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Original Article

RP-HPLC METHOD DEVELOPMENT AND VALIDATION FOR THE ESTIMATION OF DICLOFENAC SODIUM, TRAMADOL HYDROCHLORIDE AND CHLORZOXAZONE FROM THEIR COMBINED TABLET DOSAGE FORM

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ABSTRACT

Objective: The objective of the current study was to develop and validate the RP-HPLC method for the simultaneous estimation of Tramadol Hydrochloride, Chlorzoxazone and Diclofenac sodium from their combined tablet dosage form

Methods: The current method describes RP-HPLC method for the estimation of Tramadol Hydrochloride, Chlorzoxazone and Diclofenac sodium from their combined tablet dosage form. The mobile phase used was Acetonitrile: 0.05M Disodium Hydrogen Phosphate buffer pH 3.5 adjusted with 10% v/v Ortho Phosphoric acid(50:50 v/v) and Hypersil ODS C_{18} (250 mm x 4.6 mm, 5.0 µ particle sizes) was used as a stationary phase with detection wavelength of 220 nm.

Result: Linearity was obtained in the concentration range of 15-75 µg/ml, 100-500 µg/ml and 20-100 µg/ml for TRM, CHL and DIC respectively. The % recovery was found to be 99.41 -99.84%, 99.30 - 99.74 % and 99.6 - 99.97 % for DIC, TRM and CHL respectively. The LOQ was found to be 3.33, 3.95 and 36.71 µg/ml for DIC, TRM and CHL respectively. The proposed method has been validated as per ICH Q2R1 guidelines and successfully applied to the estimation of TRM, DIC and CHL from their combined Tablet dosage form.

Conclusion: The method was found to be simple, accurate, precise, and suitable for the estimation of Tramadol Hydrochloride, Chlorzoxazone and Dicofenac sodium from their combined tablet dosage form.

Keywords: Diclofenac sodium, Tramadol Hydrochloride, Chlorzoxazone, RP-HPLC method, Analytical method validation.

INTRODUCTION

Tramadol Hydrochloride (TRM) is chemically (lRS,2RS)-2-[(dimethyl amino) methyl]-I-(3-methoxyphenyl) cyclohexanol hydrochloride [1] (Figure 1). TRM belongs to analgesic and narcotic category. TRA and its O-desmethyl metabolite (M1) are selective, weak OP3-receptor agonists. It is indicated in the treatment of moderate to severe pain. TRM is used to treat postoperative, dental, cancer, and acute musculosketetal pain and as an adjuvant to NSAID therapy in patients with osteoarthritis. Tramadol Hydrochloride is official in Indian Pharmacopoeia [1], British Pharmacopoeia [2] and United States Pharmacopoeia[3].

Diclofenac sodium (DIC) is chemically Sodium 2-[(2,6-dichlorophenyl)amino] phenyl acetate[4] (Figure 2). DIC is used in acute and chronic treatment of signs and symptoms of osteoarthritis and rheumatoid arthritis. Diclofenac sodium is official in Indian Pharmacopoeia[4], British Pharmacopoeia[5] and United States Pharmacopoeia[6]. Chlorzoxazone (CHL) is chemically 2(3H)-Benzoxazolone, 5-Chloro-2-benzoxazolinone[6] (Figure 3). It is indicated for the relief of discomfort associated with acute painful musculoskeletal conditions. Chlorzoxazone is official in United States Pharmacopoeia [6]. The review of literature revealed that many analytical methods involving UV Spectrophotometric [7-8], RP-HPLC[9-11], HPTLC[12] and UPLC[13] have been reported for TRM individually and in combination with other drugs. Several methods have been reported for the estimation of DIC individually and in combination with other drugs such as UV Spectrophotometric[14], RP-HPLC[15-17], HPTLC[18] and LC-MS[19]. Several methods have been reported for Chlorzoxazone individually and in combination with other drugs namely UV Spectrophotometric[20], RP-HPLC[21] and HPTLC[22]. But no method has been reported for the estimation of TRM, DIC and CHL from their combined dosage form.

So, the present article describes RP-HPLC method for the estimation of TRM, DIC and CHL in their tablet dosage form. The developed method was validated applying ICH Q2R1 guidelines[23] and was used for the assay of three drugs in their tablet dosage form.

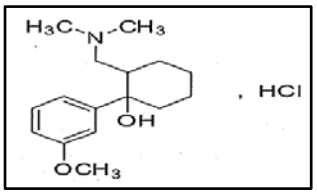

Fig. 1: It shows the chemical structure of Tramadol Hydrochloride

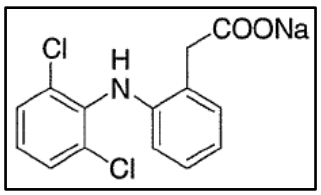

Fig. 2: It shows the chemical structure of Diclofenac Sodium

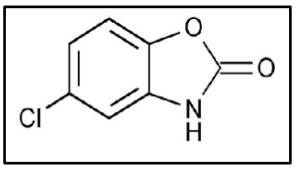

Fig. 3: It shows the chemical structure of Chlorzoxazone

MATERIALS AND METHODS

Materials

Analytically pure samples of TRM, DIC and CHL were procured from Medico Lab, Vatva, Ahmedabad; Medico Lab, Vatva, Ahmedabad and Baroque Pharmaceuticals ltd., Khambhat respectively.

Orthophosphoric acid (A.R. Grade) was purchased fromSD Fines chemicals, Bombay; Water (HPLC Grade) from RFCL limited, New Delhi; Acetonitrile (HPLC Grade) from RFCL limited, New Delhi and Disodium Hydrogen Phosphate (A. R. Grade) from SD Fines chemicals, Bombay. Tablet of TRM, DIC and CHL in combined dosage form (TRICARE-MR) was purchased from the local market.

Instrument and experimental condition

RP-HPLC analysis was carried out on the instrument of Analytical Technologies limited with the injector loop of 20 µl on the isocratic mode. The pump used was P2230 plus HPLC pump and the detector UV 2230 plus detector.

The data was analyzed using Analchrom 2006 software. The column used was Hypersil ODS C_{18} (250 × 4.6 m, 5 µm particle size). The pH meter used was Chemiline Digital pH meter CL-110.

Preparation of Mobile Phase

Accurately measured 7.089 gm Disodium Hydrogen Phosphate was dissolved in 1000 ml of HPLC grade water. A mixture of 50 ml Acetonitrile and 50 ml of 0.05M Disodium Hydrogen phosphate was prepared and the pH of the mixture was adjusted to 3.5 pH with 10 % orthophosphoric Acid and sonicated for 10 minutes, filtered through 0.45 µm filter paper and sonicated for 10 minutes to degas the mixture and used as mobile phase.

Preparation of standard stock solution

A 100 mg of standard TRM, DIC and CHL were accurately weighed and transferred to each of 100 ml volumetric flask and dissolved in 50 ml methanol. The flask was sonicated for 10 min. The flask was shaken and volume was made up to the mark with methanol to give solutions containing 1000 µg/ml TRM, DIC and CHL.

Analytical Method validation

(1) Linearity and Range

The linearity of analytical method is its ability to elicit test results that are directly proportional to the concentration of analyte in sample within a given range. The range of analytical method is the interval between the upper and lower levels of analyte that have been demonstrated to be determined within a suitable level of precision, accuracy and linearity.

The range were found to be 20-100 µg/ml for DIC, 15-75 µg/ml for TRM and 100-500 µg/ml for CHL. All the chromatogram was repeated for 6 times. Calibration curves were constructed by plotting average Area versus concentrations for all the three drugs. Straight line equations were obtained from these calibration curves.

(2) Repeatability

Standard solution mixture containing TRM (15-75 µg/ml), DIC (20-100 µg/ml) and CHL (100-500 µg/ml) were prepared and chromatograms were recorded and area were measured and C.V. were calculated. Sample solution containing 30 µg/ml TRM, 40 µg/ml DIC and 200 µg/ml CHL was prepared and chromatogram was recorded. Area was measured of the same concentration solution six times and CV was calculated.

(3) Precision (Inter-day and Intra-day)

The precision of an analytical method is the degree of agreement among individual test results when the method is applied repeatedly to multiple samplings of homogenous samples. It provides an indication of random error results and was expressed as Coefficient of Variance (CV).

Variations of results within the same day (intra-day), variation of results between days (inter-day) were analyzed. Intraday precision was determined by analyzing TRM, DIC and CHL for three times in the same day. Inter day precision was determined by analyzing the three drugs daily for three days. For this study standard solution mixture of 15, 45 and 75 µg/ml for TRM; 20, 60 and 100 µg/ml for DIC and 100, 300, 500 µg/ml for CHL were prepared and chromatograms were recorded.

(4) Accuracy

Accuracy is the closeness of the test results obtained by the method to the true value. To study the accuracy sample solution of formulation was prepared and analysis of the same was carried out. Recovery studies were carried out by addition of standard drug to the sample solution at 3 different concentration levels 50, 100 and 150 %, taking into consideration percentage purity of added bulk drug samples. Each concentration was analyzed three times and average recoveries were measured.

(5) Reproducibility

The areas were measured using same instrument by twoanalysts for sample solution and the values obtained were evaluated using t-test to verify their reproducibility.

(6) Specificity and Selectivity

Specificity is a procedure to detect quantitatively the analyte inthe presence of component that may be expected to be present in the sample matrix, while selectivity is the procedure to detect qualitatively the analyte in presence of components that may be expected to be present in the sample matrix. Specificity of an analytical method is ability to measure specifically the analyte of interest without interferences from blank and placebo. It was checked for interference from blank.

(7) Limit of detection & Limit of quantification

The limit of detection (LOD) and the limit of quantification (LOQ) of the drug were derived by using the following equations as per International Conference on Harmonization (ICH) guidelines which is based on the calibration curve.

$LOD = 3.3 \times \sigma / S$

$LOQ = 10 \times \sigma / S$

Where σ = the standard deviation of y-intercepts of regression lines

S = Slope of calibration curve.

(8) Assay of marketed formulation

Twenty tablets were weighed individually and the average weight of the single tablet was found to be 752 mg. Powder equivalents to 250 mg CHL, 50 mg DIC and 37.5 mg TRM was accurately weighed and transferred to 25 ml volumetric flask. 15 ml of methanol was added to same volumetric flask and sonicated for 20 min. The flask was shaken and volume was made up to the mark with methanol. The above solution was filtered through whatmann filter paper (0.45µm). 5 ml of aliquot was taken and transferred to volumetric flask of 50 ml capacity and volume was made up to the mark with the methanol. Further 2 ml of this solution was transferred to volumetric flask of 10 ml capacity. Volume was made up to the mark with the mobile phase to give a solution containing 30 µg/ml TRM, 40 µg/ml DIC and 200 µg/ml CHL. This solution was sonicated for 5 min. This solution was used for the estimation of TRM, DIC and CHL.

RESULTS AND DISCUSSION

Method optimization

For the selection of mobile phase, various mobile phase systems were tried for thechromatographic separation.Finally, the system containing Acetonitrile and 0.05M Disodium Hydrogen phosphate with a pH 3.5 adjusted with 10 % Ortho Phosphoric Acid (50:50 %v/v) gave well resolved peaks of all the three drugs. The average retention time of TRM, CHL and DIC were found to be 2.11 min., 3.82 min. and 12.39 min. respectively. For the selection of analytical wavelength, the overlain spectra of 50 µg/ml TRM, 50 µg/ml DIC and 50 µg/ml CHL revealed that at 220 nm all the three drugs possess absorbance (Fig. 4).

Analytical method validation

1) Linearity and Range

The method was found to be linear at the concentration range of 15-75 µg/ml for TRM, 100-500 µg/ml for CHL and 20-100 µg/ml for DIC.

Calibration data for TRM, CHL and DIC are shown in Table 2-4 respectively. The calibration curves for TRM, DIC and CHL were prepared by plotting area and concentration(Fig. 6-8).

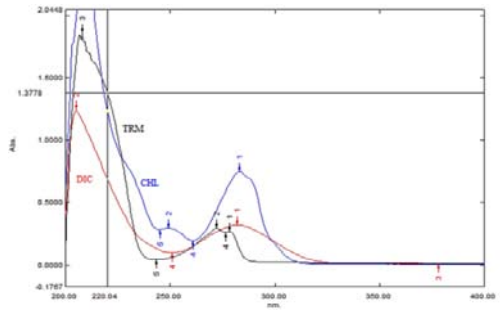

Fig. 4: It shows the overlain spectra of 50 µg/ml TRM, 50 µg/ml DIC and 50 µg/ml CHL in Methanol

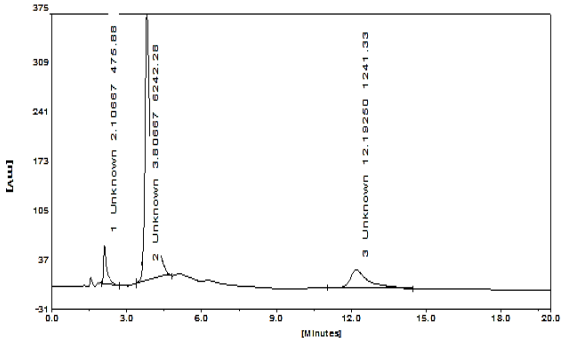

Fig. 5: It shows the chromatogram of mixed standard solution containing 60 µg/ml TRM, 400 µg/ml CHL and 80 µg/ml DIC, using mobile phase Acetonitrile: 0.05M Disodium Hydrogen Phosphate buffer pH 3.5 (50:50 v/v) (Proposed Method).

Table 1: It shows the optimized Chromatographic conditions for TRM, CHL and DIC

Parameter	Conditions
Mobile phase	Acetonitrile: 0.05M Disodium Hydrogen Phosphate buffer pH 3.5 adjusted with 10 % Ortho Phosphoric acid
Pump Mode	Isocratic
Column	Hypersil ODS C_{18}, 250 x 4.6 mm (5 µm)
Detection wavelength (nm)	220
Flow rate	1.5 ml/min
Run Time (min)	20.0
Diluent	Mobile phase
Volume of Injection (µl)	20.0
Retention time	TRM: 2.11 min
	CHL: 3.82 min
	DIC: 12.39 min

Table 2: It shows the result of calibration readings for TRM by HPLC method

Concentration (µg/ml)	Area Mean ± S.D.	C.V
15	109.16 ± 1.4454	1.32
30	231.05 ± 2.1030	0.91
45	353.11 ± 3.9710	1.12
60	476.16 ± 4.3070	0.90
75	568.97 ± 4.4632	0.78

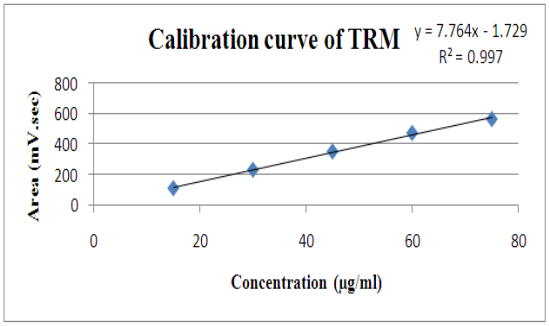

Fig. 6: It shows the calibration curve of TRM by RP-HPLC method.

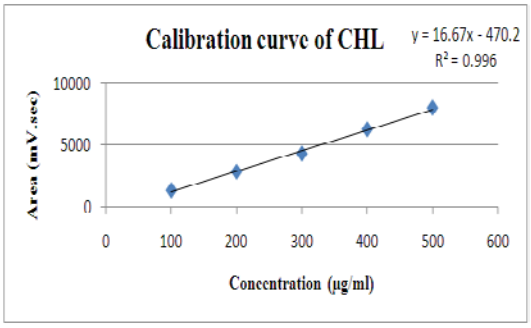

Fig. 7: It shows calibration curve of CHL by RP-HPLC method

Table 3: It shows the result of calibration readings for CHL by HPLC method

Concentration (µg/ml)	Area Mean ± S.D.	C.V
100	1340.11 ± 14.8804	1.11
200	2827.63 ± 21.1644	0.74
300	4278.32 ± 29.2475	0.68
400	6243.63 ± 60.5147	0.97
500	7968.69 ± 87.2778	1.09

Table 4: It shows the result of calibration readings for DIC by HPLC method

Concentration (µg/ml)	Area Mean ± S.D.	C.V
20	174.065 ± 2.0449	1.17
40	601.018 ± 5.9305	0.98
60	926.337 ± 8.8130	0.95
80	1242.75 ± 9.9257	0.79
100	1547.98 ± 13.402	0.86

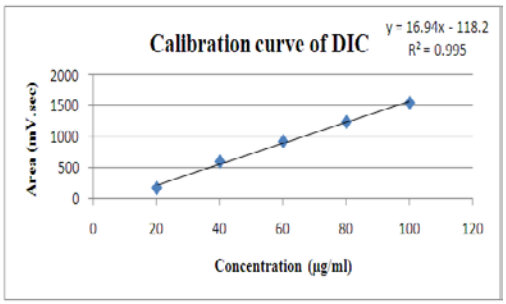

Fig. 8: It shows calibration curve of DIC by RP-HPLC method

2) Repeatability:

The developed method was found to be repeatable since C.V. was found to be less than 2 for all the drugs.

3)Limit of Detection (LOD) and Limit of Quantitation (LOQ)

The LOD for TRM, DIC and CHL was found to be 1.30 µg/ml, 1.10 µg/ml and 12.11 µg/ml respectively. The LOQ for TRM, DIC and CHL was found to be 3.95 µg/ml, 3.33 µg/ml and 36.71 µg/ml respectively

4) Accuracy

Accuracy was determined by calculating the % recovery. The method was found to be accurate with % recovery 99.30 - 99.74 % for TRM, 99.41 – 99.84% for DIC and 99.6 - 99.97 % for CHL (Table 5).

5) Precision

Precision was calculated as repeatability and intra- and inter-day variation for all the threedrugs. The method was found to be precise with C.V. 0.86 – 0.99 for intra-day (n=3) and C.V. 0.95 – 1.15 for inter-day (n=3) for TRM; C.V. 0.77 – 0.97 for intra-day (n=3) and C.V. 0.98 – 1.17 for inter-day (n=3) for DIC and C.V. 0.69 – 0.97 for intra-day (n=3) and C.V. 0.97 – 1.31 for inter-day (n=3) for CHL (Table 6-8).

Table 5: It shows the accuracy studies for TRM, CHL and DIC by HPLC method (n=3)

% Level of Recovery	Amount of drug in sample (µg/ml)	Amount of standard added (µg/ml)	Total amount of Drug (µg/ml)	Amount of drug recovered (µg/ml) ± SD	% Recovery ± SD
	TRM (µg/ml)	TRM (µg/ml)	TRM (µg/ml)	TRM (µg/ml)	% TRM
Unspiked	30	0	30	29.55 ± 0.4574	-
50 %	30	15	45	14.89 ± 0.1327	99.30 ± 0.8722
100 %	30	30	60	29.82 ± 0.1681	99.39 ± 0.5572
150 %	30	45	75	44.88 ± 0.1543	99.74 ± 0.3349
	DIC (µg/ml)	**DIC (µg/ml)**	**DIC (µg/ml)**	**DIC (µg/ml)**	**% DIC**
Unspiked	40	0	40	39.82 ± 0.1541	-
50 %	40	20	60	19.95 ± 0.0989	99.75 ± 0.4949
100 %	40	40	80	39.76 ± 0.1840	99.41 ± 0.4581
150 %	40	60	100	59.91 ± 0.0917	99.84 ± 0.1541
	CHL (µg/ml)	**CHL (µg/ml)**	**CHL (µg/ml)**	**CHL (µg/ml)**	**% CHL**
Unspiked	200	0	200	199.31 ± 0.7764	-
50 %	200	100	300	99.67 ± 0.5800	99.67 ± 0.5800
100 %	200	200	400	199.93 ± 0.6759	99.97 ± 0.3362
150 %	200	300	500	298.79 ± 0.6308	99.6 ± 0.2118

Table 6: It shows the precision study for TRM by RP-HPLC method (n=3)

Concentration (µg/ml)	Intra-day (Area ± S.D)	C.V.	Inter-day (Area ± S.D)	C.V.
15	110.1 ± 0.9543	0.86	109.95 ± 1.2744	1.15
45	352.71 ± 3.3369	0.94	349.71 ± 3.3459	0.95
75	565.17 ± 5.5827	0.99	567.70 ± 5.6119	0.98

Table 7: It shows the precision study for CHL by RP-HPLC method (n=3)

Concentration (µg/ml)	Intra-day (Area ± S.D)	C.V.	Inter-day (Area ± S.D)	C.V.
100	1340.13 ± 13.0147	0.97	1342.22 ± 17.6192	1.31
300	4274.11 ± 37.462	0.87	4258.01 ± 42.0006	0.98
500	7928.8 ± 55.2941	0.69	7927.32 ± 77.3596	0.97

Table 8: It shows the precision study for DIC by RP-HPLC method (n=3)

Concentration (µg/ml)	Intra-day (Area ± S.D)	C.V.	Inter-day (Area ± S.D)	C.V.
20	173.55 ± 1.6879	0.97	174.05 ± 2.0439	1.17
60	926.78 ± 7.2179	0.77	925.53 ± 9.0790	0.98
100	1538.37 ± 13.8437	0.89	1537.92 ± 16.0222	1.04

6) Reproducibility

The method was found to be reproducible (Table 9-11).

Table 9: It shows the reproducibility data for TRM (30 µg/ml)

Analyst 1 Area ± S.D. (n=3)	Analyst 2 Area ± S.D. (n=3)	Result of t test*	Inference
232.79 ± 1.9567	231.64 ± 2.5402	0.341	No significant difference

* At 95% confidence interval, (t-Tabulated = 4.30)

Table 10: It shows the reproducibility data for CHL (200 µg/ml)

Analyst 1 Area ± S.D. (n=3)	Analyst 2 Area ± S.D. (n=3)	Result of t test*	Inference
2860.9 ± 5.9568	2857.97 ± 4.0887	0.333	No significant difference

* At 95% confidence interval, (t-Tabulated = 4.30)

Table 11: It shows the reproducibility data for DIC (40 µg/ml)

Analyst 1 Area ± S.D. (n=3)	Analyst 2 Area ± S.D. (n=3)	Result of t test*	Inference
559.72 ± 3.1038	559.21 ± 4.5717	0.923	No significant difference

* At 95% confidence interval, (t-Tabulated = 4.30)

7) System suitability parameters

Table 12: It shows the system suitability parameters for developed method

Parameter	Diclofenac sodium	Chlorzoxazone	Tramadol Hydrochloride	Range	Inference
Retention time (min)	12.392 ± 0.3045	3.828 ± 0.0348	2.116 ± 0.0174	-	-
Resolution (Rs)	12.142 ± 0.8527	6.292 ± 0.0591	3.18 ± 0.101	> 2	Criteria met
Tailing Factor	1.684 ± 0.1335	1.3 ± 0.1264	1.38 ± 0.1166	< 2	Criteria met
Theoretical Plates (Plates/Meter)	8783.42 ± 1409.2	7620.15 ± 228.291	7678.8 ± 261.282	> 2000	Criteria met

Table 13: It shows the assay result of marketed formulation (n=3)

Formulation	Drug	Amount Taken (µg/ml)	Amount Found (µg/ml) (n=3)	Labeled claim (mg)	Amount found per Tablet (mg)	%Assay ± SD
Tricare-MR	DIC	40	39.95	50	49.9379	99.88 ± 0.2027
	TRM	30	29.91	37.5	37.3833	99.69 ± 0.4573
	CHL	200	199.78	250	249.725	99.89 ± 0.5466

8) Specificity

The method was found to be specific as no interferencewas observed when the chromatogram was recorded in the presence of excipients.

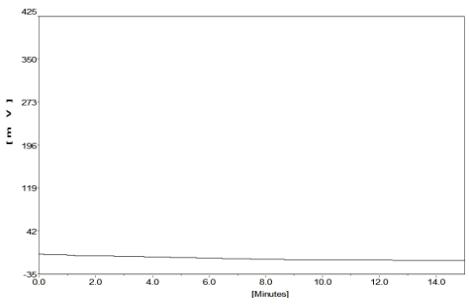

Fig. 9: It shows the chromatogram of specificity study.

9) Assay

Formulation was analyzed by the proposed method and assay result of formulation is shown in Table 12

CONCLUSION

The RP-HPLC method for the estimation of TRM, CHL and DIC from their combined tablet dosage form was successfully developed. The method was validated as per ICH Q2R1 guidelines for Specificity, Linearity, Accuracy, Precision, Limit of Detection (LOD), Limit of Quantitation (LOQ) and Reproducibility. The method can be successfully applied for the routine analysis of the formulation.

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