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"RP-HPLC METHOD DEVELOPMENT AND VALIDATION FOR SIMULTANEOUS ESTIMATION OF IRBESARTAN, AMLODIPINE BESYLATE AND HYDROCHLOROTHIAZIDE IN TABLET"

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ABSTRACT

The simple, specific, accurate & precise RP-HPLC method have been developed and validated for simultaneous estimation of triple dose combination which is used to treat hypertension. The triple combination is of Hydrochlorothiazide, Amlodipine besylate and Irbesartan. In this method the chromatographic system was equipped with Phenomenax C18 column (25cm x 0.46 cm, 5µ) as stationary phase and UV detector set at 224 nm, in conjunction with a mobile phase of Phosphate Buffer(pH 4.5): Acetonitrile in theration of 55:45% v/v ratio at a flow rate of 1.0ml/min. the retention time of Hydrochlorothiazide was 3.489 min, for Amlodipine besylate it was 4.181 min & the retention time of Irbesartan was 11.376 min. This method was linear with correlation coefficient 0.9970, 0.9974 & 0.9973 respectively. Also there was no any interference of diluent with chromatogram of standard & sample. The % recovery were 99.37, 100.70 & 100.28%. Also it was statistically validated for accuracy, precision, specificity & robustness as per ICH guidelines. After analyzing the data of validation, we can say that this method can be used for further analysis. As this proposed method is rapid, specific, accurate, and robust with good resolution & short run time. So, the method can be used for routine analysis.

KEYWORDS: Hydrochlorothiazide, Amlodipine besylate & Irbesartan, simultaneous RP-HPLC, Method validation.

INTRODUCTION

Chemically Hydrochlorothiazide is 6-chloro-1, 1-dioxo-3, 4-dihydro-2H-1λ^6, 2, 4-

benzothiadiazine-7-sulfonamide which is diuretic class of antihypertensive drug. It reduces blood volume by acting on the kidneys to reduce sodium (Na^+) reabsorption in the distal convoluted tubule. The major causes of edema is congestive heart failure, kidney or liver problem and different medications such as corticosteroid and estrogen. Thiazides increase the reabsorption of calcium in this segment in a manner unrelated to sodium transport. Ultimately HCTZ works by making more urine and helps the body to get rid of extra salt and water also it reduces extra fluid (edema) in the body.

Amlodipine besylate is chemically Benzenesulphonic acid; 3-O-ethyl 5-O-methyl 2-(2-aminoethoxymethyl)-4-(2-chlorophenyl)-6-methyl-1, 4-dihydropyridine-3, 5-dicarboxylate. It is besylate salt of Amlodipine used as Calcium channel blocker. It inhibits the movement of calcium ions into vascular and cardiac muscle cells, with a greater effect. This causes vasodilation thus lowering blood pressure. Amlodipine besylate is used with or without other medications to treat high blood pressure. It works by relaxing blood vessels so blood can flow more easily.

The chemical name of Irbesartan is 2-butyl-3-({4-[2-(2H-1, 2, 3, 4-tetrazol-5-yl) phenyl] phenyl} methyl)-1, 3-diazaspiro [4.4] non-1-en-4-one. It is Angiotensin Receptor Blocker/antagonist (ARB) which works by blocking a substance in the body that causes blood vessels to tighten. As a result, Irbesartan relaxes the blood vessels. This lowers blood pressure and increases the supply of blood and oxygen to the heart.

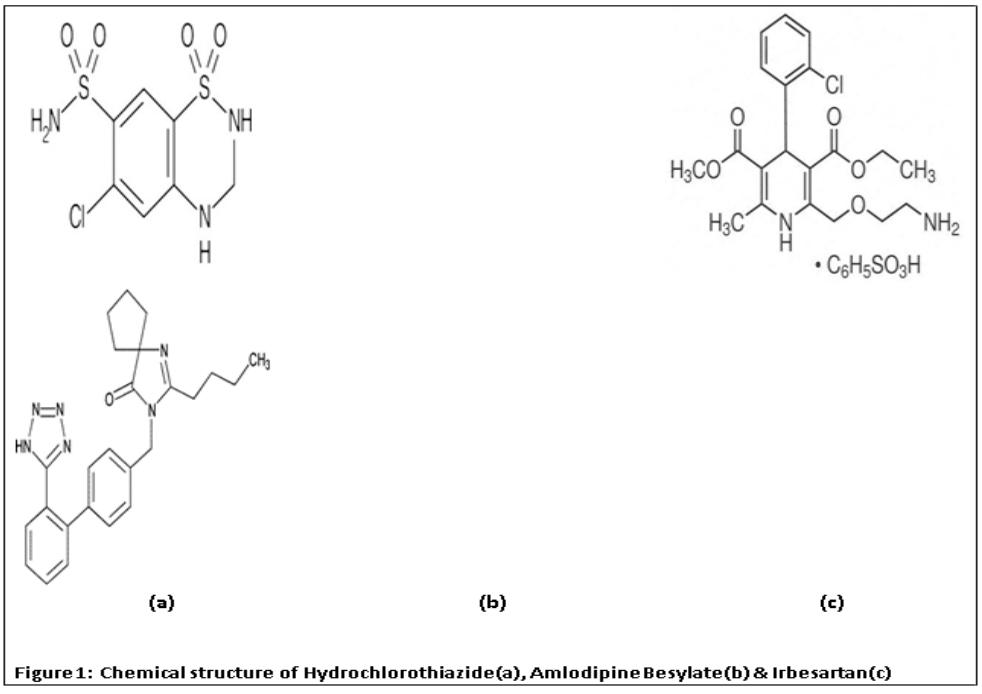

Figure 1: Chemical structure of Hydrochlorothiazide(a), Amlodipine Besylate(b) & Irbesartan(c)

Literature review reveals different analytical methods for this triple dose combination. These methods include UV-Visible spectrophotometry, LC, HPLC, HPTLC, GC, Potentiometric titration, LC-MS fir estimation of either single drug or in combination with other drugs but there is no any RP-HPLC method for simultaneous estimation for this stated combination. Therefore, attempt was made to develop and validate simple, precise, accurate, specific and robust RP- HPLC method for estimation of simultaneous estimation of these three drug in their combined dosage form as a tablet. The method was developed and validated as per ICH guideline Q2 (R1).

MATERIALS AND METHOD

Instruments

HPLC analysis carried out using RP-HPLC system (SHIMADZU) equipped with a UV detector, C18 column (25cm, 0.4cm), and LC solution a software. Analytical balance (Citizen Balance), pH meter (Expo Hitech pH-361), Ultrasonicatir (Analeb Sonicator), Corning volumetric flasks, pipettes of borosilicate glass were used in the study.

Material and Reagent

Hydrochlorothiazide Amlodipine besylate and Irbesartan were obtained from RL Fine Chem, CTX Lifescience and Mapromax Lifescience respectively. Water For Injection [HPLC grade] procured from Finar chemicals; Acetonitrile [HPLC grade]; Methanol [HPLC grade] and Sodium hydroxide [HPLC grade] procured from Ranchem; Potassium dihydrogenortho phosphate [AR grade] & Ortho Phosphoric acid [AR grade] were obtained from Merck & company.

METHOD DEVELOPMENT

Selection of Wavelength

Suitable wavelength for the HPLC analysis was determined by recording UV spectrums in the range 200-400 nm. Here solution of 25µg/ml Hydrochlorothiazide, 13.92µg/ml Amlodipine besylate and 300µg/ml Irbesartan was prepared in methanol and measured. Suitable wavelength selected for the estimation of this combined drug is 224nm.

Chromatographic Condition

Table 1 chromatographic condition	
Stationary phase	C18 (25cm x 0.46 cm, 5µm) Phenomenax
Mobile phase	Phosphate Buffer (pH 4.5) : Acetonitrile (55:455v/v)
Detection Wavelength	224 nm
Injection volume	10 µl
Flow rate	1.0 ml/min
Column Temperature	25°C
Run time	15 minutes
Concentration Of API in standard solution (throughout all trials)	Irbesartan :- 300 µg/ml Amlodipine Besylate :- 13.92 µg/ml & Hydrochlorothiazide :- 25 µg/ml

Preparation of Mobile Phase

3.40 gm Potassium dihydrogen ortho phosphate (KH_2PO_4) was weighed and dissolved in 1000 ml Water. Adjusted pH 3.40 ± 0.05 with Diluted OPA and filtered with 0.45µ membrane filter. Mix thoroughly 550 ml Phosphate Buffer pH 3.40 and 450 ml Acetonitrile In ratio of 55:45% v/v. Degassed by Sonicator for 15 Min.

a) Preparation of Diluent

3.40 gm Potassium dihydrogen ortho phosphate (KH_2PO_4) was weighed and dissolved in 1000 ml Water. Adjusted pH 6.00 ± 0.05 with Diluted NaOH solution and filtered with 0.45µ membrane filter. Mix thoroughly 400 volume of Phosphate Buffer and 600 volume of Acetonitrile as diluent.

b) Standard Stock Solution – 1 [Irbesartan-3000 µg / ml]

300 mg of Irbesartan WS was weighed and transferred to a 100 ml volumetric flask. Add 20 ml Methanol & volume was made up to the mark with diluent.

c) Standard Stock Solution – 2 [Amlodipine besylate- 348 µg / ml]

87 mg of Amlodipine besylate WS was weighed and transferred to a 250 ml volumetric flask. Add 10 ml Methanol & volume was made up to the mark with diluent**.**

d) Standard Stock Solution – 3 [Hydrochlorothiazide -250 µg / ml]

50 mg of Hydrochlorothiazide WS was weighed and transferred to a 200 ml volumetric flask. Add 10 ml Methanol & volume was made up to the mark with diluent**.**

e) Working Standard Solution

Take 5 ml of solution (1), 2 ml of solution (2), and 5 ml of solution (3) into a dried 50 ml

volumetric flask, add 20 ml of diluent, mix and make up the volume with diluent.

f) Preparation of Sample Solution

Take 5 whole Tablets and was transferred to 1000 ml volumetric flask, Add 10 ml water and sonicate to disperse. Now add 200 ml of methanol, sonicate. Add 600 ml of diluent further sonicate & cool. Make the volume up to mark with diluent.

g) Working Sample Preparation

Take 10 ml from sample solution and transferred to 50 ml volumetric flask and make up volume up to the mark with diluent.

METHOD VALIDATION

Validation of the developed RP-HPLC method was carried out as per ICH guidelines Q2 (R1).

1) Specificity

Specificity of method can be termed as absence of any interference at retention times of samples. Specificity was performed by injecting blank and standard preparations. Chromatograms were recorded and retention times from sample and standard preparations were compared for identification of analytes.

2) Preparation of calibration curve sample (linearity of the method)

A series of standard solutions were prepared. An aliquot of each solution was injected 3 times and peak area was observed. Plot of average peak area versus the concentration is plotted and from this data the regression coefficient and regression equation were generated. Calibration curves were generated by plotting peak area vs. concentration.

3) Precision

The method was validated in terms of intra-day inter-day precision. The solution containing 12.5, 25 & 37.5 µg/ml Hydrochlorothiazide, 6.69, 13.92 & 20.88 µg/ml Amlodipine besylate and 150, 300& 450 µg/ml Irbesartan were injected for inter-day and intra-day study. Repeatability study was performed by injecting standard sample solutions six times.

4) Accuracy

Accuracy was determined by calculating recovery of drugs by placebo method. Known amounts of standard solutions of Hydrochlorothiazide (20, 25 & 30 µg/ml), Amlodipine

besylate (11.15, 13.92, and 16.70 µg/ml) and Irbesartan (240, 300 and 360µg/mL) were added to a pre quantified placebo test solutions of Hydrochlorothiazide, Amlodipine besylate and Irbesartan. Each solution was injected in triplicate and the recovery was calculated by measuring peak areas.

5) Robustness

The robustness study was performed to evaluate the influence of small but deliberate variation in the chromatographic condition. The robustness was checked by making three small changes in chromatographic parameters. The mobile phase ratio was changed by ±2 ml, the wavelength was changed by ±2 nm and the flow rate was changed by ±0.02 ml/min. After each changes sample solution was injected and system suitability parameters were observed.

RESULT AND DISCUSSION

- **Method development and optimization**

The detection was carried out in the UV region at 224 nm. Different composition of mobile phase was tested the sample peak was identified by comparing retention time with the standard solution.

Composition giving retention time of 3.489 min for Hydrochlorothiazide, 4.181 min for Amlodipine besylate and 11.376 min for Irbesartan with good resolution and theoretical plates was selected, that optimized mobile phase was Buffer: ACN (55:45% v/v pH 3.40 with OPA). SST parameters were within the accepted limits and was ideal for the injected sample. A chromatogram of the mixture in optimized conditions is shown Figure 3 and the system suitability parameters are shown in Table3.

Table 2: Results for System suitability parameters

Drug	HCTZ	AMLO	IRBE
Retention Time	3.489	4.181	11.376
Tailing Factor	1.273	1.296	0.999
Resolution	-	3.745	24.578
Theoretical Plates	6734.000	7048.018	13876.122

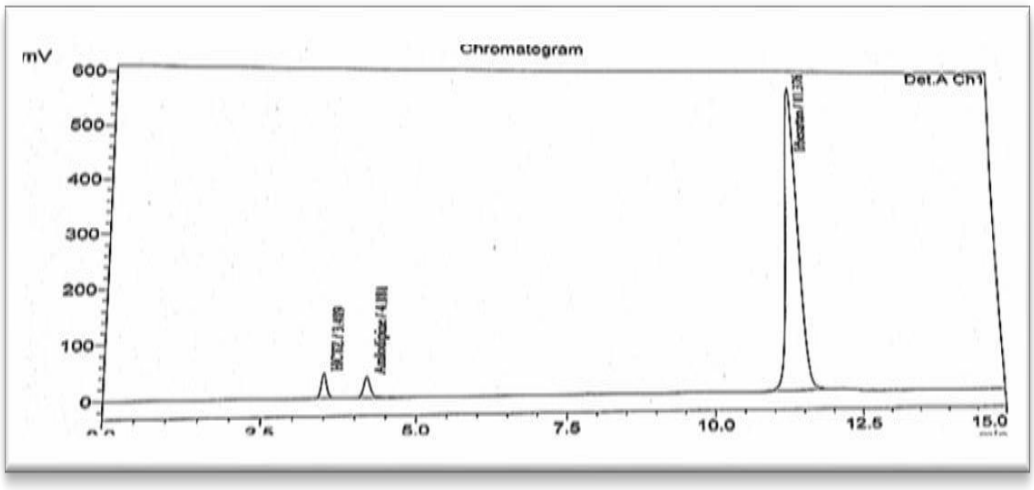

Figure 2: Optimized condition chromatogram of HCTZ(25 µg /ml), AMLO(13.92 µg /ml) & IRBE (300 µg /ml).

Method validation

1. Linearity

Linearity is ability of method to provide results directly proportional to the concentration of analyte. The calibration data of Hydrochlorothiazide, Amlodipine besylate and Irbesartan is given in Table 3.

Table 3: Calibration Curve data for Linearity

Sr. No	% con	Aliquot (ml)			Make Up (ml)	Conc. (µg /ml)		
		HCTZ	IRBE	AMLO		HCTZ	IRBE	AMLO
1	70	3.5	3.5	1.4	50	17.5	210	9.74
2	80	4.0	4.0	1.6	50	20.0	240	11.13
3	90	4.5	4.5	1.8	50	22.5	270	12.52
4	100	5.0	5.0	2.0	50	25.0	300	13.92
5	110	5.5	5.5	2.2	50	27.5	330	15.31
6	120	6.0	6.0	2.4	50	30.0	360	16.70
7	130	6.5	6.5	2.6	50	32.5	390	18.09

- Mean area and RSD values are as follow.

Table 4: Mean Area And %RSD for Linearity.

Sr. No	Hydrochlorothiazide		Amlodipine besylate		Irbesartan	
	Mean Area ± SD	% RSD	Mean Area ± SD	% RSD	Mean Area ± SD	% RSD
1	187424.6667± 1374.164	0.73	190684.6667 ±2396.38488	1.25	6038215.667± 48521.2219	0.81
2	214214± 699.98	0.33	218421.6667± 492.853426	0.22	6888854.667± 59982.7475	0.87
3	238938± 542.1291	0.22	243872.3333± 4159.90919	1.7	7672910.333± 80293.0118	1.05
4	267287.3333± 1811.014	0.67	275289.3333± 2494.67272	0.91	8546420.667± 2368.65876	0.29
5	298299± 149.3687	0.05	309297.6667± 1129.95664	0.36	9578910.667± 74425.9294	0.78
6	323262± 2068.228	0.639	329759.3333± 4265.97521	1.29	10372937.33± 45891.8774	0.44
7	344219.6667± 3128.718	0.91	355320.3333± 1966.46396	0.56	11036090.33± 56833.4226	0.52

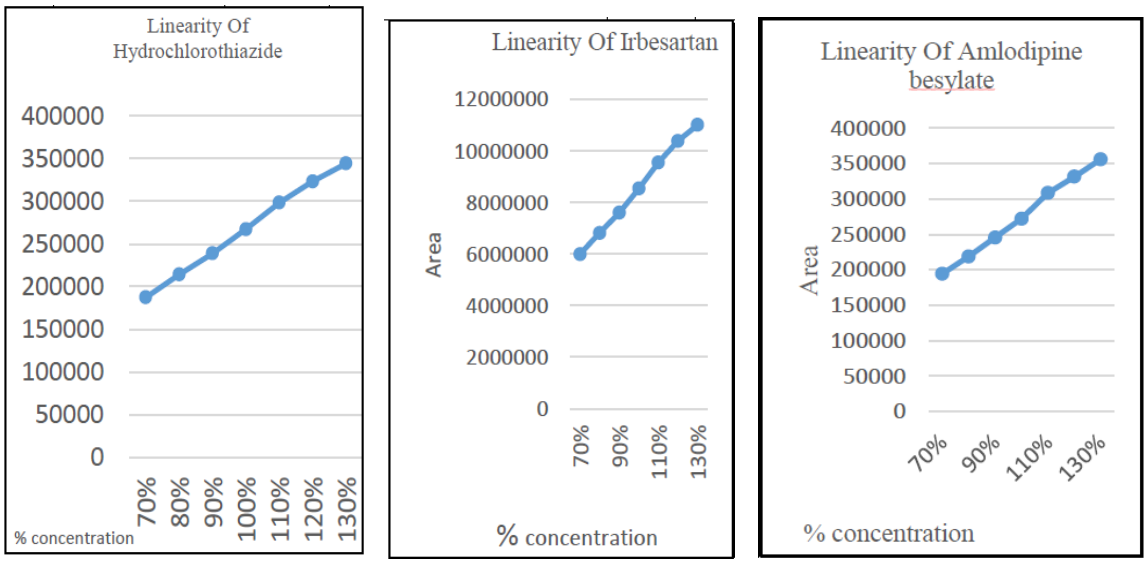

Figure 3: Linearity curve of HCTZ, AMLO & IRBE.

Analysis of above curves are as follow.

Table 5: Regression analysis of calibration curves.

Drug	HCTZ	AMLO	IRBE
Concentration Range (µg/ml)	17.5 – 32.5	9.74-18.09	210-390
slope	266774.6429	276063.2143	8623393.5714
Intercept	680.7857	684.9286	56855.0000
(R^2)	0.9970	0.9974	0.9973
Regression equation	y = 266774.6429x + 680.7857	y = 276063.2143x – 684.9286	y = 8623393.5714x – 56855.0000

2. Precision

Repeatability was studied by calculating %RSD for six replicate injections on same day under same experimental conditions. The obtained %RSD value for Hydrochlorothiazide was 0.05%, 0.824% for Amlodipine besylate and 0.098% for Irbesartan. It was found to be <2%, which indicate repeatability of proposed method.

Interday precision study reveals % RSD 0.71-1.07 for Hydrochlorothiazide, 0.27-0.47 for Amlodipine besylate and 0.30-0.69 for Irbesartan respectively. While the % RSD for intraday was 0.12-1.70 for Hydrochlorothiazide, 0.59-1.01 for Amlodipine besylate & 0.10-0.57 for Irbesartan. Which is also <2%. So, the method is precise and reproducible. Data for all there three parameters are given in Table 6.

Table 6 Precision study for HCTZ,AMLO, & IRBE

Parameter	Conc.(µg/ml)			%RSD		
	HCTZ	AMLO	IRBE	HCTZ	AMLO	IRBE
Repeatability*	25	13.92	300	0.005	0.8249	0.0985
Inter-day Precision**	12.5	6.69	150	0.71	0.27	0.45
	25	13.92	300	0.79	0.47	0.69
	37.5	20.88	450	1.07	0.46	0.30
Intra-day Precision**	12.5	6.69	150	0.79	0.99	0.1
	25	13.92	300	0.12	1.01	0.11
	37.5	20.88	450	1.70	0.59	0.57

*= average of six determination

** = average of three determinations

3. Accuracy

It is estimated by placebo method for three replicates of three different solution containing 80% 100% and 120% of target concentration. The obtained recovery of 80%, 100% & 120% range for Hydrochlorothiazide were 101.46, 99.37 & 101.46; for Amlodipine besylate they were 101.64, 100.70 & 100.81 while for Irbesartan they were 101.75, 100.28 & 100.16 respectively. The values of recovery reveal accuracy of method.

Table 7 Recovery data for HCTZ & AMLO AND IRBE by Placebo method				
Drug	**Accuracy Level**	**Amount Added (µg/ml)**	**Total Amount found* (µg/ml)**	**% Recovery ± SD**
HCTZ	80%	20.00	20.33	101.46±0.287
	100%	25.00	24.78	99.37±0.268
	120%	30.00	29.71	101.46±0.287
AMLO	80%	11.15	11.17	101.64±0.252
	100%	13.92	14.01	100.70±0.780
	120%	16.70	16.82	100.81±0.165
IRBE	80%	240.00	241.81	100.75±0.124
	100%	300.00	300.12	100.28±0.310
	120%	360.00	360.23	100.16±0.150

*= average of six determination

4. Robustness

The result and range of three different variables selected for robustness testing are given in Table 6. The values indicate no significance changes in chromatographic pattern when small deliberate modifications were made in the experimental conditions, thus the developed method to be robust.

Table 8 Data of Robustness for Hydrochlorothiazide, Amlodipine besylate & Irbesartan				
Parameter	**Change Level**	**Area**		
		Hydrochlorothiazide (25µg/ml)	**Amlodipine besylate (13.92 µg/ml)**	**Irbesartan (300 µg/ml)**
Detection wavelength (±2.0 nm)	222	266525	278734	8498032
	224	268581	276681	8548309
	226	265597	279556	8411223
	Mean±SD	266901 ±1363.12	278323.67 ±1480.7722	8485854.7 ±69349.538
	%RSD	**0.51**	**0.53**	**0.82**
Flow rate (±0.02 ml/min)	0.98	269056	278632	8601836
	1.00	270525	276823	8501662
	1.02	267989	280123	8463226
	Mean±SD	269190 ±1273.299	278526 ±1652.5517	8522241.3 ±71559.868
	%RSD	**0.47**	**0.59**	**0.84**
Mobile Phase ratio (±2 ml in organic phase)	43:57	269821	277246	8301795
	45:55	268140	270983	8416018
	47:53	261163	271632	8503794
	Mean±SD	266374.7 ±4591.028	273287 ±3443.9165	8407202.3 ±101287.64
	%RSD	**1.72**	**1.25**	**1.2**

- **Summary of all the parameters including RSD values are given following.**

Table 9 Result of Validation Parameter					
Sr. No.	**Parameter**		**Result**		
	DRUG		**HCTZ**	**AMLO**	**IRBE**
1.	Specificity		Specific		
2.	Linearity	Regression equation	y = 266774.6429x + 680.7857	y = 276063.2143x – 684.9286	y = 8623393.5714x – 56855.0000
		R2	0.9970	0.9974	0.9973
3.	Accuracy (% recovery)	80%	101.46	101.64	100.75
		100%	99.37	100.70	100.28
		120%	101.46	100.81	100.16
4.	Precision (%RSD)	Repeatability	0.05	1.82	0.098
		Interday	0.71-1.07	0.27-0.47	0.30-0.69
		Intraday	0.12-1.70	0.59-1.01	0.10-0.57
5.	Robustness		%RSD for all the parameter were found well within acceptance criteria. So, the method was found to be robust.		

CONCLUSION

All the parameters and results were found within the accepted limits. So it could be concluded that the developed RP-HPLC method for simultaneous estimation of combined dosage form of Hydrochlorothiazide, Amlodipine besylate and Irbesartan was simple, selective, precise, linear, accurate, robust and sensitive. Thus the method can be applied for routine quality control sample testing.

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