobile Phase-A	% Mobile Phase-B
0	80	20
10	75	25
25	75	25
40	50	50
50	45	55
55	35	75
56	80	20
60	80	20

Table 2: Retention times, Relative retention times and Resolutions Values

S.No	Component Name	Retention time	RRT	Resolution
Lysine clonixinate impurity RRTs calculated with respect to Lysine clonixinate RT				
1	Lysine clonixinate	23.339	1	13.73
2	5-Chloro clonixin	36.14	1.55	6.76
3	Chloramide	37.868	1.62	4.69
4	Hydroxamide	33.977	1.46	7.39
5	Dechloro clonixin	6.736	0.29	NA
Cyclobenzaprine impurity RRTs calculated with respect to Cyclobenzaprine HCl RT				
6	Cyclobenzaprine HCl	28.313	1.00	1.99
7	CBP Related compound A	15.234	0.54	28.12
8	CBP Related compound B	26.734	0.94	4.66
9	Amitriptyline	31.117	1.10	4.54
10	CBP N-Oxide	31.734	1.12	2.02
11	Dibenzocycloheptenone	47.9	1.69	25.95

Table 3: System suitability Results

Concentration 0.2 %	Injection	Peak Area	Theoretical Plates	Tailing factor	Similarity factor
Lysine Clonixinate	1	477901	17127	1.44	0.98
	2	486306	16900	1.4	
Statistical Analysis	Mean	482104	--	--	--
Cyclobenzaprine Hydrochloride	1	39046	31071	0.97	0.97
	2	40316	32035	0.93	
Statistical Analysis	Mean	38386	--	--	--

Table 4 Forced Degradation Conditions and Results

Degradation Mechanism / Condition	% Degradation	Mass balance
Undegraded Sample	0.22	99.1
Acid/1N HCl Heat on water bath at 60°C – 2 hrs	0.29	98.5
Base/1N NaOH Heat on water bath at 60°C – 2 hrs	0.8	97.8
Peroxide/3%H_2O_2 kept on bench top – 2 hrs	22.3	96.8
Thermal/ at 105°C – 24 hrs	2.58	98.1
Photolytic/1.2 Million Lux hours & 200 watts hours per square meter	0.25	97.6

Table 5 Relative Response Factor (RRF), Limit of Detection (LoD), Limit of Quantification (LoQ) and Correlation Coefficient (r) Results

Component Name	RRF	LoD	LoQ	r
5-Chloro clonixin	0.83	0.002	0.005	0.994
Chloramide	0.9	0.0003	0.001	0.999
Hydroxamide	0.85	0.0004	0.001	0.997
Dechloro clonixin	1.98	0.0004	0.001	0.999
Dibenzocycloheptenone	2.21	0.0017	0.005	0.995
Amitriptyline	0.65	0.01	0.03	0.998
CBP Related Compound A	0.18	0.02	0.04	0.993
CBP N-oxide	0.79	0.01	0.03	0.998
CBP Related Compound B	0.92	0.0028	0.008	0.995
Cyclobenzaprine HCl	NA	0.003	0.01	0.999
Lysine Clonixinate	NA	0.001	0.005	0.999

Table 6 Precision, Intermediate Precision and Accuracy Results

Component Name	Precision % RSD	Intr. Precision % RSD	% Recovery
5-Chloro clonixin	2.8	2.7	97
Chloramide	4.5	3.5	99
Hydroxamide	3.2	2.8	101
Dechloro clonixin	2.7	3.4	98
Dibenzocycloheptenone	3.1	2.8	100
Amitriptyline	4.1	3.5	97
CBP Related Compound A	2.9	3.7	98
CBP N-oxide	2.6	3.4	99
CBP Related Compound B	3.5	2.9	100
Cyclobenzaprine HCl	NA	NA	98
Lysine Clonixinate	NA	NA	100

REFERENCES

1. http://www.emedexpert.com/facts/cyclobenzaprine-facts.shtml
2. http://www.uspnf.com/uspnf/pub/index/USP37-NF32
3. http://www.drugs.com/search.
4. Krymchantowski AV, Barbosa JS, Cheim C, Alves LA. Arq Neuropsiquiatr. 2001; 59(1): 46-9
5. http://www.masterfarma.com/dorixyl_clonixinato.html
6. http://www.roemmers.com.ar/uploads/DorixinaRelaxComprimidosEN.pdf
7. Heinitz ML, J Pharm Sci, 1982;71 (6):656-8
8. Tatiane Maria de Lima SB, Simone GS, Eunice KK, Eunice Emiko MK, Ting HC, Cristina HS, Valentina P. Biomed Res Int, 2013: Article ID 281392, 6 pages.
9. Jian-Chun L, Fei-Hu C, Jun-Dong Z, Hai-Jun D, Shu G. Afr J Pharm Pharmacol, 2012; 6(10): 708-716
10. Pallapies D, Muhs A, Bertram L, Rohleder G, Nagyivanyi P, Peskar BA. Eur J Clin Pharmacol, 1996; 49(5): 351-4
11. Bica A, Farinha A, Blume H, Barbosa CM. J Chromatogr A, 2000; 889(1-2): 135-41
12. Pineros I, Ballesteros P, Lastres JL. J Pharm Biomed Anal, 2002; 27(5): 747-54
13. http://clinicaltrials.gov/show/NCT01421433
14. http://www.epharmapedia.com/medicine/search
15. http://www.medschat.com/Drugs/Amrix
16. http://chemdraw-pro.software.informer.com/12.0
17. ICH, Validation of Analytical Procedures: Text and Methodology (Q 2(R1)): International Conference on Harmonization, Current step 4 version, 2005.
18. ICH, Stability Testing of New Drug Substances and Products (Q1A(R2)): International Conference on Harmonization, Current Step 4 version, February 6, 2003.