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Original Article

DEVELOPMENT AND VALIDATION OF A STABILITY INDICATING LIQUID CHROMATOGRAPHIC METHOD FOR THE SIMULTANEOUS ESTIMATION OF PAROXETINE AND CLONAZEPAM IN BULK AND ITS PHARMACEUTICAL FORMULATIONS

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ABSTRACT

A novel stability indicating reversed-phase liquid chromatographic method has been developed and validated for simultaneous estimation of paroxetine and clonazepam in combined pharmaceutical dosage form. An Agilent zorbax sb-c18 (250mmx4.6mmx5 µm) column with the mobile phase containing 0.2 % Orthophosphoric acid and Methanol (60:40 v/v) was used. The flow rate was maintained at 0.8 ml/min, column temperature was 30°C and effluents were monitored by using a photodiode array detector at 270 nm. The retention times of paroxetine and clonazepam were found to be 3.478min and 3.964 min, respectively. Correlation co-efficient for paroxetine and clonazepam were found to be 0.99 and 0.99, respectively. The proposed method was validated with respect to linearity, accuracy, precision, specificity, and robustness. Recovery of paroxetine and clonazepam in formulations was found to be in a range of 97-103% and 97-103% respectively. Paroxetine and clonazepam were also subjected to the stress conditions of oxidative, acid, base, hydrolytic, thermal and photolytic degradation. The degradation products were well resolved from and peak purity test results confirmed that paroxetine and clonazepam peaks were homogenous and pure in all stress samples, thus proving stability-indicating power of the method. Due to its simplicity, rapidness and high precision, this method can be applied for regular analysis.

Keywords: Paroxetine and clonazepam, Liquid chromatography, Method validation, Forced degradation.

INTRODUCTION

Paroxetine: (3S, 4R}3- [(1, 3-benzodioxol-5-vloxy) methyl] -4-(4-flurophenyl) piperidine (PRX) is a new generation antidepressant drug[1]. It exerts its antidepressant effect through a selective inhibition for the reuptake of the neurotransmitter serotonin by the presynaptic receptors. PRX is comparable to the tricyclic antidepressants in their clinical efficacy, however, PRX is safer and has greater acceptance by the patients. It is also prescribed in the treatment of relateddisorders, suchasobsessive - compulsive Disorder, panic fits, social phobia, and posttraumatic stress[2]. Clonazepam (Merck Index, 13th edition, 2002, 2413) [5-(o-chlorphenyl)-7-nitro-1H-1,4-benzodiazepin-2(3H)-one] is mainly used as anticonvulsant, muscle relaxant and anxiolytic agent. Clonazepam is slightly soluble in acetone, chloroform, acetic anhydride, hardly soluble in methanol, isopropanol, ether, almost insoluble in water. Chemical structures of paroxetine and clonazepam are presented in Figure I.

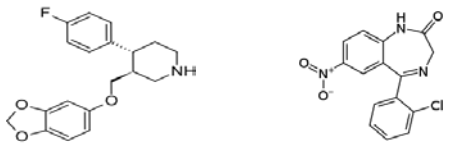


Fig. 1: Structures of (A) Paroxetine and (B) Clonazepam

A literature survey revealed few liquid chromatography (LC) assay methods that have been reported for the determination of clonazepam in bulk drug and pharmaceutical dosage forms, but there are no reported methods for simultaneous estimation of paroxetine and clonazepam in combined pharmaceutical dosage forms [3-13].

The present International Conference on Harmonization (ICH) drug stability guidelines suggest that stress studies should be conducted on the drug product to establish its inherent stability characteristics, and the analytical method should able to separate all degradation impurities formed under stress studies to prove its stability-indicating power. In order to monitor possible changes to a product over time, the applied analytical chromatographic method must be stability-indicating. The best case for testing the suitability of a method is using real-time stability samples containing all relevant degradation products that might occur. But due to product development timelines, process characteristics, excipients, and other environmental factors, a forced degradation study (stress test) can serve as an alternative.

In a typical study, relevant stress conditions are light, heat, humidity, hydrolysis (acid / base influence) and oxidation or even a combination of described parameters. If it is necessary to form degradation products, the strength of stress conditions can vary due to the chemical structure of the drug substance, the kind of drug product, and product specific storage requirements. An individual program has to be set up in order to reach a target degradation of 5 to 20%. A higher level of degradation will be out of the scope of product stability requirements and therefore unrealistic. The scope of the test is to generate degradation products in order to facilitate a method development for determination of the relevant products. Therefore, samples will be stressed in a solid form and/or in solution. Typically, stress tests are carried out on one batch of material. For drug products the placebo should be stressed in a similar way in order to exclude those impurities that are not degradation products (e.g. impurities arising from excipients). The stability studies were determined by applying the physical stress (acid, base, peroxide, heat and light) to the product [14-19].

The aim of the present work is to focus on the development of an efficient stability indicating liquid chromatographic method for simultaneous estimation of paroxetine and clonazepam in combined pharmaceutical dosage form such as capsule in presence of its excipients and degradation products in a short chromatographic run.

The present work concerns the method development, method validation and forced degradation studies of paroxetine and clonazepam in combined pharmaceutical dosage form. The developed Liquid Chromatographic method was validated with respect to specificity, limit of detection (LOD), limit of quantification (LOQ), linearity, precision, accuracy and robustness. Forced degradation studies were performed on the placebo and drug products to show the

stability-indicating nature of the method. These studies were performed in accordance with established ICH guidelines.

Experimental

Instrumentation

Samples were analyzed on Waters alliance 2695 HPLC system (Waters Corporation, Milford, MA) equipped with a with binary HPLC pump, Waters 2998 PDA detector and Waters Empower 2.0 software. The separation was achieved on Agilent zorbax sb-c18 (250 mm x 4.6 mm x 5 µm) column.

Chemicals and Reagents

Paroxetine and Clonazepam standards were supplied by Dr. Reddy's Laboratories Ltd., Hyderabad. Methanol of HPLC grade was purchased from E. Merck (India) Ltd., Mumbai. Orthophosphoric acid of AR grade was obtained from S.D. Fine Chemicals Ltd., Mumbai and milli Q water. Paroxetine and Clonazepam capsules (ZAPTRA 25 - Intas Company) were procured from Local market.

HPLC Conditions

The mobile phase consisting of 0.2% v/v ortho phosphoric acid and methanol (HPLC grade) were filtered through 0.45 µm membrane filter before use, degassed and were pumped from the solvent reservoir in the ratio of 60:40 v/v into the column at a flow rate of 0.8 ml/min. The column temperature was maintained at 30ºC. The detection was monitored at 270 nm and the run time was 6.0 minutes. The volume of injection loop was 10 µl prior to injection of the drug solution.

Preparation of standard solution

Accurately weighed quantity, 100 mg of Paroxetine and 2 mg of Clonazepam was transferred into 50 ml of volumetric flask and diluted to the volume with mobile phase. From this stock, 5 ml of a solution was taken into a 10 ml volumetric flask and diluted to the volume with mobile phase (Concentration of Paroxetine: 1 mg/ml, concentartion of Clonazepam: 20µg/ml).

Preparation of sample (drugs from marketed formulations) solution

Twenty tablets were weighed and the average weight was calculated and crushed in to the fine powder, Tablet powder (Equivalent to four tablets) was transferred into 50 ml of volumetric flask and diluted to the volume with mobile phase. From this stock solution 5 ml was transferred into a 10 ml volumetric flask and diluted to the volume with mobile phase.(Concentration of Paroxetine : 1mg/ml, Concentartion of Clonazepam : 20 µg /ml).

Forced degradation studies

Forced degradation studies were performed at a 1048 mg of paroxetine and clonazepam in capsules to provide an indication of the stability-indicating property and specificity of the proposed method. A peak purity test was conducted for paroxetine and clonazepam peaks by using a PDA detector on stress samples. All solutions used in forced degradation studies were prepared by dissolving the drug product in a small volume of stressing agents. After degradation, these solutions were diluted with mobile phase to yield a stated concentration approximately. Conditions employed for performing the stress studies are described below.

Acid degradation

Tablet powder equivalent to 1048 mg was accurately weighed and dissolved in 5 ml of mobile phase, 5 ml 5 N Hcl was added and the mixture was kept at 70^{0}C for 5 min. The solution was brought to ambient temperature, neutralized by the addition of 5 ml 5 N NaOH and diluted to 25 ml with mobile phase.

To prepare the blank, 5 ml of 5 N HCl and 5 ml of 5 N NaOH were diluted to 25 ml with mobile phase.

Base degradation

Tablet powder equivalent to 1048 mg was accurately weighed and dissolved in 5 ml of mobile phase, 5 ml 5N NaOH was added and the mixture was kept at 700C for 5 min. The solution was brought to ambient temperature, neutralized by the addition of 5 ml 5 N HCl and diluted to 25 ml with mobile phase.

To prepare the blank, 5 ml of 5 N NaOH and 5 ml of 5 N HCl were diluted to 25 ml with mobile phase.

Oxidation degradation

Tablet powder equivalent to 1048 mg was accurately weighed and dissolved in 5 ml of mobile phase, 5 ml of 3% hydrogen peroxide was added and the mixture was kept at 70^{0}C for 10 min. The solution was brought to ambient temperature and diluted to 25 ml with mobile phase.

To prepare the blank, 5 ml of 3% hydrogen peroxide was diluted to 25 ml with mobile phase.

Thermal degradation

Tablet powder equivalent to 1048 mg was stored at 105^{0}C for 9 hr, dissolved and diluted to 25 mL with mobile phase.

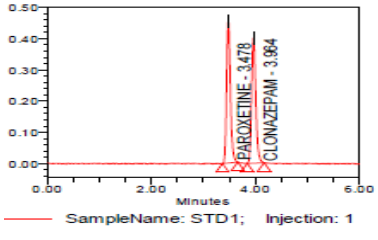
Photolytic degradation

The susceptibility of the drug product to the light was studied. Tablet powder for photo stability testing was placed in a photo stability chamber and exposed to a white florescent lamp with an overall illumination of 1.2 million lux hours and near UV radiation with an overall illumination of 200 watt/m^2/h at 25^{0}C. Following removal from the photo stability chamber, the sample was prepared for analysis as previously described.

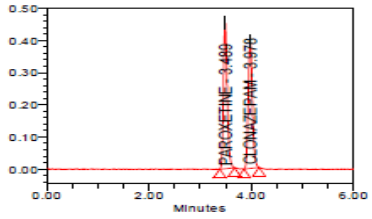
RESULTS AND DISCUSSION

Method Development

The analytical procedure for the estimation of paroxetine and clonazepam in marketed formulation was optimized with a view to develop a precise and accurate assay method. Agilent Eclipse XDB (4.6*150mm*3.5mic), Agilent Zorbax C8 (4.6*150mm*5mic) and Inertsil-ODS (4.6*250mm*5mic) were used to provide an efficient separation but appropriate chromatographic separation was achieved on An Agilent zorbax sb-c18 (250mmx4.6mmx5mic). Various mobile phase systems were prepared and used to provide an appropriate chromatographic separation, but the proposed mobile phase containing 0.2% v/v Orthophoshoric acid: Methanol in the ratio of 60:40 (v/v) gave a better resolution. Using UV-visible PDA detector at 270 nm carried out the detection. Amongst the several flow rates tested, the flow rate of 1 ml/min was the best suited for both the drugs with respect to location and resolution of peaks. The retention time of paroxetine and clonazepam was found to 3.478min and 3.964 min respectively. The chromatograms of standard and sample solution of paroxetine and clonazepam were shown in Figure II. The asymmetry factor of paroxetine and clonazepam was 1.246 and 1.196 found to be respectively, which indicates symmetrical nature of the peak. The USP resolution of 3.361was achieved between paroxetine and clonazepam. The USP plate count of paroxetine and clonazepam was 10704 and 11407 found to be respectively, which indicates column efficiency for separation. System suitability parameters such as Peak asymmetry, Resolution and Number of theoretical plates are meeting ICH requirements. The percentage label claim of individual drugs found in formulations were calculated and provided in Table I. The results of analysis shows that the amounts of drugs estimated were in good agreement with the label claim of the formulations.



(A)



(B)

Fig. 2: Typical chromatograms of Paroxetine and Clonazepam (A) Standard (B) Formulation

Table 1: Assay results

Sample	Label claim (mg/tablet)	Amount present (mg/tablet)	Percentage Label claim (% w/w)
Paroxetine	25 mg	24.79	99.1
Clonazepam	0.5 mg	0.5	100

Method Validation

System Suitability Studies

System suitability was determined before sample analysis from duplicate injections of the standard solutions of paroxetine and clonazepam.The column efficiency, resolution and peak asymmetry were calculated for the standard solutions. Resolution between paroxetine and clonazepam peaks was found to be 3.361. USP tailing (Peak Asymmetry) for paroxetine and clonazepam were found to be 1.246 and 1.196 respectively. Number of theoretical plates (USP plate count) for paroxetine and clonazepam were found to be 10704 and 11407 respectively.

The values obtained demonstrated the suitability of the system for the analysis of this drug combinations, system suitability parameters may fall within ± 3 % standard deviation range during routine performance of the method.

Specificity

Specificity is the ability to assess unequivocally the analyte in presence of components which may be expected to be present. Typically these might include impurities, degradants, matrix, etc. Placebo interference was evaluated by analyzing the placebo prepared by the test method. No peak due to placebo was detected at the retention time of paroxetine and clonazepam. The specificity of the developed method was also conducted in presence of its degradation products.

Precision

The precision of method was verified by repeatability and intermediate precision. Repeatability was checked by injecting six individual sample preparations of paroxetine and clonazepam capsule. Percent relative standard deviation (RSD) of the area for each drug was calculated. The intermediate precision of the method was also evaluated using different analysts and different instruments and performing the analysis on different days. The results of precision study are provided in Table II.

Accuracy

The accuracy of the method was determined by recovery experiments. The recovery studies were evaluated in triplicate using three concentration levels 50%, 100% and 150%. The percentage recovery data was obtained, added recoveries of standard drugs were found to be accurate (Table III & IV).

Linearity and Range

The linearity of the method was determined at five concentration levels (50%, 75%, 100%, 125% and 150%). Linearity test solutions were prepared by diluting the stock solutions to the required concentrations. The calibration curves were plotted between the responses of peak area versus concentration of analyte. The slope and intercept value for calibration curve was y = 16616 x (r2=0.99) for paroxetine and y = 19288 x (r2=0.99) for clonazepam. The result (Table V) shows that an excellent correlation exists between areas and concentration of drugs within the concentration range. Calibration curves are presented in Figure III.

Limit of detection & Limit of quantification (LOD & LOQ)

Limit of quantification and detection were predicted by plotting linearity curve for different nominal concentrations of paroxetine and clonazepam (Table V).

Relative standard deviation (σ) method was applied, the LOQ and LOD values were predicted using following formulas. Precision was established at these predicted levels.

(a) LOQ = 10 σ / S

(b) LOD = 3.3 σ / S

Where σ = Residual standard deviation of response;

S = slope of the calibration curve.

LOQ and LOD values for paroxetine and clonazepam were found to be 9.501, 8.064 and 2.850, 2.419 respectively.

Robustness

Robustness of the method was determined by making slight changes in the chromatographic conditions and system suitability parameters for paroxetine and clonazepam standard and the resolution, USP Tailing and USP Plate count were recorded. The variables evaluated in the study were column temperature (±5°C), flow rate (±0.2 mL/min). It was observed that there were no marked changes in the chromatograms, which demonstrates that the method developed is rugged and robust (Table VI & VII)

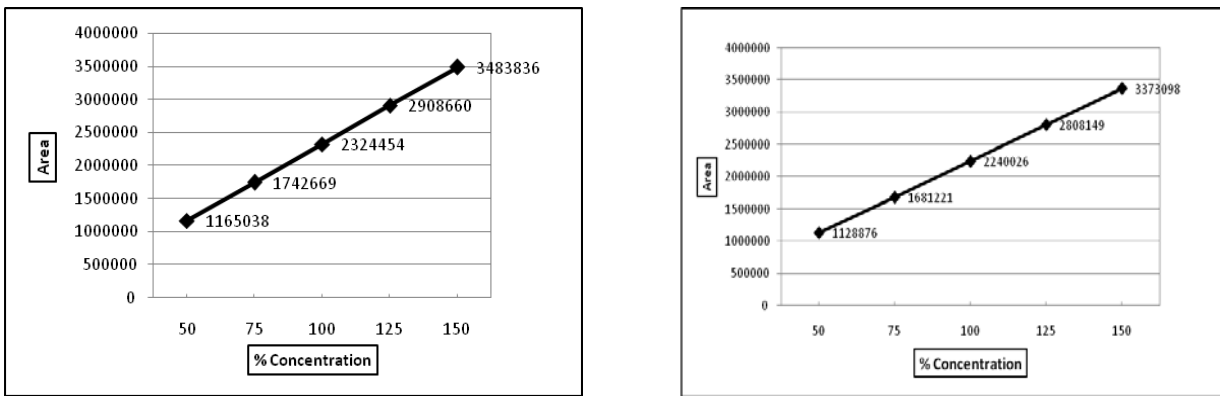


Fig. 3: Linearity graphs of Paroxetine and Clonazepam

Table 2: Precision Studies of Paroxetine and Clonazepam

S. No.	Sample Wt	Area of Paroxetine	Area of Clonazepam	% Assay of Paroxetine	% Assay of Clonazepam
1	1047.84	2329638	2244561	99	100
2	1047.84	2327634	2249193	99	100
3	1047.84	2320550	2248507	99	100
4	1047.84	2322042	2247725	99	100
5	1047.84	2321371	2244102	99	100
6	1047.84	2325815	2241087	99	99
Average				99	100
STD				3725.18	3135.38
%RSD				0.16	0.14

Table 3: Accuracy for Paroxetine

Spiked Level	Sample Weight	Sample Area	µg/ml added	µg/ml found	% Recovery	Mean
50%	523.92	1164679	198.000	198.59	100	100
50%	523.92	1161393	198.000	198.03	100	
50%	523.92	1163728	198.000	198.42	100	100
100%	1047.84	2328234	396.000	396.98	100	
100%	1047.84	2324841	396.000	396.40	100	
100%	1047.84	2322363	396.000	395.98	100	100
150%	1571.76	3486485	594.000	594.47	100	
150%	1571.76	3485959	594.000	594.38	100	

Table 4: Accuracy of Clonazepam

Spiked level	Sample weight	Sample Area	µg/ml added	µg/ml found	% Recovery	Mean
50%	523.92	1124728	4.00	3.99	100	100
50%	523.92	1127607	4.00	4.00	100	
50%	523.92	1128025	4.00	4.00	100	
100%	1047.84	2244332.00	8.00	7.96	100	100
100%	1047.84	2244473.00	8.00	7.96	100	
100%	1047.84	2241524.00	8.00	7.95	99	
150%	1571.76	3374707	12.00	11.98	100	100
150%	1571.76	3374280	12.00	11.97	100	
150%	1571.76	3377070	12.00	11.98	100	

Table 5: Linearity of Paroxetine and Clonazepam

Paroxetine					Clonazepam				
% Conc.	*Area*	*ug/ml*	*LOD*	*LOQ*	*% Conc.*	*Area*	*ug/ml*	*LOD*	*LOQ*
50	1165038	200	S/N	421	50	1128876	4	S/N	9.92
75	1742669	300	2.850	9.501	75	1681221	6	2.419	8.064
100	2324454	400			100	2240026	8		
125	2908660	500			125	2808149	10		
150	3483836	600			150	3373098	12		

Table 6: Robustness of Paroxetine

Sample Name	RT	Area	USP Tailing	USP Plate count	S/N
TEMP-1	1	3.485	2353377	1.223	10195
TEMP-2	1	3.453	2305010	1.169	10854
FLOW-1	1	4.634	3134093	1.271	11944
FLOW-2	1	2.797	1903663	1.198	8117

Table 7: Robustness of Clonazepam

Sample Name	RT	Area	USP Tailing	USP Plate count	S/N
TEMP-1	3.897	7425147	1.177	7989	635.11
TEMP-2	3.802	6964485	1.174	8811	670.73
FLOW-1	4.548	10059395	1.171	8911	816.17
FLOW-2	3.433	7024245	1.193	7317	688.34

Forced Degradation Studies

Based on the results of the stress studies, the degradation behavior of paroxetine and clonazepam is as follows.

Acid degradation

Paroxetine and Clonazepam were undergoing degradation in 5 N HCl at 70^{0}C for 10 min moderately. The impurities formed during this

study are well separated from main drug peaks and mass balance is found to be in acceptable limit. Peak purity of drugs also matches (Table VIII, Figure IV (A).

Base degradation

Paroxetine and Clonazepam were found to be slightly unstable in 5 N NaOH at 70^{0}C for 5 min. The major degradation peaks are well separated from drug peaks and well resolved. Mass balance is found to be in acceptable limit. Peak purity of drugs also matches (Table VIII, Figure IV (B).

Oxidation degradation

Paroxetine and Clonazepam were found to be slightly unstable under conditions of 3% hydrogen peroxide at 70^{0}C for 10 min. The major impurities in the study were resolved with drug peaks. Mass balance is found to be in acceptable limit. Peak purity of drugs also matches (Table VIII, Figure IV (C).

Thermal degradation

Paroxetine and Clonazepam were found to be stable to thermal exposure. Partial degradation was take place. Impurities formed well resolved from main drug peaks. Mass balance is found to be in acceptable limit. Peak purity of drugs also matches (Table VIII, Figure IV (D).

Photolytic degradation

Upon subjecting the Paroxetine and Clonazepam sample to both UV and visible light, only partial degradation of sample was observed.

Testing of a placebo containing preservative leads to formation of number of different impurities with respect to an unstressed placebo. The amount of preservative decreased mainly by influence of oxidation, light and acid. Mass balance of preservative shows almost 100%. The active ingredients remain almost stable within tested period and mass balance matches (Table VIII, Figure IV (E).

Table 8: Degradation studies for Paroxetine and Clonazepam

Stress condition	Sample weight	Paroxetine			Clonazepam		
		Area	*% Assay*	*% Deg.*	*Area*	*% Assay*	*% Deg.*
Acid	1048	2028376	86	-13	1885379	84	-16
Base	1048	2096111	89	-10	1884672	84	-16
Peroxide	1048	2112722	90	-9	1967354	88	-12
Heat	1048	2197396	94	-5	2185996	97	-3
Light	1048	2155038	92	-7	2085936	93	-7

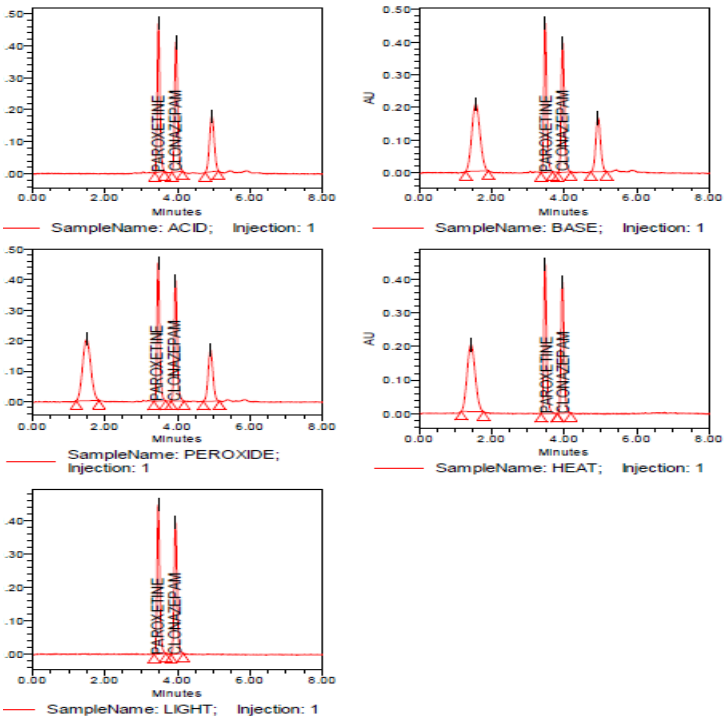


Fig. 4: Typical Chromatograms (A) Acid degradation (B) Alkali degradation (C) Oxidative degradation (D) Thermal degradation (E) Photolytic degradation

CONCLUSION

The The proposed HPLC method for the simultaneous estimation of paroxetine and clonazepam in pharmaceutical dosage forms was found to be simple, sensitive, precise, accurate, linear, robust and rugged during validation. Further this method is stability indicating and can be used for routine analysis of production samples. Hence, this method can be easily and conveniently adopt for routine quality control of paroxetine and clonazepam in pure and its pharmaceutical dosage forms.

CONFLICT OF INTERESTS

Declared None

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