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# Development and Validation of Stability Indicating HPLC Method for Simultaneous Estimation of Clonazepam and Propranolol HCl in their Combined Marketed Dosage Form

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**ABSTRACT:**

A simple, rapid, economical, precise and accurate HPLC method for simultaneous estimation of Propranolol HCl and Clonazepam in Their Combined Dosage Form has been developed. A Stability indicating reverse phase high performance liquid chromatographic method was developed for the simultaneous estimation of Propranolol HCl and Clonazepam in their combined dosage form. The separation was achieved by LC- 20 AT C18 (250mm x 4.6 mm x 2.6 µm) column and Buffer (Potassium Phosphate, pH 4.5): Methanol (85:15) at a flow rate of 1 ml/min. Detection was carried out at 240 nm. Retention time 4.080 min and 5.343 for Propranolol HCl and Clonazepam respectively. The method has been validated for linearity, accuracy and precision. Linearity observed for Propranolol HCl 40-120 µg/ml and for Clonazepam 1-3 µg/ml. Developed method was found to be accurate, precise and rapid for simultaneous estimation of Propranolol HCl and Clonazepam in their combined dosage form. The proposed method was successfully applied for the simultaneous estimation of both the drugs in commercial combined dosage form. The drug was subjected to stress condition of hydrolysis, oxidation, photolysis and Thermal degradation, Considerable Degradation was found in Thermal degradation. The proposed method was successfully applied for the simultaneous estimation of both the drugs in commercial Combined dosage form.

**KEY WORDS:** Propranolol HCl, Clonazepam, Simultaneous Estimation, HPLC Method, Validation

## INTRODUCTION:

**Clonazepam:** Clonazepam (Fig.2) which is chemically 5-(2-Chlorophenyl)-7-nitro-1,3-dihydro-1,4-benzodiazepin-2-one. CNZ is an anticonvulsant used for several types of seizures[1], including myotonic or atonic seizures, photosensitive epilepsy, and absence seizures, although tolerance may develop .which is Cytochrome P-450 CYP2E1 Inhibitors[2].

**Propranolol Hydrochloride :** Propranolol hydrochloride (Fig.1) which is chemically 1-(naphthalen1yloxy)-3-[(propan-2-yl)amino]propan-2-ol, Hydrochloride[4]. PRH is a widely used non cardio selective beta adrenergic antagonist mainly used in treatment of high blood pressure. Also used in treatment of acute myocardial infraction, angina pectoris, anxiety.

This Combination of Clonazepam and Propranolol HCl used For the treatment, control, prevention, & improvement of the following diseases, conditions and symptoms: Hypertension; Treatment of panic disorder; Treatment of seizure disorder;Fast heartbeat. Clonazepam

is official in IP-2010(RP-HPLC)[5], USP30-NF25(RP-HPLC)[6], BP-2009(RP-HPLC)[7].Propranolol HCl is official in IP-2010[8], USP30-NF25(Liquid Chromatography)[9], BP-2009(Potentiometric Titration)[10].A review of the literature states that the quantity of their analytical methods is available for the estimation of its combination in the form of clonazepam and propranolol HCl. But no method has been reported for stability indicating simultaneous estimation of Clonazepam and Propranolol HCl in combined pharmaceutical dosage form by RP-HPLC. So it is develop stability indicating reverse phase high performance liquid chromatographic method for simultaneous estimation of Clonazepam and Propranolol HCl in their Combined Dosage form.Therefore, the goal of the present work is to develop simple, accurate, fast, Specialized, sensitive and selective stability indicating HPLC method for simultaneous estimation of clonazepam and propranolol HCl in their combine dosage form.

**MATERIAL AND METHADOLOGY:**

**Material:**

The reference samples of Propranolol HCl and Clonazepam were obtain Form the Yash pharma Pvt Ltd. Purified water was obtain from the Merck specialties pvt, Ltd., Mumbai. HPLC grade methanol and acetonitrile also obtain from the Merck specialties pvt, Ltd., Mumbai were used for the preparing the mobile phase and the diluent. Potassium Dihydrogen Phosphate analytical grade obtain from Merck specialties pvt, Ltd. Petril-Beta, tablet a combination of Clonazepam(0.25mg) and Prapronalol HCl(10mg) manufactured by Micro Labs Limited was purchased from local firms.

**Mobile phase:** Potassium Dihydrogen Phosphate buffer and methanol (adjust to pH 4.5 orthophosphoric acid) in the ration (15:85) v/v was used for separation of this drug. The mobile phase was filttered through the 0.22µ membrane filter after sonication of each solvent for 20 min.

**Diluent:** mixture of Potassium Dihydrogen Phosphate buffer and methanol (adjust to pH 4.5 orthophosphoric acid) in the ratio of 85:15v/v was used.

**Method development:**

**Selection of wavelength**

The sensitivity of HPLC method that uses UV detection depends upon proper selection of detection wavelength. An ideal wavelength is the one that gives good response for the drugs that are to be detected. In the present study drug solutions of Propranolol HCl (80 ppm) and Clonazepam (2 ppm) were prepared in Methanol over 200nm to 400nm.Both the components show the good response at 240nm.therefore wavelength 240nm was selected for further study.

**Selection of Mobile Phase**

Trail contains various mobile phase which are considered of Methanol, Water and Acetonitrile in different proportions and different volumes at different flow rate were tried.

**Optimization of flow rate**

1ml/min flow rate, proved to be better than the other in terms of resolution, peak shape and shorter retention time

**Chromatographic separation:**

Standard solutions of 800 µg/ml of propranolol HCl and 20µg/ml of Clonazepam were injected in column with 20 µl micro-syringe. The chromatogram was run for appropriate minutes with mobile phase Buffer (pH 4.5): Methanol (85:15).The detection was carried out at wavelength 240 nm. The chromatogram was stopped after separation achieved completely. Data related to peak like area, height, retention time, resolution etc were recorded using software.

**Preparation of standard solution of mixtures of Propranolol HCl (80 ppm) and Clonazepam (2 ppm)**

**(A) Propranolol HCl standard stock solution: (800 µg/mL)**

A 80 mg of Propranolol HCl was weighed and transferred to a 100 mL volumetric flask. volume was made up to the mark with mobile phase.

**(B) Clonazepam standard stock solution: (20 µg/mL)**

A 20 mg of Clonazepam was weighed and transferred to a 100 mL volumetric flask. volume was made up to the mark with mobile phase. Take 10ml from this Solution and transfer to 100ml Volumetric flask and Volume was made up with the Mobile phase

**(C) Preparation of standard solution of binary mixtures of Propranolol HCl (80 µg/mL) and Clonazepam (2 µg/mL)**

Take 1 mL from the Propranolol HCl stock solution and 1mL from Clonazepam stock solution and transferred to 10 mL volumetric flask and volume made up to the mark by mobile phase which was used in particular trials.

**(D) Sample Stock Solution (Propranolol HCl 800 µg/ml, Clonazepam 20 µg/ml )**

Take Tablet Powder equivalent to 80 mg of Propranolol HCl, and 2 mg of Clonazepam was transferred to a 100 ml volumetric flask, Add 60 ml Mobile phase, Shake well for 15 minutes and make up volume with Mobile phase. The solution was filtered through Whatman filter paper no. 42.

**System suitability test:**

These tests are used to verify that the resolution and reproducibility of the system are adequate for the analysis to be performed. System suitability tests are based on the concept that the equipment, electronics, analytical operations and samples constitute an integral system that can be evaluated as a whole. System suitability testing provides assurance that the method will provide accurate and precise data for its intended use.

**STABILITY STUDY:**

**Acid degradation**

Acid decomposition studies were performed by Transferring 1ml of stock solution in to 10 ml of volumetric flask. Two ml of 0.1 N HCl solutions was added and mixed well and put for 5 hrs at Room temperature. After time period volume was adjusted with diluent to get 2 µg/ml for Clonazepam and 80 µg/ml for Propranolol HCl.

**Base degradation**

Basic decomposition studies were performed by transferring 1ml of stock solution in to 10 ml of volumetric flask. Two ml of 0.1 N NaOH solutions was added and mixed well and put for 3 hrs at Room temperature. After time period volume was adjusted with diluent to get 2µg/ml for Clonazepam and 80µg/ml for Propranolol HCl.

**Thermal degradation**

Thermal decomposition studies were performed by transferring 1ml of stock solution in to 10 ml of volumetric flask and were kept in an Oven at $105^0$C for 24 hrs. After time period volume was adjusted with diluent to get 2µg/ml for Clonazepam and 80µg/ml for Propranolol HCl.

**Oxidation degradation**

Oxidative decomposition studies were performed by transferring 1ml of stock solution in to 10 ml of volumetric flask. Two ml of 30% $H_2O_2$ solutions was added and mixed well and put for 4 hrs at Room temperature. After time period volume was adjusted with diluent to get 2µg/ml for Clonazepam and 80µg/ml for Propranolol HCl.

**Photo degradation**

Photo decomposition studies were performed by transferring 1ml of stock solution in to 10 ml of volumetric flask. ant was kept in UV Chamber for 10 hrs. After time period volume was adjusted with diluent to get 2µg/ml for Clonazepam and 80µg/ml for Propranolol HCl.

**VALIDATION OF RP-HPLC METHOD**

**Specificity:**

No interference is found in Chromatograms of Propranolol HCl and Clonazepam std and Propranolol HCl and Clonazepam sample with the Chromatogram of Propranolol HCl and Clonazepam Blank, which shows that method which is developed is specific.

**Linearity:**

The linearity for Clonazepam and Propranolol HCl were assessed by analysis of combined standard solution in range of 1-3 µg/ml and 40-120 µg/ml respectively, 5,7.5,10,12.5,15 ml solutions were pipette out from the Stock solution of Clonazepam (20 µg/ml) and Propranolol HCl (800 µg/ml) and transfer to 100 ml volumetric flask and make up with mobile phase to obtain 1,1.5,2,2.5 and 3 µg/ml, and 40,60,80,100 and 120 µg/ml for Clonazepam and Propranolol HCl respectively

In term of slope, intercept and correlation co-efficient value. The graph of peak area obtained verses respective concentration was plotted. Correlation co-efficient for calibration curve Clonazepam and Propranolol HCl was found to be 0.999 and 0.999 respectively. The regression line equation for Clonazepam and Propranolol HCl are as following:

For Clonazepam **y = 102.27x - 2.633** and For Propranolol HCl: **y = 12.55x - 1.193**

**Precision**

**I. Repeatability** The data for repeatability of peak area measurement for Clonazepam (2 µg/ml) and Propranolol HCl (80 µg/ml) based on six measurements of same solution of Clonazepam (2 µg/ml) and Propranolol HCl (80 µg/ml).The % RSD for Clonazepam and Propranolol HCl was found to be 0.551 and 0.665 respectively.

**II. Intraday precision** Standard solution containing (40,80,120 µg/ml) of Propranolol HCl and (1,2,3 µg/ml) of Clonazepam were analyzed three times on the same day and % R.S.D was calculated.

**III. Interday precision** Standard solution containing (40,80,120 µg/ml) of Propranolol HCl and (1,2,3 µg/ml) of Clonazepam were analyzed three times on the different day and % R.S.D was calculated.

**Accuracy:**

**For Propranolol HCl**

40 µg/ml drug solution was taken in three different flask label A, B and C. Spiked 80% , 100%, 120% of standard solution in it and diluted up to 10ml. The area of each solution peak was measured at 240 nm. The amount of Propranolol HCl was calculated at each level and % recoveries were computed

**For Clonazepam**

1 µg/ml drug solution was taken in three different flask label A, B and C. Spiked 80% , 100%, 120% of standard solution in it and diluted up to 10ml. The area of each solution peak was measured at 240 nm. The amount of Clonazepam was calculated at each level and % recoveries were computed.

**Analysis of marketed formulation by developed method**

**Sample Stock Solution (Propranolol HCl 800 µg/ml, Clonazepam 10 µg/ml )**

Take Tablet Powder equivalent to 80 mg of Propranolol HCl, and 2 mg of Clonazepam was transferred to a 100 ml volumetric flask, Add 60 ml Mobile phase, Shake well for 15 minutes and make up volume with Mobile phase. The solution was filtered through Whatman filter paper no. 42.

**Working Sample Preparation (Propranolol HCl 80 µg/mL, and Clonazepam 2 µg/mL):**

Take 1 mL from standard stock solution and transferred to 10 ml volumetric flask and made up volume up to the mark with the mobile phase Inject above Solution 20 µl for Assay Analysis.

**Table 2: Results of System Suitability Parameter**

| Parameters | Propranolol HCl | Clonazepam |
|---|---|---|
| Retention Time | 4.080 | 5.343 |
| Theoretical Plates | 7180 | 3369 |
| Asymmetry | 1.385 | 1.264 |
| Resolution | 4.505 | |

**Table 3: Clonazepam and Propranolol HCl standard for stability**

| Drugs | Area |
|---|---|
| **Clonazepam** | 188.104 |
| **Propranolol HCl** | 916.253 |

**Table 4: Clonazepam % Degradation**

| Clonazepam | | | | |
|---|---|---|---|---|
| Parameter | **Standard** | | **Sample** | |
| | **Area** | **%Degradation** | **Area** | **%Degradation** |
| **Acid** | 150.515 | 19.983 | 153.131 | 18.592 |
| **Base** | 155.049 | 17.573 | 145.5 | 22.649 |
| **Oxidation** | 152.928 | 18.7 | 149.068 | 20.752 |
| **Photo** | 152.282 | 19.044 | 153.976 | 18.143 |
| **Thermal** | 158.013 | 15.997 | 163.108 | 13.288 |

**Table 5: Propranolol HCl % Degradation**

| **Propranolol HCl** | | | | |
|---|---|---|---|---|
| **Parameter** | **Standard** | | **Sample** | |
| | **Area** | **%Degradation** | **Area** | **%Degradation** |
| **Acid** | 798.943 | 12.803 | 787.87 | 14.012 |
| **Base** | 662.278 | 27.719 | 682.565 | 25.505 |
| **Oxidation** | 749.528 | 18.196 | 738.145 | 19.439 |
| **Photo** | 815.743 | 10.97 | 806.221 | 12.009 |
| **Thermal** | 638.708 | 30.291 | 619.163 | 32.424 |

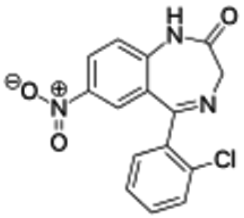

Figure 1: clonazepam

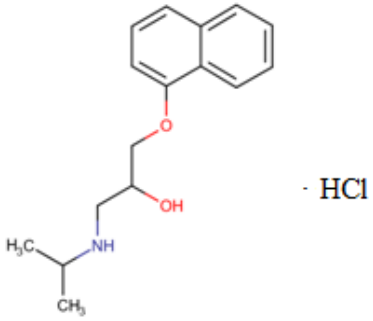

**Figure 2: Propranolol HCL**

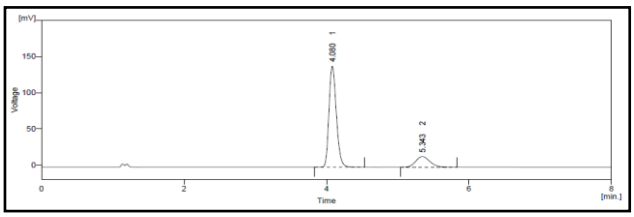

**Figure 3 : HPLC Chromatogram of Propranolol HCl 80 ppm and Clonazepam 2 ppm in Buffer (pH 4.5): Methanol (85:15) (Final)**

**Table: 6 Linearity data for Propranolol HCl**

| Sr.No | Concentration (µg/ml) | Area |
|---|---|---|
| **1** | 40 | 510.871 |
| **2** | 60 | 735.164 |
| **3** | 80 | 1008.459 |
| **4** | 100 | 1251.952 |
| **5** | 120 | 1507.44 |

**Table: 7 Linearity data for Clonazepam**

| Sr.No | Concentration (µg/ml) | Area |
|---|---|---|
| **1** | 1 | 102.27 |
| **2** | 1.5 | 147.49 |
| **3** | 2 | 202.41 |
| **4** | 2.5 | 251.36 |
| **5** | 3 | 306.01 |

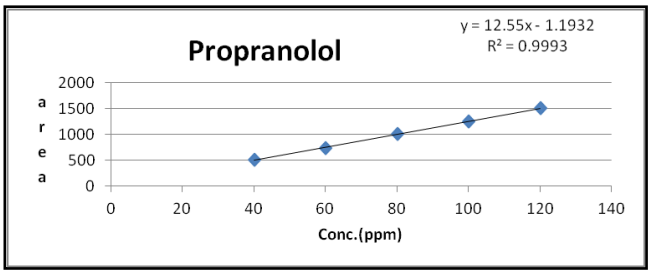


**Figure 4 : Calibration Curve of Propranolol HCl (40-120 µg/ml)**

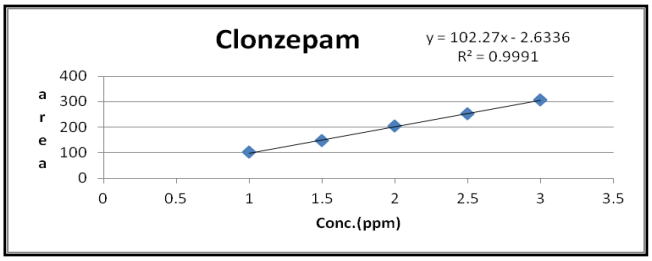


**Figure 5 : Calibration Curve of Clonazepam (1-3 µg/ml)**

**Table 8: Repeatability data for Propranolol HCl**

| Sr No. | Conc (µg/ml) | Area | Mean ± S.D (n=6) | % R.S.D |
|---|---|---|---|---|
| **1.** | 80 | 1011.478<br>1009.442<br>996.977<br>1009.389<br>1007.360<br>1017.485 | 1008.689±6.710 | 0.665 |

**Table 8: Repeatability data for Clonazepam**

| Sr No. | Conc (µg/ml) | Area | Mean ± S.D (n=6) | % R.S.D |
|---|---|---|---|---|
| **1** | 2 | 203.033<br>202.648<br>200.811<br>202.615<br>202.2<br>204.242 | 202.592 ±1.118 | 0.552 |

**Table 9: Intraday precision data for estimation of Propranolol HCl**

| SR. NO. | Conc. (µg/ml) | Area Mean ± S.D. (n=3) | % R.S.D |
|---|---|---|---|
| **1** | 40 | 507.460 ± 2.414 | 0.476 |
| **2** | 80 | 1020.596 ± 4.406 | 0.432 |
| **3** | 120 | 1510.421± 4.710 | 0.312 |

**Table 10 : Intraday precision data for estimation of Clonazepam**

| SR. NO. | Conc. (µg/ml) | Area Mean ± S.D. (n=3) | % R.S.D |
|---|---|---|---|
| **1** | 1 | 101.769 ± 0.177 | 0.174 |
| **2** | 2 | 203.844 ± 1.446 | 0.709 |
| **3** | 3 | 301.321 ± 3.841 | 1.274 |

**Table 11: Interday precision data for estimation of Propranolol HCl**

| SR. NO. | Conc. (µg/ml) | Area Mean ± S.D. (n=3) | % R.S.D |
|---|---|---|---|
| **1** | 40 | 509.291 ± 4.371 | 0.858 |
| **2** | 80 | 990.636± 12.191 | 1.231 |
| **3** | 120 | 1516.091± 7.122 | 0.470 |

**Table 12: Interday precision data for estimation of Clonazepam**

| SR. NO. | Conc. (µg/ml) | Area Mean ± S.D. (n=3) | % R.S.D |
|---|---|---|---|
| **1** | 1 | 101.345 ± 1.913 | 1.888 |
| **2** | 2 | 199.053± 2.104 | 1.057 |
| **3** | 3 | 304.791 ± 0.914 | 0.300 |

**Table 13: Recovery data for Propranolol HCl**

| SR. NO. | Conc. Level (%) | Sample amount (µg/ml) | Amount Added (µg/ml) | Amount recovered (µg/ml) | % Recovery | % Mean Recovery ± S.D |
|---|---|---|---|---|---|---|
| **1** | 80 % | 40 | 32 | 32.388 | 101.214 | 100.312 ± 1.191 |
| **2** | | 40 | 32 | 32.244 | 100.762 | |
| **3** | | 40 | 32 | 31.668 | 98.962 | |
| **4** | 100 % | 40 | 40 | 39.926 | 99.814 | 100.889 ± 0.948 |
| **5** | | 40 | 40 | 40.499 | 101.247 | |
| **6** | | 40 | 40 | 40.642 | 101.606 | |
| **7** | 120 % | 40 | 48 | 48.358 | 100.747 | 101.296 ± 0.663 |
| **8** | | 40 | 48 | 48.975 | 102.032 | |
| **9** | | 40 | 48 | 48.532 | 101.109 | |

**Table 14: Recovery data for Clonazepam**

| SR. NO. | Conc. Level (%) | Sample Amount | Amount Added | Amount recovered (µg/ml) | % Recovery | % Mean Recovery ± S.D |
|---|---|---|---|---|---|---|
| **1** | 80 % | 1 | 0.8 | 0.807 | 100.823 | 100.383 ± 0.973 |
| **2** | | 1 | 0.8 | 0.808 | 101.058 | |
| **3** | | 1 | 0.8 | 0.794 | 99.268 | |
| **4** | 100 % | 1 | 1 | 1.001 | 100.122 | 100.937 ± 1.205 |
| **5** | | 1 | 1 | 1.023 | 102.321 | |
| **6** | | 1 | 1 | 1.004 | 100.367 | |
| **7** | 120 % | 1 | 1.2 | 1.213 | 101.048 | 100.388 ± 0.594 |
| **8** | | 1 | 1.2 | 1.203 | 100.219 | |
| **9** | | 1 | 1.2 | 1.199 | 99.896 | |

**Table15 : Analysis on marketed formulation**

| Tablet | Petril-Beta | |
|---|---|---|
| Label claim | **Propranolol HCl (10mg)** | **Clonazepam (0.25mg)** |
| Assay (% of label claim*) Mean ± S. D. | 98.53±0.950 | 101.33±1.041 |

**RESULTS AND DISCUSSION:**

The goal of this study was developing a sensitive, precise and accurate stability indicating HPLC method for simultaneous estimation of Propranolol HCL and clonazepam in their combined marketed dosage form. In order to achieve the optimum separation of the component peak, various proportions of buffer with methanol were tested as mobile phase on C18 (25cm x 0.46 cm) Hypersil BDS coloumn. Mobile phase containing a mixture of Potassium Dihydrogen Phosphate buffer and methanol (adjust to pH 4.5 orthophosphoric acid) in the ration(15:85) v/v was selected as it resulted in peak with good symmetry and resolution. The flow rate of 1.0mL/min was found to be optimum in the 0.5 to 1.0mL/min range resulting in the short retention time, baseline stability and minimum noise. The retention time of Propranolol HCL and Clonazepam were found to be 4.080min and 5.343min respectively showing the proposed method is time saving (figure 3) The standard are of Clonazepam(188.104) and Propranolol HCL(916.253) for stability is mention in Table 3. The % of degredation of both Clonazepam and Propranolol HCl in Acidic, Basic, Oxidative, Thermal, Photolytic condition show the both drug are stable in all Conditions which is show in Table no 4 and Table 5.the calibaration curve show the linearity in the concentration rang 40-120**(µg/ml)** for Propranolol HCl and 1 to 3**(µg/ml)**for Clonazepam (figure 4 and 5). The regression equation of concentration over their peak area were found by Clonazepam **y = 102.27x - 2.633** and For Propranolol HCl: **y = 12.55x - 1.193.**The results of intraday and interday precision values are represented in (Table 9 to 12).the RSD % for assay of drugs during intra day and inter day were 0.709 and 1.231 for clonazepam and propranolol HCL.The percentage mean recovery of individual analys was high, satisfactory and indicate the purpose method is acuurate. The no of theoretical plate was determined to be 7180 for propranolol HCl and 3369 for clonazepam.

**CONCLUSION**:

A simple, specific, accurate and precise Stability indicating RP-HPLC method has been developed and validated as per ICH guideline for Simultaneous Estimation of Propranolol HCl and Clonazepam in their combined dosage form. Validation parameters like Linearity, Accuracy, Precision, Robustness, System suitability, Specificity were tested. Observation of all these parameters leads to the point that developed Stability indicating RP-HPLC method is linear, accurate, precise, specific. It can be successfully adopted for routine quality control analysis of Propranolol HCl and Clonazepam in Combined dosage form without any interference from common excipients and impurity. This method can now transfer to utilize for routine laboratory analysis and assay of Propranolol HCl and Clonazepam in their combined dosage form.

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