



ISSN: 0975-766X  
Research Article

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**DEVELOPMENT AND VALIDATION OF STABILITY INDICATING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD FOR SIMULTANEOUS DETERMINATION OF ALPRAZOLAM AND PROPRANOLOL IN COMBINED DOSAGE FORMS**

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*Received on 28-04-2011*

*Accepted on 09-05-2011*

**Abstract**

The Objective Of the current study was to develop a validated stability indicating high performance liquid chromatographic method for alprazolam and propranolol in combined dosage form. The method was validated by subjecting the drugs to forced decomposition under hydrolysis, Oxidation, photolysis, and thermal stress conditions prescribed in international Conference on Harmonization. The drugs were successfully separated from major and minor degradation products on a reversed –phase C18 column by using phosphate buffer (PH 4)-ACN-Methanol (55+35+10 %V/V/V) as the mobile phase with determination at 254 nm. The flow rate was 1 ml/min. The method was validated with respect to linearity, precision, accuracy, Sensitivity, robustness. The response were linear over the range of 1-10 and 8-800 µg/ml for Alprazolam and Propranolol, respectively. The recoveries of both the drugs from a mixture of degradation products were in the range of 97-101%. The utility of the procedure was verified by its application to marketed formulations that were subjected to accelerated stability studies. The method distinctly separated the drugs and degradation products, even in actual sample. The products formed in marketed tablets were similar to those formed during stress studies.

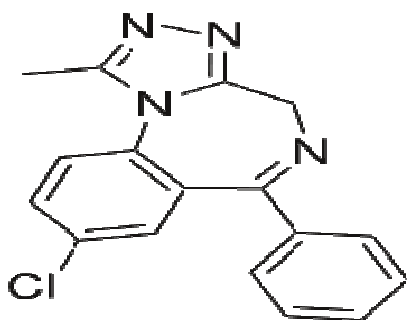
**Key words:** Propranolol hydrochloride, Alprazolam, Simultaneous, High performance liquid chromatography.

**Introduction**

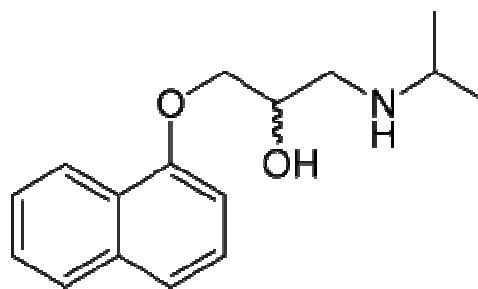
Stability studies of drug substances via acid hydrolysis, base hydrolysis, oxidation, and thermal and photolytic stress testing are a part of development strategy under the International Conference on Harmonization (ICH) requirements(1). These studies provide information on a drug's inherent stability and help to validate analytical methods to be used for evaluation of stability. Stability-indicating assays are currently being developed (2,3) by

using the stress-testing approach of the ICH guidelines, Q1A [R2](1). The approach has been further extended to stress tests of drug combinations(4,5). These tests allow accurate and precise quantification of drugs, their degradation products, and their interaction products.

Alprazolam (ALP), 8-chloro-1-methyl-6-phenyl-4H-triazolo[4,3- $\alpha$ ][1,4]benzodiazepine, is a benzodiazepine derivative that is used in the treatment of generalized anxiety, panic attacks with or without agoraphobia, and depression(6); Propranolol hydrochloride is a non-selective beta-blocker mainly used in the treatment of hypertension(7). It was the first successful beta-blocker developed. Chemically it is (R,S)-1-(isopropyl amino)-3-(1-naphthoxy) propan-2-ol Fig 1a.



**Alprazolam**



**Propranolol**

Several high-performance liquid chromatographic (HPLC) methods have been reported for the determination of ALZ and PRL individually. To our knowledge, no stability-indicating HPLC assay method has been reported for the simultaneous determination of ALP and PRL in tablets in the presence of their degradants by using the ICH approach of stress testing. The focus of the present study is to develop a simple, rapid, precise, and accurate isocratic reversed-phase stability-indicating HPLC method for the simultaneous determination of ALP and PRL in tablet dosage form.

## **Experimental**

### **Apparatus**

Chromatography was performed with The HPLC system (Shimadzu Corporation, Japan), model Shimadzu VP, consisted of a system controller (CLASS-VP), on-line degasser (LC 2010C, Shimadzu), low pressure gradient valve (LC 2010C, Shimadzu), solvent delivery module (LC 2010C, Shimadzu), auto injector (LC 2010C, Shimadzu), column oven (LC 2010C, Shimadzu), and CLASS – VP software version = SPI, binary pump, auto injector (SIL-10AD VP, Shimadzu),

column oven (CTO-10AS VP, Shimadzu) and PDA detector (PDA-SPD-M10A VP, Shimadzu Diode Array Detector) and Chem station (software). The separation was performed at room temperature on a Kromacil C18(250 × 4.6 mm, 5 μm) column. The mobile phase was degassed by Oscar Ultra Sonics, OU- 72 (SPL) sonicator. The standard substances were weighed on analytical balance (AX 205, METTLER TOLEDO). Stability studies were carried out in Proto – Tech oven.

### **Reagent and Material**

Pure ALP and PRP were gift samples from Merrill Pharma (Roorakee, Uttarkhand) with 99.7 and 99.5% purity, respectively. Combination products, ANZAL-PLUS label claim: 0.25 mg ALP and 0.20 mg PRP per tablet, Merrill pharmaceutical LTD were purchased from a local shop.

Acetonitrile, Methanol, and water (HPLC grade) were purchased from Spectrochem Pvt Ltd (Mumbai, India). Potassium Dihydrogen Phosphate, Disodium Hydrogen Phosphate, hydrochloric acid, sodium hydroxide and hydrogen peroxide were purchased from Rankem (Mumbai); phosphoric acid was purchased from Spectrochem PVT Ltd., Mumbai.

### **Preparation of standard solutions**

Individual standard stock solutions of ALP (100 μg/ml) and PRP (800 μg/ml) were prepared in methanol. For the calibration plot of ALP and PRP, various dilutions were prepared from the stock solution in a range of 1-10 μg/ml and 80-800 μg/ml, respectively. All the dilutions were made with methanol.

### **Forced degradation studies of standard drug solution and their binary mixtures**

Standard stock solutions of ALP (10000 μg/ml) and PRP (10000 μg/ml) were individually prepared by dissolving 1 gm standard ALP and 1 gm standard PRP in 100 ml methanol. 5 ml of ALP stock solution was transferred in series of 50 ml volumetric flask containing 5 ml 0.01 N HCL, 5 ml 1 N NaOH and 5 ml 3% H<sub>2</sub>O<sub>2</sub>. Flask containing 0.01 N HCL was refluxed for 2 hours at 80 °C and flask containing 1 N NaOH was refluxed for 8 hours at 80 °C. Flask containing H<sub>2</sub>O<sub>2</sub> was allowed to react for 20 min at room temperature. For the PRP 5 ml stock solution was transferred in series of 50 ml volumetric flask containing 5 ml 0.001 N HCL, 5 ml 0.001 N NaOH, 5 ml 3% H<sub>2</sub>O<sub>2</sub>. Flask containing 0.001 N HCL was refluxed at 80 °C for 45 minutes and flask containing 0.001 N NaOH was refluxed at 80 °C for 45 minutes. Flask containing 3% H<sub>2</sub>O<sub>2</sub> was allowed to react for 20 min. Binary mixtures of these drugs were prepared from the above stock solution by combining 10 ml ALP stock solution and 10 ml PRP stock solution in 100 ml volumetric flask to get 1000 μg/ml of both the drugs. From this stock solution 5 ml were transferred to series of volumetric flask containing 5 ml 0.01 N HCL, 5 ml 1 N NaOH, 5 ml 3% H<sub>2</sub>O<sub>2</sub>. Flask

containing HCL was refluxed for 2 hours at 80°C and flask containing NaOH was refluxed for 5 hours at 80°C.

Degradation was also carried out in the solid state by exposing pure drug to dry heat at 80°C for 48 hours. For photolytic condition pure drug was exposed to UV light for 36 hours.

### **Forced Degradation Studies of Tablets**

The tablets were exposed to thermal stress at 80°C for 48 hours and for photolytic degradation tablets were exposed to UV light for 36 hours. An amount of degraded tablet powder equivalent to 4 tablets was transferred in 100 ml volumetric flask and dissolved in 50 ml methanol and sonicated for 15 minutes and diluted up to the mark with methanol.

### **Chromatographic separation**

HPLC studies were carried out with all the reaction solution individually, and with a mixture of the solution in which decomposition was observed. The stressed samples were initially analyzed by HPLC using a reversed-phase C18 column and the mobile phase buffer-acetonitrile (55+45, V/V). Because the separation and peak shape were not good, methanol was added as organic modifier, the further trials optimizing the ratio of buffer and solvents. Eventually, a phosphate buffer (pH 4, adjusted with 0.05% phosphoric acid)-acetonitrile-methanol (55+45+10, V/V/V) gave the best results. During these studies, the injection volume was 20 µl and the mobile phase flow rate was constant at 1 ml/min; the analytical wavelength was 254 nm.

### **Method Validation**

Linearity was established by triplicate injection of solution containing standard ALP and PRP in the concentration ranges of 1-10 and 8-800 µg/ml, respectively. The system precision was established by making 6 replicate injection of standard solution containing 2 µg/ml and 160 µg/ml of ALP and PRL, respectively. The method precision was established by making 6 replicate injection of test solution containing 2 µg/ml and 160 µg/ml of ALP and PRL respectively. Accuracy was determined by fortifying the mixture of test solution with 3 standard solution containing known concentration of ALP (0.5, 1 and 1.5 µg/ml) and PRP (40, 80 and 120 µg/ml) and percent recoveries of added drug were determined. The specificity of the method was established through study of resolution factor of drug peaks from the nearest peaks, and also from all other peaks. The specificity of the method toward the drug also established through determination of purity of ALP and PRP peaks from HPLC analysis of mixture of stressed samples through study of purity plots by using a PDA detector.

Robustness of the method was determined by deliberately varying certain parameter like flow rate (ml/min), PH, Temperature, concentration of acetonitrile (ml) in mobile phase. Each parameter was evaluated at 3 level (-1, 0, +1).

## Results and Discussions

### Forced degradation studies

Condition used for forced degradation were attenuated to achieve degradation in the range of 20-80 % . The following behavior of drugs was observed during the HPLC studies.

Acidic condition:- The individual drug and their combination were heated in HCL for varies conditions compared with the ALP, PRL was more susceptible to the degradation process. About 20-30% degradation of PRP was observed. Whereas minute degradation of APL was seen. The major degradation products formed from PRL in the combination product were at retention times of 3.3 min.

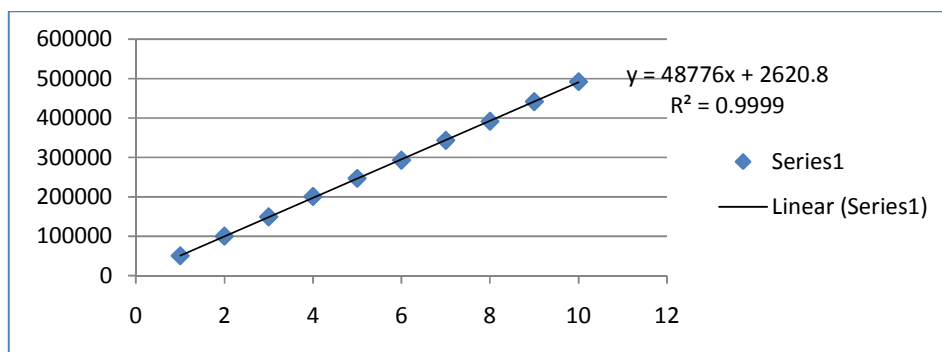


Figure-1. Linearity Calibration Curve of Alprazolam

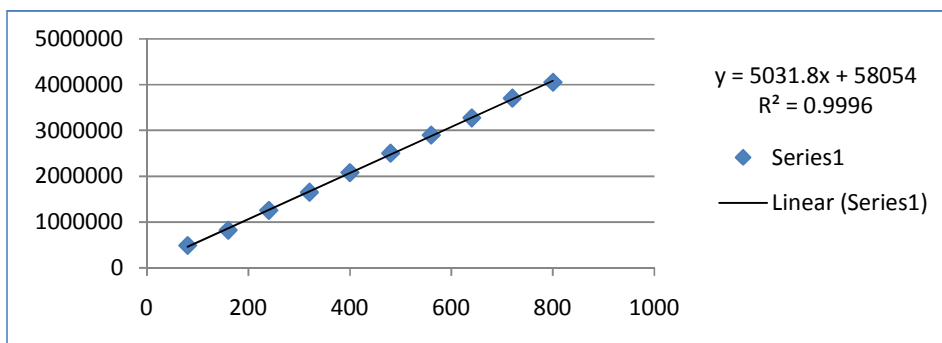


Figure-2. Linearity Calibration Curve of Alprazolam

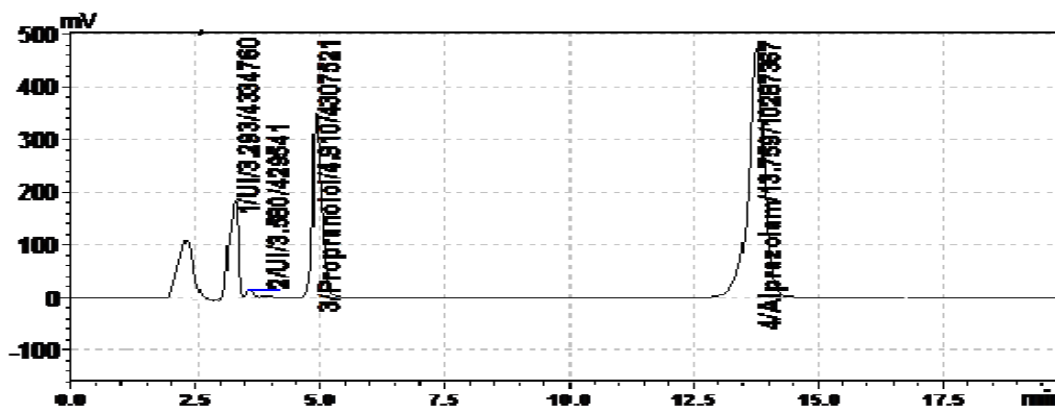


Figure-3. Chromatogram showing the separation of alprazolam and Propranolol in stressed sample of the synthetic mixture subjected to acid Hydrolysis.

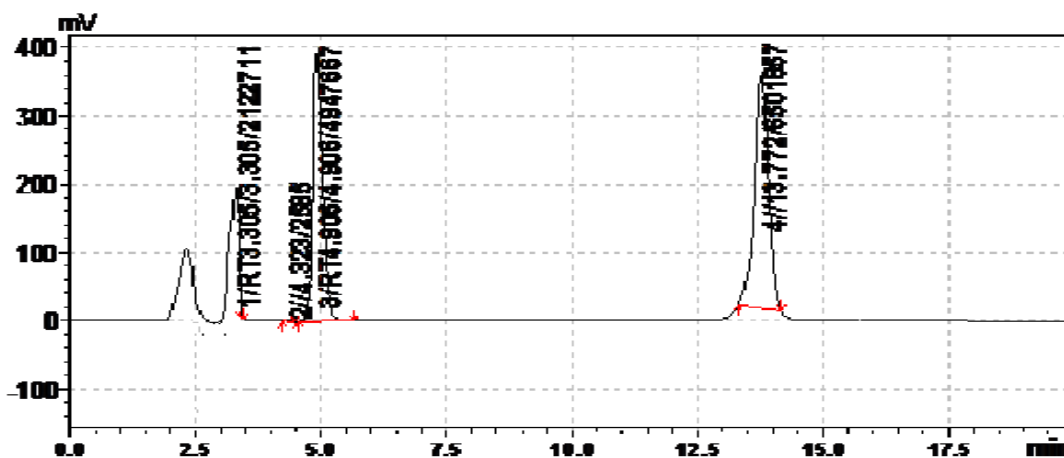


Figure-4. Chromatogram showing the separation of alprazolam and Propranolol in stressed sample of the synthetic mixture subjected to alkali Hydrolysis.

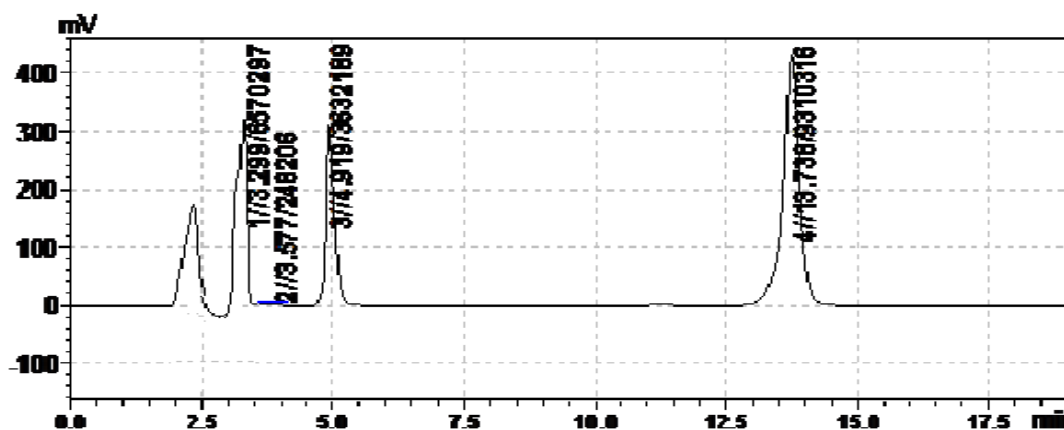


Figure-5. Chromatogram showing the separation of alprazolam and Propranolol in stressed sample of the synthetic mixture subjected to Oxidative Hydrolysis.

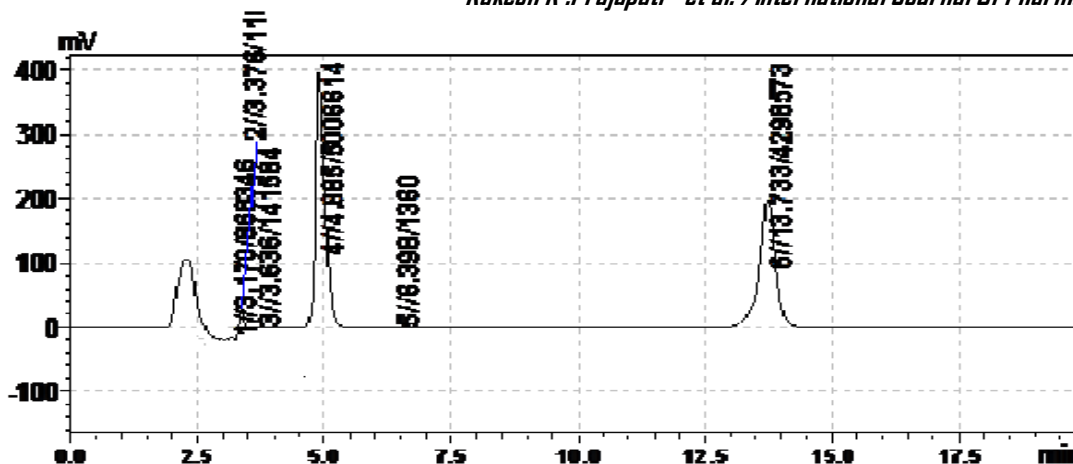


Figure-6. Chromatogram showing the separation of alprazolam and Propranolol in stressed sample of the synthetic mixture subjected to Thermal condition.

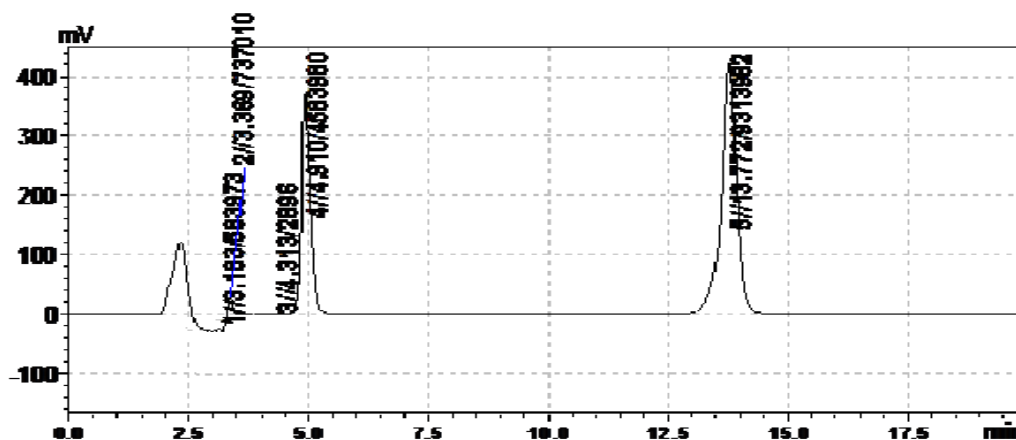


Figure-7. Chromatogram showing the separation of Alprazolam and Propranolol in stressed sample of the synthetic mixture subjected to Photolytic condition.

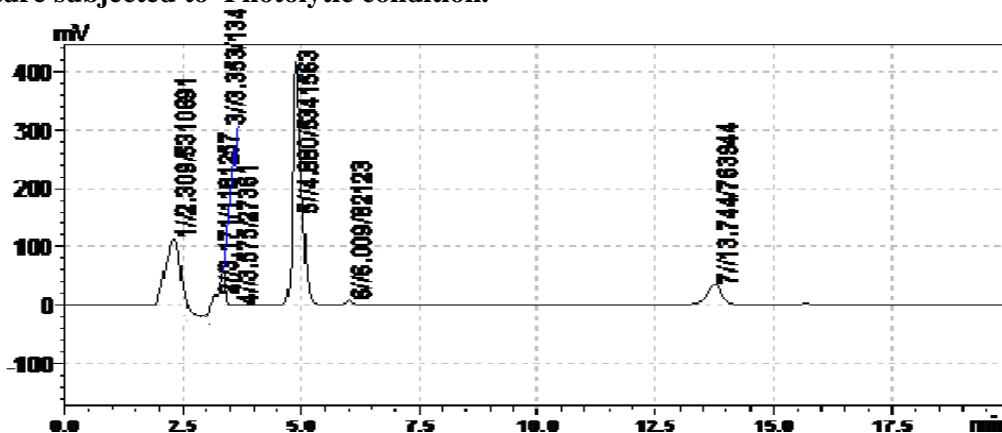


Figure-8. Chromatogram showing the separation of Alprazolam and Propranolol in degraded formulation subjected to Thermal condition.

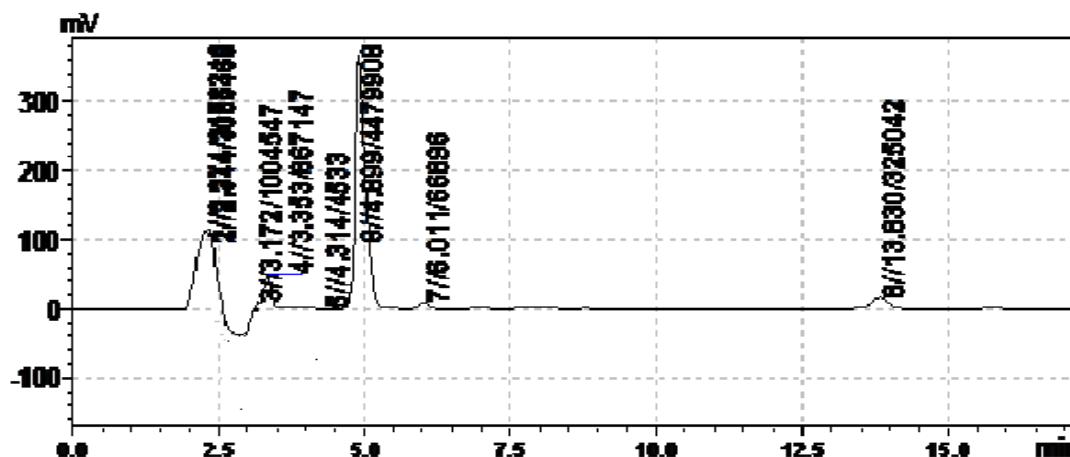


Figure 9 Chromatogram showing the separation of Alprazolam and Propranolol in degraded formulation subjected to Photolytic condition.

### Degradation in alkali

PRP underwent alkali hydrolysis but the rate of hydrolysis was slower than that under acidic condition. The degradation patterns was similar to that seen under acidic condition . Degradation peaks for PRP were at 3.3 min.

### Oxidative Condition

Both the drugs were highly liable to oxidative hydrolysis in 3% H<sub>2</sub>O<sub>2</sub> , PRP was comparatively more labile than ALP . Around 48% degradation was observed in the case of PRP and 15-20% in the case of ALP .Degradation peaks for PRP were at 3.3 min.

### Thermal degradation

Both the drugs relatively stable when exposed to dry heat at 80 °C for 48 hours. The percentage of both drugs remaining after 48 hours of exposure to dry heat were in the range of 95-98%. Degradation peaks of PRP were at 3.3 min and for ALP were at 3.1, 3.3 min.

### Photolytic conditions

Both the drugs relatively stable when exposed to UV for 36 hours. The percentage of both drugs remaining after 36 hours of exposure to UV were in the range of 95-98%. Degradation peaks of PRP were at 3.3 min and for ALP were at 3.1, 3.3 min.



**Establishment of a Stability-indicating Method for a mixture of Stressed Solutions**

The mobile phase Phosphate buffer –acetonitrile – methanol (55+35+10,V/V/V) was first used to analyze individual standard drug sample for linearity study(figure 1). It was then applied to stressed sample of individual drugs and subsequently used to analyze synthetic mixture, which shows recognizable degradation (figure 2-6).

The method was then successfully applied to degradation studies of formulation(figure 7 and 8)

**Validation of Proposed Method**

The method was validated with respect to the following parameters given below;

Linearity:- Linearity calibration plot for above method was obtained in the calibration ranges of 1-10 µg/ml and 80-800 µg/ml, respectively, and the correlation coefficient obtained were >0.999(Table 1).

**Table-1. System suitability parameters for the determination of alprazolam(ALP) and propranolol(PRP) by proposed HPLC method.**

Parameter	ALP	PRP
Linearity Range	1-10 µg/ml	80-800 µg/ml
Regression Coefficient	0.999	0.999
Slop	48776	5031
LOD	0.052	1.2
LOQ	0.32	0.4
Retention Time	4.78	13.73
Tailing Factor	1.2	0.92
Resolution Factor	8.95	-
Theoretical Plates	3708.04	11248.11

\*Average of Six Replicate

LOD and LOQ – The LOD value for ALP and PRP were 0.032µg/ml and 0.4 µg/ml, respectively , and the LOQ values foe ALP and PRP were 0.107 µg/ml and 1.2µg/ml, respectively.

**Precision:** Data obtained from analysis of the sample on the same day (n=6) and on consecutive days(n=6) are given in the table-4. The RSD values obtained were well below 2%. The RSD value indicate that the method is sufficiently precise. The intermediate precision established for the method showed that similar resolution was obtained when the experiment was conducted with 2 different days(Table 4).

**Table-4. Statistical evaluation of Precision for determination of alprazolam and propranolol by proposed HPLC method.**

	Alprazolam		Propranolol	
	Repeatability	Intermediate Precision	Repeatability	Intermediate Precision
% Assay Mean ± SEM	99.87 ± 0.5090	100.01 ± 0.3214	100.01 ± 0.3767	100.03 ± 0.5380
% RSD	1.2	0.8	0.4	1.3
% Difference of two Means	0.14		0.02	

Accuracy:- Percent recoveries were calculated from the differences between the peak area obtained for the fortified and unfortified solutions. Good recoveries were obtained for each fortification level(Table 3) ,indicating that method is accurate.

Table-3. Recovery studies for determination of alprazolam and propranolol by proposed HPLC method.

Drug	Added, µg/ml	Recovery ± SD	% RSD
ALP	1	100.36 ± 1.67	1.6
	2	100.20 ± 0.761	0.76
	3	99.56 ± 0.0134	0.134
	80	100.50 ± 1.19	0.99
PRP	160	100.76 ± 2.0	0.99
	240	99.26 ± 0.231	0.23

\*n = 3

Specificity :- The specificity of the HPLC method was shown by the complete separation of ALP and PRP from their degradation products. The degradation products of ALP and PRP were found to be similar for both the tablets and active pharmaceutical ingredients(API) standards. Resolution Factor values for the acidic , alkaline, oxidative, thermal and photolytic degradation products were always >1.9, which ensured the complete separation of ALP and PRP from their degradation products. Studies using PDA detection to determine the purity of ALP and PRP peaks showed peak purity index were always >0.998 , thereby indicating that the ALP and PRP peaks were free from any coeluting peaks.

**Robustness:** The method remained robust even with small variation in flow rate(±0.1 ml/min) and the concentration of acetonitrile (±5 ml) in the mobile phase. There was no significant influence on the determination. Insignificant differences in peak area and less variability in retention times were observed(table 5)

Table-5. Robustness studies for determination of alprazolam and propranolol by proposed HPLC method.

Compound	% RSD (n= 5)		
	Normal Condition	Changed Condition	
<b>Temperature</b>	<b>Normal</b>	<b>(-5°C)</b>	<b>(+5°C)</b>
Alprazolam	0.8	0.7	0.7
Propranolol	1.2	1.0	1.12
<b>Ph</b>	<b>Normal</b>	<b>(-0.2 unit)</b>	<b>(+0.2 unit)</b>
Alprazolam	0.8	1.0	0.1
Propranolol	1.2	1.4	0.6
<b>Flow Rate</b>	<b>Normal</b>	<b>(0.9ml/min)</b>	<b>(1.1ml/min)</b>
Alprazolam	0.8	0.1	0.2
Propranolol	1.2	0.4	0.52
<b>Mobile phase ratio</b>	<b>Normal</b>	<b>(-5%)</b>	<b>(+5%)</b>
Alprazolam	0.8	0.1	0.2
Propranolol	1.2	0.8	0.7

### Applicability of Developed Method to Marketed Formulations

A clear separation of the drugs and degradation products was obtained for tablets with no interference from excipients( figures 7 and 8).This result indicate that the method could be extended to the study of available drug content in commercial products(Table 2)

Table-2. Assay result obtained for combined dosage form by proposed HPLC method.

Tablet	Drug,mg/Tablet	Drug found $\pm$ SD	% RSD
ANZAL-PLUS	ALP 0.20 mg	97.54 $\pm$ 1.16	1.156
	PRP 20 mg	99.01 $\pm$ 0.15	0.107

\*n = 3

### Conclusions

The combination of ALP and PRP has gained popularity for treatment of panic disorders. The major constraint in the determination of these drugs in combination is the ratio in which they are incorporated in tablets( ALP:PRP = 1:80).The present study examined the stability behavior of ALP and PRP individually and in combination according to ICH guidelines. PRP was found to be more susceptible under stress conditions in comparison with ALP.

The work described in this paper has shown that the developed method is precise, accurate, linear and stability indicating. The method was found to be specific to the drugs, because the peaks of degradation products did not interfere with the drugs peaks. Application of this method to the determination of ALP and PRP in tablet dosage form shows that neither the degradation nor the excipients interfere with the analysis. The finding indicates that the proposed method could be used as a stability-indicating method for simultaneous determination of ALP and PRP either in the bulk drug or in pharmaceutical formulations.

### Acknowledgments

We are thankful to the Merrill pharma ( Roorakee , Uttarkhand) for supplying gift sample of ALP and PRP.

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