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Development and Validation of a Stability-Indicating HPLC Method for determination of Temazepam and its Related Substance

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ABSTRACT

A new HPLC method was developed for selective and simultaneous determination of Temazepam. The developed method is also applicable for the related substances determination in Temazepam. The chromatographic separation was achieved on a Zorbax SB C-18, 4.6 x 250mm, and 5µ column. The mobile phase consisted of buffer and acetonitrile (60:40, V/V) delivered at a flow rate of 2.0 mL min^{-1}. Buffer consisted of 0.03M of dipotassium hydrogen orthophosphate and 2 mL of triethylamine, adjusted to a pH 3.0 with ortho phosphoric acid. The mobile phase was pumped at a flow rate of 2.0 mL min^{-1} and UV detector at 245 nm was used. In the developed HPLC method, the resolution between Temazepam and its potential impurities, namely Impurity-A, Impurity-B, Impurity-C, and Impurity-G was found to be satisfactory. Accuracy, found by recovery experiments varied between from 99.2-100.5 at 80.0% to 120.0% level. The curves between concentration is response were linear with coefficient of corrolation (r) not less than 0.99. The drug was subjected to stress conditions of hydrolysis, oxidation, photolysis and thermal degradation. Considerable degradation was found to occur in acid hydrolysis condition. The stress samples were assayed against a qualified reference standard and the mass balance was found close to 95% - 105%. The developed Reverse Phase-LC method was validated with respect to specificity, linearity, accuracy, precision and robustness. The validation was performed according to the current requirements as laid down in the ICH guidelines.

Key words: Temazepam, impurities, HPLC, Stability-indicating, Method development.

INTRODUCTION

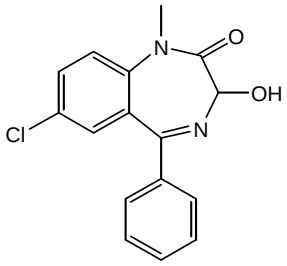
Temazepam (marketed under brand names Normison, Temtabs, Euhypnos, Restoril, Remestan, Tenox, Temaze and Norkotral) is an intermediate-acting 3-hydroxy benzodiazepine. It is generally prescribed for the short-term treatment of sleeplessness in patients who have difficulty maintaining sleep. In addition, Temazepam has anxiolytic (anti-anxiety), anticonvulsant, and skeletal muscle relaxant properties [1-3]. Temazepam was first synthesized in 1964, but it first came into use in 1969 when its ability to counter insomnia was realized [4]. By the late 1980s, Temazepam was one of the most popular and widely prescribed hypnotics on the market and it

became one of the most widely prescribed drugs. Temazepam is a hypnotic agent. In sleep laboratory studies, Temazepam significantly decreased the number of nightly awakenings [5] but has the drawback of distorting the normal sleep pattern [6]. Temazepam is officially indicated for severe insomnia and other severe or disabling sleep disorders. The prescribing guidelines in the UK limit the prescribing of hypnotics to two-to-four weeks due to concerns of tolerance and dependence [7].

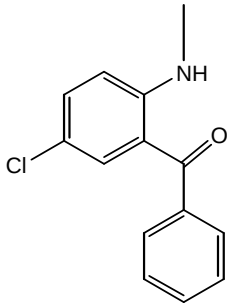
Temazepam should not be used in pregnancy, as it may cause harm to the fetus. The safety and effectiveness of Temazepam has not been established in children; therefore Temazepam should generally not be given to individuals under 18 years of age, and should not be used at all in children under 6 months old. Benzodiazepines also require special caution if used in the elderly, alcohol or drug-dependent individuals and individuals with comorbid psychiatric disorders [8]. The smallest possible effective dose should be used in elderly or very ill patients, as there is a risk of apnea and/or cardiac arrest. This risk is increased when Temazepam is given concomitantly with other drugs that depress the central nervous system [9].

Chronic or excessive use of Temazepam may cause drug tolerance, which can develop rapidly [10], so this drug is therefore not recommended for long-term use. In 1979 the Institute of Medicine (USA) and the National Institute on Drug Abuse stated that most hypnotics lose their sleep-inducing properties after about 3 to 14 days [11]. In use longer than 1–2 weeks, tolerance will frequently develop towards the ability of Temazepam to maintain sleep, so that the drug loses effectiveness [12]. Some studies have observed tolerance to Temazepam after as little as one week's use[13]. Another study examined the short-term effects of the accumulation of Temazepam over 7 days in elderly inpatients, and found that little tolerance developed during the accumulation of the drug[14]. Other studies examined the use of Temazepam over six days and saw no evidence of tolerance [15,16].

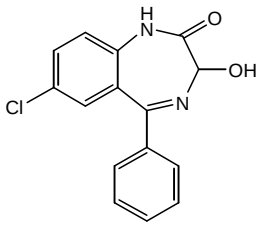
Fig. 1. Chemical structure of Temazepam and it`s impurities



Temazepam



Impurity A



Impurity B

The objectives of the present manuscript are to study the degradation behaviour of Temazepam under hydrolysis (acid, base and neutral), oxidation, thermal and photolysis conditions, to optimize the liquid chromatography conditions to separate the drug from it's degradation products on a reverse phase C_{18} column and to establish a validated stability-indicating assay HPLC method by UV detection at 245 nm. These studies provide precious information about drug's inherent stability and assist in the validation of analytical methods to be used in stability studies [17]. The developed HPLC Assay method was validated as per the International Conference on Harmonization (ICH) guidelines [18, 22, 23]. Several HPLC methods have been reported for the determination of Temazepam in biological samples[19-21]. Furthermore, to the best of our knowledge, no stability-indicating assay method for this drug is reported in the literature.

MATERIALS AND METHODS

Reagents and Chemicals:

Analytical grade reagents and HPLC grade solvents were used. Milli-Q water (Millipore Corporation, USA) was used. Acetonitrile and methanol, triethylamine, anhydrous potassium dihydrogen orthophosphate, ortho phosphoric acid (85 %), hydrochloric acid, sodium hydroxide and hydrogen peroxide were purchased from Merck Research Laboratories, India. Temazepam drug was obtained as a gift sample from a local manufacturing unit in Hyderabad.

Chromatographic conditions:

Chromatographic system consisted of a Waters Model Alliance 2489 separation module equipped with auto sampler, photodiode array and ultraviolet (UV) detector. The data were recorded using empower software. HPLC analytical column; Zorbax SB C-18, 4.6 x 250 mm, particle size 5 µm; was used along with UV detector at 245 nm.

Preparation of standard solution:

All the samples were prepared using mobile phase as diluent. The products, obtained from acid and base hydrolysis, were neutralized with base and acid of same strength respectively. Neutral hydrolysis, thermal and photolytic samples were diluted by mobile phase to obtained 100 µg/mL solutions. The Oxidative stress sample was diluted by mobile phase to obtained 10 µg/mL solution. All the prepared samples were filtered through 0.45µm nylon membrane.

About 100 mg of Temazepam standard was transferred into a 50 mL volumetric flask. Dissolved in methonal and diluted to volume with diluent methanol. Diluted 5.0 mL of this solution was diluted to 10 mL with diluent methanol. The standard solution was prepared in duplicate.

Validation of HPLC method:

In order to confirm method suitability during routine quality control use, the proposed method was checked critically for the following validation characteristics as per ICH guidelines.

Linearity:
Linearity for Temazepam was determined in the concentration range about 50-150 % of working concentration of standard. The peak area responses were plotted against the corresponding concentrations and the r values were calculated.

Precision:
System precision: The system precision was performed by analysing system suitability standard solution six times. Results of Peak area of the impurities and Temazepam were recorded. The peak area variation observed for Temazepam and impurities was less than 5%.

Method precision:
Six samples of the drug were analyzed as per the method. Each named impurity and total impurities were calculated on these replicates.

Intermediate precision or inter-day precision:
The intermediate or inter-day precision of the method was determined by six replicate analysis of Temazepam from sample, as per the proposed method by different instruments (Waters Alliance 2489 and Shimadzu), by same analyst on different days. The average drug content and the % RSD were calculated in each case.

Accuracy (recovery studies):
Recovery studies were performed by standard addition method at three levels i.e. 80%, 100% and 120%. Known amounts of standard Temazepam were added to pre-analyzed samples and they were subjected to proposed HPLC method. Results of recovery studies are shown in Table 1.

Stability of analytical solution:
A sample solution of Temazepam was prepared as per the proposed method. To this sample all known impurities were quantitatively spiked at specification limit concentration and stored at 10°C. The sample was injected into the system initially and at various time intervals. Sample solution spiked with impurities was found to be stable up to 600 minutes at 10°C.

Robustness:
Robustness of the method was determined by making slight and deliberate changes in experimental conditions. The effect of change in flow rate (-10% to +10%), % of organic modifier in mobile phase (-2% to +2%) while the amounts of the other mobile phase components were held constant, column oven temperature (-2°C to +2C°), pH of the buffer (-0.2 units to +0.2 units) and the detection wavelength (-2 nm to +2nm) was studied. For all the above deliberately varied experimental conditions, there is no change in the chromatographic performance. It indicates the robustness of the method parameters as indicated in Table 7.

Table 6: Robustness for Temazepam

Parameters	Actual	Low level	High level
Flow variation	2.0 mL/min	1.9 mL/min	2.1 mL/min
Column oven temperature	30^0C	28^0C	32^0C
Buffer pH variation	3.0	2.8	3.2
Buffer composition variation	60:40	62:38	58:42
Measurement Wavelength (nm)	245	243	247

Evaluation of system suitability:

Blank (diluent) was injected, followed by reference solution six times into the chromatograph and recorded the chromatograms. The system is suitable for analysis, if and only if, the resolution between Temazepam and intermediate peaks is not less than 2.0, the tailing factor for Temazepam peak not more than 2.0, the number of theoretical plates for Temazepam peak not less than 3000. Sample solutions were injected into the chromatograph and recorded the chromatograms.

Specificity:

Each known impurity and Temazepam solutions were prepared individually at a concentration of 0.12 mg/ml and a solution of all known impurities spiked with Temazepam (Figure 1) was also prepared. A test solution of Temazepam (Figure 2), solutions of impurities A, B, C, G and solutions of the Temazepam spiked with the impurities were also prepared. The good selectivity of the method is apparent from Figure 2 and 3 Shows that blank.

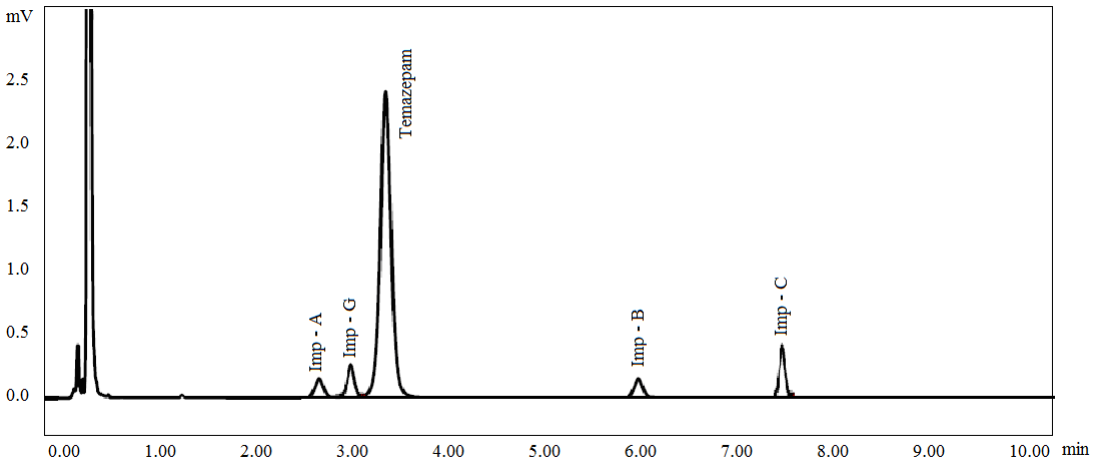


Fig. 2. Chromatograph of Temazepam spiked with impurities

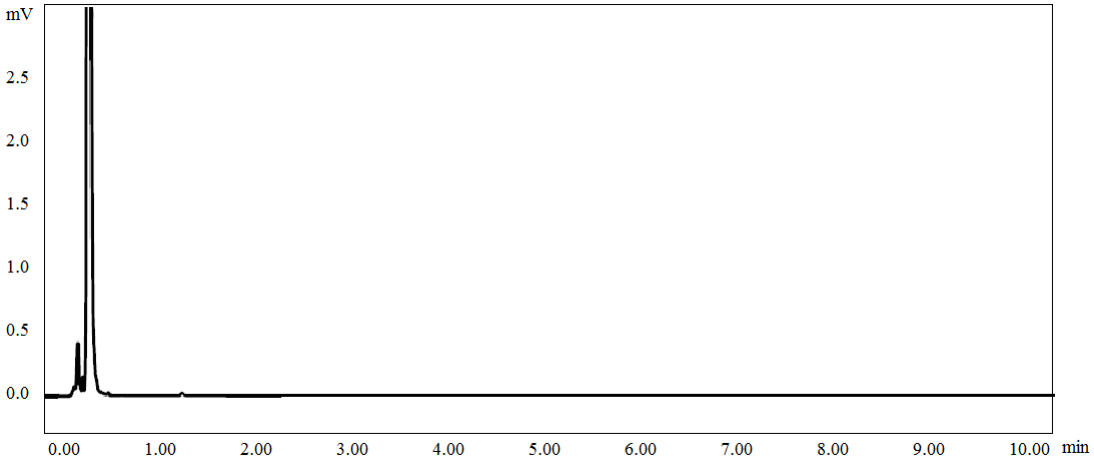


Fig. 3. Blank chromatogram of mobile

RESULTS AND DISCUSSION

The linearity of response for each known impurity was determined in the concentration range of limit of quantitation (LOQ) to about 150% of specification limit for each known impurity. Acceptance criteria squared correlation coefficient is not less than 0.99. The data are tabulated in Tables 3, 4, 5, and 6. The linearity of graphs is shown in Figure. 4. In system precision study, the % RSD for Temazepam was found to be 0.12. The % RSD observed on the replicate indicates the precision of the system.

Precision

In method precision study, the mean % drug content for Temazepam was found to be 99.2 % and 99.6 %. The % RSD for Temazepam was found to be 0.16. The results indicate that the method is validated for method precision. No interference from other components or excipient was found during determination.

In intermediate or inter-day precision study, the mean % drug content for Temazepam found to be 99.2 and 99.9. The % RSD for Temazepam was found to be 0.16 and 0.24. There is no significant difference by same analyst by different instruments on different days. Therefore the intermediate or inter-day precision of the method can be considered to be acceptable as shown in Table 1.

Table 1: Assay results of intermediate precision.

Sample	Analyst 1 Day1	Analyst 2 Day2	Bias
Sample-1	99.4	99.9	-0.5
Sample-2	99.2	99.6	-0.7
Sample-3	99.6	99.4	+0.2
Sample-4	99.2	99.7	-0.5
Sample-5	99.3	99.5	-0.2
Sample-6	99.5	99.9	-0.4
% RSD	0.16	0.24	

Accuracy

Recovery studies of the results are shown in Table 2. The overall % of recovery and % RSD for Temazepam in marketed formulation indicated that there is no significant difference in percentage of recovery. Therefore, accuracy of the method considered acceptable as it was well within 99.2 to 100.5 %.

A sample solution of Temazepam was prepared as per the proposed method. To this sample all known impurities were quantitatively spiked at specification limit concentration and stored at 10°C. The sample was injected into the system initially and at various time intervals. Sample solution spiked with impurities was found to be stable up to 600 minutes at 10°C.

Table 2: Result of recovery studies.

Recovery levels	Mean % of recovery	% RSD
80 %	100.1	0.25
100 %	100.5	0.15
120 %	99.2	0.05

Robustness

In robustness or system suitability study, there was no significant impact on the % RSD, resolution, theoretical plates and tailing factor. The results of the robustness study also indicated

that the method is robust and is unaffected by small variations in the chromatographic conditions.

Degradation study:
Impurities and Temazepam were analyzed individually as per the above method to verify the retention time. In order to assess the stability indicating nature of the HPLC method, Temazepam samples were stressed by acid, base, hydrogen peroxide, heat and UV radiation. The degraded samples were analyzed using a photodiode-array detector.

Sample Preparation:
About 50.0 mg of Temazepam was accurately weighed and transferred into a 100 mL volumetric flask, dissolve in and diluted with methanol. 5.0 mL of this solution was diluted to 10 mL with diluent.

Acid hydrolysis:
At room temperature accurately weighed 100 mg of substance was transferred into a 100 mL volumetric flask, dissolved in and diluted to volume with methanol. 5.0 mL of this solution was taken and transferred into a 10 mL volumetric flask and 0.2 mL of 1N hydrochloric acid solution. The solution was kept at room temperature for 3 hours, then neutralized with 0.2 mL of 1N sodium hydroxide solution and diluted to 10 mL with methanol. Another was prepared and transferred about 100 mg of substance into a 50 mL volumetric flask, dissolved in and diluted to volume with methanol. 5.0 mL of this solution was transferred into a 10 mL volumetric flask and added 0.2 mL of 1N hydrochloric acid solution. The solutions were kept at 60°C for 3 hours, then neutralized with 0.2 mL of 1N sodium hydroxide solution and diluted to 10 mL with diluent methanol. Figure 5. Shows that degraded occured in acid hydrolysis.

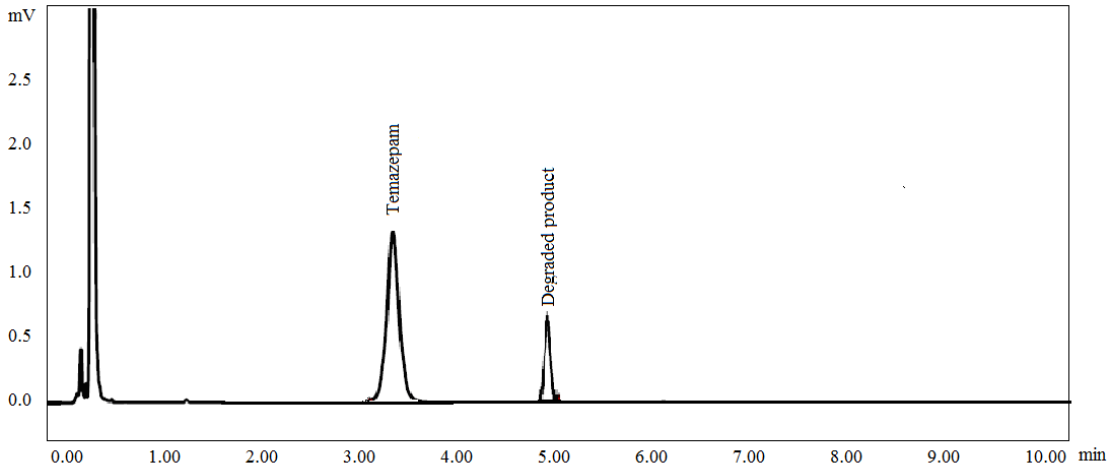


Fig 5. Degradation product obtained in acid hydrolysis

Base hydrolysis:
At room temperature four solutions were prepared individually by transferring about 100 mg of substance into a 50 mL volumetric flask, dissolved in and diluted to volume with methanol. 5.0 mL of each of these solutions were transferred into a 10 mL volumetric flasks and added 0.2 mL of 1N sodium hydroxide solution. The solutions were kept at room temperature for 3 hours, 6 hours, 12 hours and 24 hours, then neutralized with 0.2 mL of 1N hydrochloric acid solution and diluted to 10 mL with methanol.

Oxidation Degradation:
At room temperature four solutions were prepared individually by transferring about 100 mg of substance into a 50 mL volumetric flask, dissolved in and diluted to 100 mL diluent of methanol. 5.0 mL of these solutions were transferred into a 10 mL volumetric flasks and added 0.2 mL of 5% of hydrogen peroxide solution. The solutions were kept at room temperature for 3 hours, 6 hours, 12 hours and 24 hours and diluted to 10 mL with diluents, methanol.

Thermal and UV degradation:
Four solutions were prepared individually by transferring about 100 mg of substance into a 50 mL volumetric flask, dissolved in and diluted to 100 mL diluent of methanol. 5.0 mL of these solutions were transferred into a 10 mL volumetric flasks. The solutions were kept at 60°C for 3 hours, 6 hours, 12 hours and 24 hours and diluted to 10 mL with diluent of methanol.

UV degradation was carried out by accurately weighed and transferred about 50.0 mg of substance into a 100 mL volumetric flask, dissolve in and diluted to volume with diluents of methanol. Taken 5.0 mL of above solution into a 10 mL volumetric flask and exposed to an integrated near Ultra violet energy (UV light) of not less than 200 watt/square meter and then diluted to 10 mL with the diluent.

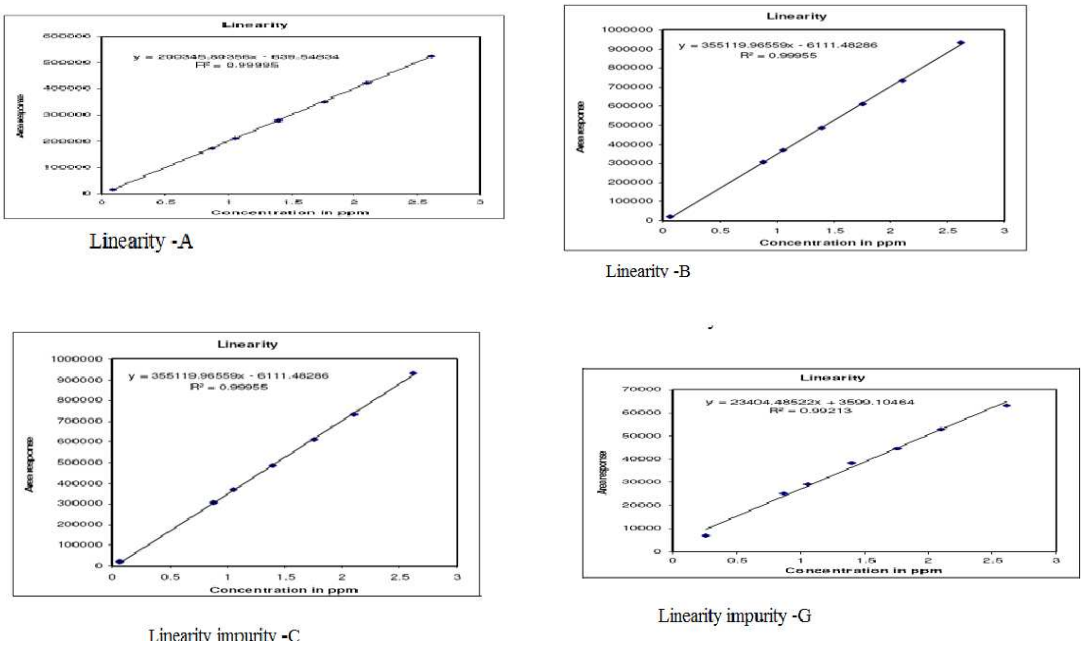


Fig. 4: Shows that linearity of Impurity-A, Impurity-B, Impurity-C and Impurity-G

Table 3: Shows that linearity of impurity A

Concentration(ppm)	Area response
0.0840	16423
0.880	175096
1.060	212568
1.400	279399
1.760	350341
2.100	422066
2.620	523855
Slope	200345.80356
Intercept	-639.54834
CC*	0.99998
Squared CC*	0.99995

Table 4: Shows that linearity of impurity B

Concentration(ppm)	Area response
0.061	21512
0.880	305989
1.060	369643
1.400	487309
1.760	610730
2.100	735980
2.620	934996
Slope	355119.96559
Intercept	-6111.482869
CC*	0.99977
Squared CC*	0.99955

Table 5: Shows that linearity of impurity C

Concentration(ppm)	Area response
0.263	7114
0.865	25214
1.051	29090
1.401	38403
1.751	44478
2.101	52885
2.616	63178
Slope	23404.48522
Intercept	3599.10464
CC*	0.99606
Squared CC*	0.99213

Table 5: Shows that linearity of impurity G

Concentration(ppm)	Area response
0.080	5642
0.880	71895
1.060	85633
1.400	110731
1.760	139057
2.100	165799
2.620	201415
Slope	77155.83239
Intercept	2332.75134
CC*	0.99949
Squared CC*	0.99898

CONCLUSION

The results of the present study indicate that the newly developed Reverse Phase-HPLC method is simple, rapid, cost-effective, linear, accurate, precise and robust over the specified range; and selective for Temazepam without any interference from other components or additives. This method can be employed conveniently, reliably and successfully for the estimation of Temazepam for routine quality control and stability studies.

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