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DEVELOPMENT AND VALIDATION OF RP-UFLC AND UV SPECTROPHOTOMETRIC METHODS FOR DETERMINATION OF 10-(4'-N-[(B-HYDROXYETHYL) PIPERAZINE] BUTYL)-1, 3-DIMETHYL-10H-ACRIDINONE IN BULK DRUG AS A POTENT DNA INTERCALATOR

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Abstract:

The present RP-UFLC and UV methods are relatively simple, rapid, robust and highly precise in the determination of 10-(4'-N-[(β-Hydroxyethyl) piperazine] butyl)-1,3-dimethyl-10H-acridinone (**HBA**) in bulk drug. The chromatographic separation of the drug was achieved on eclipse plus C_8 column (250×4.6mm i.d. 5µm) using a mobile phase of methanol: water (50:50). The flow rate was 1.0 ml/min and effluents were monitored at 267 nm. The separation was achieved within 3.0 ± 0.2 min. The method showed good linearity in the range of 0-10 µg/mL with coefficient of correlation 0.9906. The intra and inter day RSD ranged within limits. The limit of detection and limit of quantification were 0.140 and 0.424 µg/mL, respectively. UV spectrophotometric determination of HBA in bulk drug was done by using 0.1N HCL as solvent and the absorption maxima was found to be 262 nm. A linear response was observed in the range of 0-18 µg/mL with a correlation coefficient of 0.999. The method was validated as per ICH guidelines and RSD ranged within limits. These methods can be successfully employed to quantify HBA in the bulk drug and can be used for routine quality control purposes.

Keywords: 10-(4'-N-[(β-Hydroxyethyl) piperazine] butyl)-1,3-dimethyl-10H-acridinone (**HBA**), Method Validation, RP-UFLC, UV spectrophotometry.

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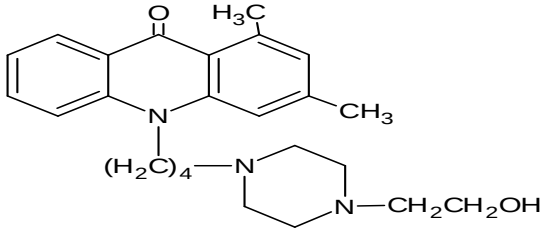
INTRODUCTION:

HBA is chemically 10-(4'-N-[(β-Hydroxyethyl) piperazine] butyl)-1,3-dimethyl-10H-acridinone, which is an 1,3-dimethyl acridone derivative having good cytotoxic activity and DNA intercalation properties. Various 9-acridone derivatives with or without an alkyl side chain attached to the N-position were found to exhibit anticancer and antibacterial activities. HBA is freely soluble in methanol, water, DMSO and 0.1N HCL. HBA showed higher DNA-binding constant (K_i) 10.32 x 10 x M^{-1} and also showed relatively better cytotoxic activity against tumor cell lines, which may be attributed, at least in part, to its intercalative association and high binding affinity with the DNA. As for the structure-activity relationship, HBA bearing the planar tricyclic ring with butyl (β-hydroxyethyl) piperazine side chain shows stronger binding property [1]. HBA exhibited potent cytotoxic activity against MCF-7 cell line, HL-60 and Ehrlich Ascites Carcinoma (EAC) cell line (Satish et al., 2010). The Structural Elucidation of synthesized HBA was confirmed by ^{1}H-NMR, ^{13}C-NMR, mass spectroscopy and elemental analysis [1].



Structure of HBA

As far there are very few numbers of analytical methods reported in the literature for the determination of 1,3-dimethyl acridone derivatives. Officially assay of HBA is not described in any pharmacopoeias. From literature survey, there has been no HPLC and UV spectroscopy method reported for the estimation of HBA in bulk drug.

In the present work, an attempt was made to provide a newer, simple, low cost, robust, precise and reliable UFLC and UV-spectrophotometric methods for the effective quantitative determination of HBA as an active pharmaceutical ingredient. The developed method can be successfully applied in estimation of HBA. The results of analysis were validated as per International Conference on Harmonization guidelines [2].

MATERIALS AND METHODS:

Instrumentation and Analytical conditions

The RP-UFLC method was performed by using Shimadzu Prominence binary gradient, UFLC instrument. The instrument was provided with a reversed-phase C-8 (250 mm X 4.6 mm, 5µm i.d.), a binary prominence LC 20AD pump and an SPD M20A prominence diode array detector. A 20 µL Hamilton injection syringe was used for sample injection. LC solutions software was used for Data acquisition. HPLC grade methanol (Rankem pvt. Ltd.) and Milli-pore water were used in the study. A freshly prepared binary mixture of methanol: water (50:50 %v/v) was used as the mobile phase. The mobile phase was filtered through a 0.45 µ membrane filter and degassed before use. The flow rate of mobile phase was maintained at 1.0 mL/min. The detection of the drug was carried out at 267 nm. The analysis was carried out at 18°C.

The UV method was performed on a Shimadzu UV-Visible Spectrophotometer 1700, and a pair of 1-cm matched quartz cells were used to measure absorbance of the resulting solution. In this UV method solvent used is 0.1N Hydrochloric acid (S d fine-chem limited) and double distilled water is used as diluents. Maximum absorbance was observed at 262 nm [3].

Drug sample

The drug sample HBA was synthesized and purified by column chromatography and its purity is checked by RP-UFLC.

Preparation of stock and working standard solutions

RP-UFLC Method

Approximately 100 mg of HBA was accurately weighed and transferred to a 100 ml volumetric flask and dissolved with minimum quantity of mobile phase. The flask was sonicated for 10 min to dissolve the solids. It was diluted to volume with mobile phase and mixed well, which yields standard stock solution of concentration 1000µg/ml (stock-1) of HBA. Pipette out 2ml from stock-1 and dissolved in 100 ml of mobile phase to get working standard stock solution of 20µg/ml (stock-2) of HBA**.**

UV Method

Approximately 40mg of HBA was accurately weighed and transferred into a 100mL volumetric flask and diluted to volume with 0.1N HCL which gave 400µg/ml (stock-1) concentration of HBA. Pipette out 5 ml from stock-1 and transferred into 100 ml volumetric flask and diluted with 0.1N HCL which yields working standard concentration of 20µg/ml (stock-2) of HBA.

Linearity and Construction of Calibration Curve

RP-UFLC Method

The quantitative determination of the drug was accomplished by using methanol: water (50:50 *v/v*). The column was equilibrated with the mobile phase for at least 30 minutes prior to the injection of the drug solution. The Linearity (Beer's law) of detector response was established by plotting a graph of concentration versus peak area of HBA standard and determining the correlation coefficient. A series of solution of HBA standard solution in the concentration ranging from about 0-10 µg/mL were prepared and injected into the UFLC system (**figure 1**). Each dilution was injected six times into the column. The drug analyte was monitored at 267 nm and the

corresponding chromatograms were obtained. From these chromatograms, a plot of concentrations over the peak areas was constructed. A representative chromatogram for the separation of HBA is given in **figure 2**. Linearity data of HBA by RP-UFLC method is depicited in **table 1**.

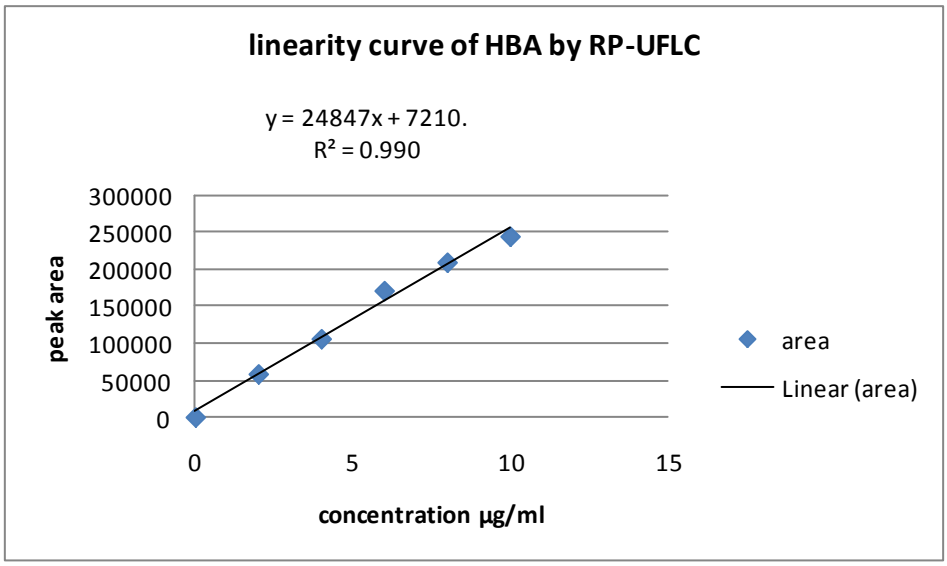


Figure 1: The linearity curve of HBA by RP-UFLC method

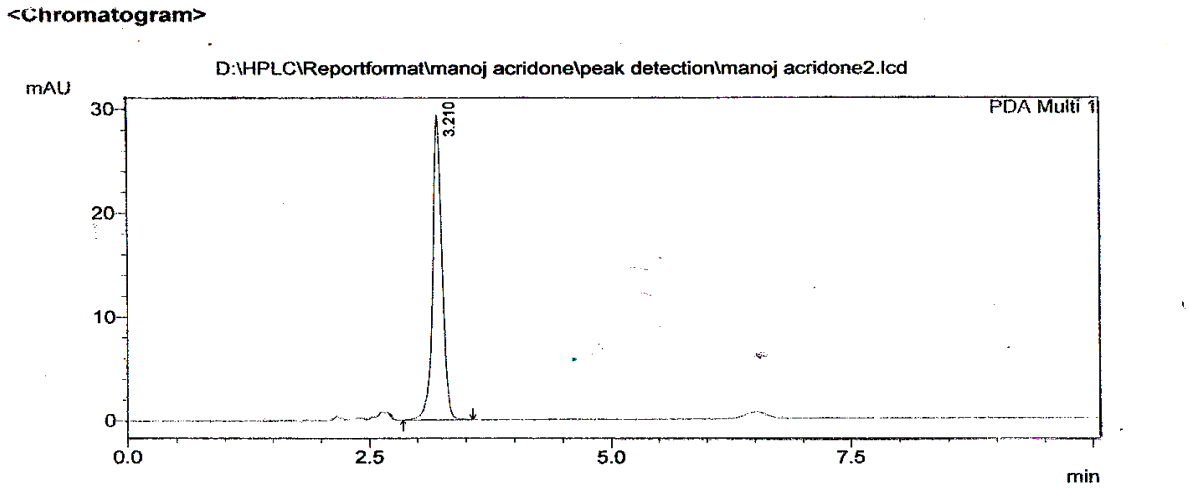


Figure 2: Chromatogram of HBA

Table 1: linearity data of HBA by RP-UFLC method

Sl. No.	Concentration (µg/ml)	Retention time (min)	Peak area
1.	0	0	0
2.	2	3.210	58328
3.	4	3.212	105887
4.	6	3.218	170905
5.	8	3.210	209254
6.	10	3.215	244299

HBA is 10-(4'-N-[(β-Hydroxyethyl) piperazine] butyl)-1,3-dimethyl-10H-acridinone.

UV Method:

Suitable aliquots of the standard solution of HBA were taken in 10 mL volumetric flasks. The volume was then made up to the mark with 0.1N hydrochloric acid to prepare a series of standard solutions containing 0-18 µg/ml. The UV spectrum for HBA is shown in **figure 3**. Absorbance was measured at 262 nm against blank. Linearity graph is shown in **figure 4.**

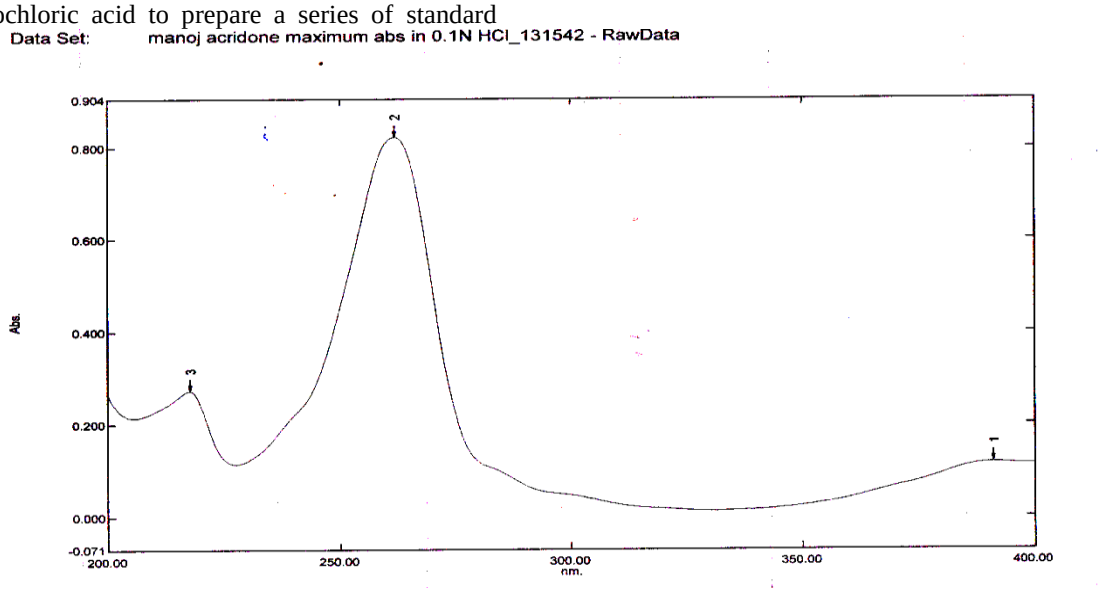


Fig 4: UV-Spectrum of HBA

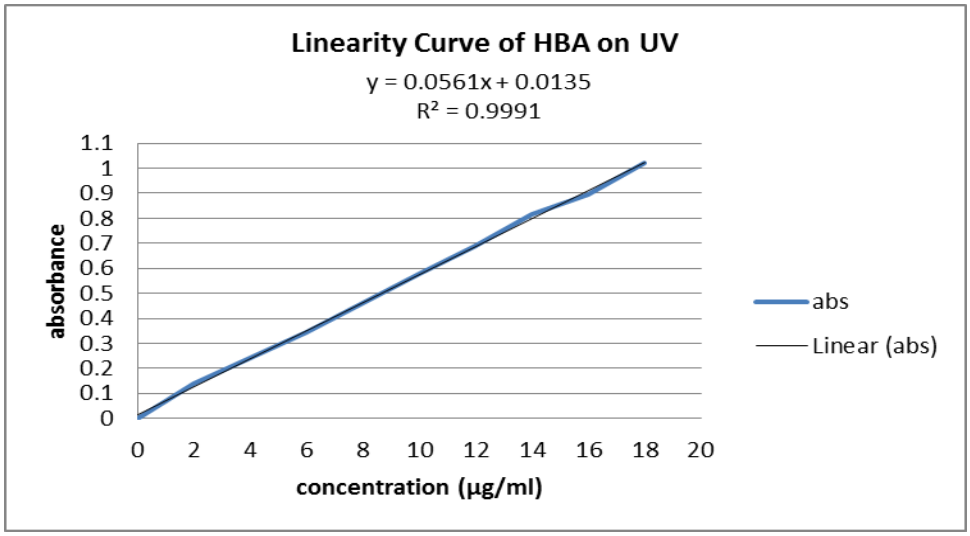


Fig 4: The linearity curve of HBA by UV spectrophotometry method

Method Validation

HPLC and UV Methods

Precision

The precision of the analytical method was studied by analysis of multiple sampling of homogeneous sample. The precision of a method was calculated by performing intra-day precision and inter-day precision studies. The average was taken and % RSD was calculated and the values were within the limit i.e. (< 2), so the method was found to be highly precise. The precision data observed were shown in **table 2 and table 3** by RP-UFLC and UV method respectively.

Linearity

The linearity of the analytical method was its ability to elicit test results which are directly proportional to analyte concentration in samples within a given range. Linearity was studied by preparing standard solution at five different concentration levels. The linearity range was found to be 0-10 µg/ml. Twenty µL of each solution was injected into the RP-UFLC system through manual injector. Peak areas were recorded for all the chromatogram. The correlation coefficient of HBA was found to be 0.9906. Peak area of linearity range and the parameters were calculated and presented in Table 1. The linearity curve of HBA was shown in **figure 3.** The linearity of the proposed UV spectroscopy method was established by preparing various aliquots of the standard solution of the HBA from stock solution and analyzed. The drug showed linearity in the range of 0-18µg/ml with correlation coefficient 0.999. Linearity data are shown in **table 4** and linearity graph is plotted, concentration in X-axis and absorbance in Y-axis shown in **figure 5.** These data clearly demonstrates that the developed method have adequate sensitivity to the concentration of the analyte in the sample.

Table 2: Results of precision of HBA by RP-UFLC method

Concentration (µg/ml)	Peak area	Statistical Analysis
4	104108	**Mean- 105097** **SD- 1054.921** **% RSD-1.00376**
4	106309	
4	104583	
4	106562	
4	104604	
4	104416	

SD is standard deviation for n = 6 observations, %RSD is relative standard deviation.

Table 3: Results of precision of HBA by UV spectrophotometry method

Concentration (µg/ml)	Absorbance	Statistical Analysis
10	0.573	Mean- 0.574
10	0.572	SD- 0.002366
10	0.572	
10	0.577	%RSD- 0.41
10	0.577	
10	0.573	

Table 4: Linearity results of HBA by UV-spectrophotometry method

Sl. No.	Concentration (µg/ml)	Absorbance
1.	0	0
2.	2	0.139
3.	4	0.243
4.	6	0.342
5.	8	0.460
6.	10	0.578
7.	12	0.692
8.	14	0.814
9.	16	0.895
10.	18	1.023

Limit of Detection (LOD) and Limit of Quantification (LOQ):

Limit of detection (LOD) is the lowest amount of analyte in the sample that can be detected. Limit of quantification (LOQ) is the lowest amount of analyte in the sample that can be quantitatively determined by suitable precision and accuracy. The LOD and LOQ were separately determined and reported, based on the calibration curve of standard solution. The relative standard deviation of the regression line or the standard deviation of y–intercepts of regression lines may be used to calculate LOD and LOQ. LOD = 3.3 × S/m and LOQ = 10 × S/m, where, S is the standard deviation of Y-intercepts of regression line and m is the slope of the calibration curve. The LOD and LOQ of the developed methods were determined by analyzing progressively low concentration of the standard solutions using the developed methods. The LOD is the lowest concentration of the analyte that gives a measurable response (signal to noise ratio of 3.3). LOD of HBA was found to be 0.140 µg / ml. The LOQ is the lowest concentration of the analyte, which gives response that can be accurately quantified (signal to noise ratio of 10). The LOQ of HBA was found to be 0.424 µg / ml.

Robustness

Robustness of the method was determined by making slight change in the chromatographic condition in RP-UFLC method. Robustness is the ability to provide accurate and precise results under a variety of conditions. It was observed that there were no marked changes in the chromatograms. Results for robustness study were shown in **table 5** which indicated that the small change in the temperature conditions did not significantly affect the determination of HBA in bulk drug. Analysis was carried out at two different temperatures, room temperature and at 18°C to determine the robustness of the method and the respective areas was measured. The results were indicated as % RSD. Robustness of the method by UV spectroscopy was determined by making slight change in the temperature condition. It was observed that there were no marked changes in the absorbance.

Table 5: Results of robustness for HBA by UV spectrophotometry method

Room Temperature			Temperature 18 ˚C		
Concentration (µg/ml)	**Absorbance**	**Statistical Analysis**	**Concentration (µg/ml)**	**Absorbance**	**Statistical Analysis**
10	0.573	Mean- 0.5758 SD- 0.002483 %RSD- 0.43	10	0.572	Mean = 0.5708 SD = 0.001169 %RSD = 0.204
10	0.574		10	0.571	
10	0.574		10	0.571	
10	0.579		10	0.569	
10	0.577		10	0.572	
10	0.578		10	0.570	

Ruggedness

Ruggedness was determined by carrying out analysis by two different analysts and the respective absorbance was noted and the results were indicated as % RSD. It was observed that there were no marked changes in the absorbance. The results for ruggedness study which indicated that the analysis by different analysts did not significantly affect the determination of HBA in bulk drug (**table 6).**

RESULTS AND DISCUSSION:

RP-UFLC Method

The present study was aimed at developing a sensitive, simple, precise and robust UFLC method for the analysis of HBA in bulk drug. In order to achieve optimum separation of the component peaks, mixtures of methanol with water and acetonitrile were tested as mobile phase on C-8 stationary phase. A binary mixture of methanol and water (50:50 %v/v) was selected, as the chromatographic peaks were well defined and resolved with no tailing. The retention time obtained for HBA was 3.2 min. Each of the samples was injected six times and the same retention times were observed in all the cases. The peak areas of HBA were reproducible as indicated by low coefficient of variation. A good linear relationship (r^2 = 0.9906) was observed over the concentration range of 0-10 µg/ml of HBA. The regression curve was constructed by linear regression and its mathematical expression was y = 24847x + 7210.4 (where y gives peak area and x is the concentration of the drug). The absence of additional peaks indicated non-interference of other impurities in the drug sample is shown in **figure 2**. The low percentage relative standard deviation indicated the reproducibility of the assay of HBA in bulk drug. The deliberate changes in the method have not much affected the tailing factor, mean peak area and the retention time. This indicated the robustness of the method. System suitability parameters were studied with six replicates standard solution of the drug and the calculated parameters are within the acceptance criteria and the results are shown in **table 7.**

Table 6: Results of ruggedness for HBA by UV spectrophotometry method

Analyst 1				Analyst 2		
Concentration (µg/ml)	**Absorbance**	**Statistical Analysis**		**Concentration (µg/ml)**	**Absorbance**	**Statistical Analysis**
10	0.576	Mean- 0.575 SD- 0.001789 %RSD- 0.311105		10	0.579	Mean = 0.57633 SD = 0.001862 %RSD = 0.323
10	0.574			10	0.578	
10	0.575			10	0.576	
10	0.574			10	0.576	
10	0.573					
10	0.578					

Table 7: Characteristic parameters of HBA for the proposed RP-UFLC method

Parameters	RP-UFLC
Linearity range (µg / ml)	0-10
Detection wavelength (nm)	267
Mobile phase (Methanol : water)	50 : 50(v / v)
Retention time (min)	3.2
Regression equation (Y)	Y = 24847x + 7210.4
Slope (m)	24847
Intercept (c)	7210.4
Correlation coefficient(r^2)	0.9906
Precision (% RSD) **Intraday Precision (n=6)** **Interday Precision (n=6)**	1.00376 0.79 1.66
Limit of detection (µg / ml)	0.140
Limit of quantitation (µg / ml)	0.424
Theoretical plates	4240.899
Tailing factor (asymmetry factor)	0.940

Table 8: Characteristic Parameters of HBA for Proposed UV Spectrophotometry Method

Parameters	RESULTS
Linearity range (µg / ml)	0-18
Detection wavelength (nm)	262
Regression equation (Y)	Y = 0.0561x + 0.0135
Slope (m)	0.0561
Intercept (c)	0.0135
Correlation coefficient(r^2)	0.9991
Precision (% RSD) **Intra-day Precision (n=6)** **Inter-day Precision (n=6)**	0.41 0.158 0.364
Limit of detection (µg / ml)	0.139
Limit of quantitation (µg / ml)	0.421

UV Method

The solubility of HBA was determined in a variety of solvents. The proposed method is simple and precise and do not suffer from any interference due to impurities. Method was validated in terms of ruggedness, robustness, precision, LOD, LOQ and linearity. The characteristic parameters of HBA for proposed UV spectrophotometry method are shown in **table 8.**

CONCLUSION:

A validated UFLC and UV methods have been developed for the determination of HBA in bulk drug. The proposed methods are simple, rapid, precise, cost-effective and specific. Its chromatographic run time of 10 minutes allows the analysis of a large number of samples in a short period of time. Therefore, it is suitable for the routine analysis of HBA in bulk drug. The simplicity of the method allows for application in laboratories that lack sophisticated analytical instruments such as LC-MS/MS or GC-MS/MS that are complicated, costly and time consuming rather than a simple HPLC and UV methods. The results for standard drug HBA in the proposed method were found to be satisfactory. In an over view the results indicate that the method is precise enough for the analysis of the drug. Considering the possible worldwide development of new drug i.e. HBA as cytotoxic agent and DNA intercalator, the proposed method could be useful for the quality control, purity and identification of HBA in bulk drug in research and development and in pharmaceutical industries.

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