CODEN [USA]: IAJPBB ISSN : 2349-7750

INDO AMERICAN JOURNAL OF
PHARMACEUTICAL SCIENCES

SJIF Impact Factor: 7.187
https://doi.org/10.5281/zenodo.7498436

Available online at: http://www.iajps.com *Research Article*

DEVELOPMENT AND VALIDATION OF A RP - HPLC METHOD FOR THE SIMULTANEOUS DETERMINATION OF AZELNIDIPINE AND CHLORTHALIDONE IN PURE AND PHARMACEUTICAL DOSAGE FORM

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Abstract:
Analytical Method Development and Validation for Azelnidipine and Chlorthalidone in bulk and Combined Dosage Form by RP-HPLC. New method was established for simultaneous estimation of Azelnidipine and Chlorthalidone by RP-HPLC method. The chromatographic conditions were successfully developed for the separation of Azelnidipine and Chlorthalidone by using Inertsil C18 (4.6mm ×250mm, 5µm particle size), flow rate was 1.0 ml/min, mobile phase ratio was (55:45% v/v) Methanol: Phosphate buffer pH 4.8 (pH was adjusted with ortho phosphoricacid), detection wavelength was 282nm. The instrument used was WATERS Alliance 2695 separation module, Software: Empower 2, 996 PDA detector. The retention times were found to be 1.688mins and 3.282mins. The % purity of Azelnidipine and Chlorthalidone was found to be 99.86%. The system suitability parameters for Azelnidipine and Chlorthalidone such as theoretical plates and tailing factor were found to be 7586, 1.69 and 6235 and 1.58, the resolution was found to be 10.85. The analytical method was validated according to ICH guidelines (ICH, Q2 (R1)). The linearity study of Azelnidipine and Chlorthalidone was found in concentration range of 100µg-500µg and 30µg-70µg and correlation coefficient (r2) was found to be 0.999 and 0.999, % recovery was found to be 100.112% and 100.16%, %RSD for repeatability was 0.1702 and 0.043 respectively. The precision study was precise, robust, and repeatable. The LOD value was found to be 2.1µg/ml and 1.28µg/ml, and LOQ value was 6.3µg/ml and 3.84µg/ml for Azelnidipine and Chlorthalidone respectively. Hence the suggested RP-HPLC method can be used for routine analysis of Azelnidipine and Chlorthalidone in API and Pharmaceutical dosage form.

Keywords: *Azelnidipine and Chlorthalidone, Method Development, Validation, Accuracy, Precision.*

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Please cite this article in press P.Priyanka et al, ***Development And Validation Of A Rp - Hplc Method For The Simultaneous Determination Of Azelnidipine And Chlorthalidone In Pure And Pharmaceutical Dosage Form.,*** *Indo Am. J. P. Sci, 2022; 09(12).*

INTRODUCTION:

Azelnidipine is a dihydropyridine calcium channel blocker. It is marketed by Daiichi-Sankyo pharmaceuticals, Inc. in Japan. It has a gradual onset of action and produces a long-lasting decrease in blood pressure, with only a small increase in heart rate, unlike some other calcium channel blockers. It is currently being studied for post-ischemic stroke management. [1]

Azelnidipine inhibits trans-membrane Ca2+ influx through the voltage-dependent channels of smooth muscles in vascular walls. Ca2+ channels are classified into various categories, including L-type, T-type, N-type, P/Q-type, and R-type Ca2+ channels. The L-type Ca2+ channels.[2] Normally, calcium induces smooth muscle contraction, contributing to hypertension. When calcium channels are blocked, the vascular smooth muscle does not contract, resulting in relaxation of vascular smooth muscle walls and decreased blood pressure. IUPAC name is 3-[1-(diphenylmethyl) azetidin-3-yl] 5-propan-2-yl 2-amino-6-methyl-4-(3-nitrophenyl)-1,4-dihydropyridine-3,5-dicarboxylate. Molecular Formula is $C_{33}H_{34}N_4O_6$. Molecular weight is 582.6. Azelnidipine is sparingly soluble in aqueous buffers. For maximum solubility in aqueous buffers, azelnidipine should first be dissolved in DMSO and then diluted with the aqueous buffer of choice. Azelnidipine has a solubility of approximately 0.25 mg/ml in a 1:3 solution of DMSO: PBS (pH 7.2) using this method.

Chlorthalidone is a thiazide-like diuretic used for the treatment of hypertension and for management of edema caused by conditions such as heart failure or renal impairment. Chlorthalidone improves blood pressure and swelling by preventing water absorption from the kidneys through inhibition of the Na+/Cl− symporter in the distal convoluted tubule cells in the kidney.[3] The exact mechanism of chlorthalidone's anti-hypertensive effect is under debate, however, it is thought that increased diuresis results in decreased plasma and extracellular fluid volume, decreased cardiac output and therefore overall reduction in blood pressure.[4] IUPAC name is 2-chloro-5-(1-hydroxy-3-oxo-2,3-dihydro-1H-isoindol-1-yl)benzene-1-sulfonamide. Molecular Formula is $C_{14}H_{11}ClN_2O_4S$. Molecular weight is 338.7. Chlorthalidone is practically insoluble in water, in ether and in chloroform; soluble in methanol; slightly soluble in alcohol.

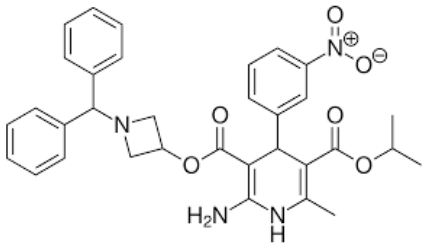

Figure 1: Structure of Azelnidipine

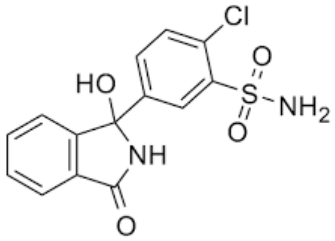

Figure 2: Structure of Chlorthalidone

The literature survey revealed that There are really few approaches reported in the literary works for evaluation of Azelnidipine and Chlorthalidone alone or in combination with various other drugs in the pure form as well as drugs formulations by RP-HPLC [5-13]. In view of the demand for an appropriate, cost-effective RP-HPLC method for routine analysis of Azelnidipine and Chlorthalidone synchronized evaluation of in pharmaceutical dose type. Attempts were made to establish easy, precise, accurate as well as cost-efficient logical method for the estimate of Azelnidipine and Chlorthalidone. The recommended approach will be validated according to ICH guidelines. The objective of the recommended work is to establish a brand-new, simple, delicate, exact and economical logical method as well as recognition for the Synchronized evaluation of Azelnidipine and Chlorthalidone in pharmaceutical dose kind by utilizing RP-HPLC. To verify the established method based on ICH standards for the desired analytical application.

MATERIALS AND METHODS:

Chemicals and Reagents:

Azelnidipine and Chlorthalidone were Purchased from Honour Lab. NaH_2PO_4 was analytical grade supplied by Finerchem limited, Orthophosphoric acid (Merck), and Water and Methanol for HPLC (Lichrosolv (Merck).

Equipment and Chromatographic Conditions:

The chromatography was performed on a Waters 2695 HPLC system, equipped with an auto sampler,

UV detector and Empower 2 software. Analysis was carried out at 282 nm with column Inertsil C18 (4.6mm ×250mm, 5µm particle size), dimensions at 35^{0}C temperature. The optimized mobile phase consists of Phosphate Buffer (pH-4.8): Methanol (55:45% v/v). Flow rate was maintained at 1 ml/min.

Preparation of solutions:
Preparation of mobile phase:
Accurately measured 500 ml (50%) of HPLC Methanol and 350 ml of Acetonitrile (35%) and 150 ml of Water (15%) were mixed and degassed in a digital ultrasonicater for 10 minutes and then filtered through 0.45 µ filter under vacuum filter.

Diluent Preparation:
Accurately measured 450 ml (45%) of HPLC Methanol and 550 ml of Phosphate Buffer (55%) were mixed and degassed in a digital ultra sonicater for 15 minutes and then filtered through 0.45 µ filter under vacuum filter.

Assay
Preparation of the Azelnidipine and Chlorthalidone standard solution:
Preparation of standard solution: (Azelnidipine)
Accurately weigh and transfer 8 mg of Azelnidipine , working standard into a 10ml of clean dry volumetric flasks add about 7ml of diluent and sonicate to dissolve and removal of air completely and make volume up to the mark with the diluent.

Preparation of standard solution: (Chlorthalidone)
Accurately weigh and transfer 12.5 mg of Chlorthalidone working standard into a 10ml of clean dry volumetric flasks add about 7ml of diluent and sonicate to dissolve and removal of air completely and make volume up to the mark with the diluent.

Further pipette 3ml of Azelnidipine , 0.5ml of Chlorthalidone from stock solutions in to a 10ml volumetric flask and dilute up to the mark with diluent.

Procedure:
Inject the samples by changing the chromatographic conditions and record the chromatograms, note the conditions of proper peak elution for performing validation parameters as per ICH guidelines.

Preparation of Sample Solution:
Take average weight of Tablet and crush in a mortar by using pestle and weight 10 mg equivalent weight of Azelnidipine , Chlorthalidone sample into a 10ml clean dry volumetric flask and add about 7ml of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent.

Procedure:
Further pipette 1.2ml of Azelnidipine, Chlorthalidone from above stock solution into a 10ml volumetric flask and dilute up to the mark with diluent.

RESULTS AND DISCUSSION:

METHOD:

The developed chromatographic method was validated for system suitability, linearity accuracy, precision, ruggedness and robustness as per ICH guidelines.

System suitability parameters:
To evaluate system suitability parameters such as retention time, tailing factor and USP theoretical plate count, the mobile phase was allowed to flow through the column at a flow rate of 1.0 ml/min to equilibrate the column at ambient temperature. Chromatographic separation was achieved by injecting a volume of 20 µL of standard into Inertsil C18 (4.6mm ×250mm, 5µm particle size), the mobile phase of composition Phosphate Buffer (pH-4.8): Methanol (55:45% v/v) was allowed to flow through the column at a flow rate of 1.0 ml per minute. Retention time, tailing factor and USP theoretical plate count of the developed method are shown in table 1.

Table 1: System suitability parameters

S. NO	Parameter	Azelnidipine	Chlorthalidone
1.	Retention Time (min)	1.688	3.282
2.	Theoretical Plates	7586	6235
3.	Tailing factor	1.69	1.58
4.	Area	1658768	426589
5.	Resolution	10.89	

Assay of pharmaceutical formulation: The proposed validated method was successfully applied to determine Azelnidipine and Chlorthalidone in their tablet dosage form. The result obtained for was comparable with the corresponding labeled amounts and they were shown in Table-2.

Table 2: Assay results for Azelnidipine and Chlorthalidone

	Label Claim (mg)	% Assay
Azelnidipine	8	99.86
Chlorthalidone	12.5	99.86

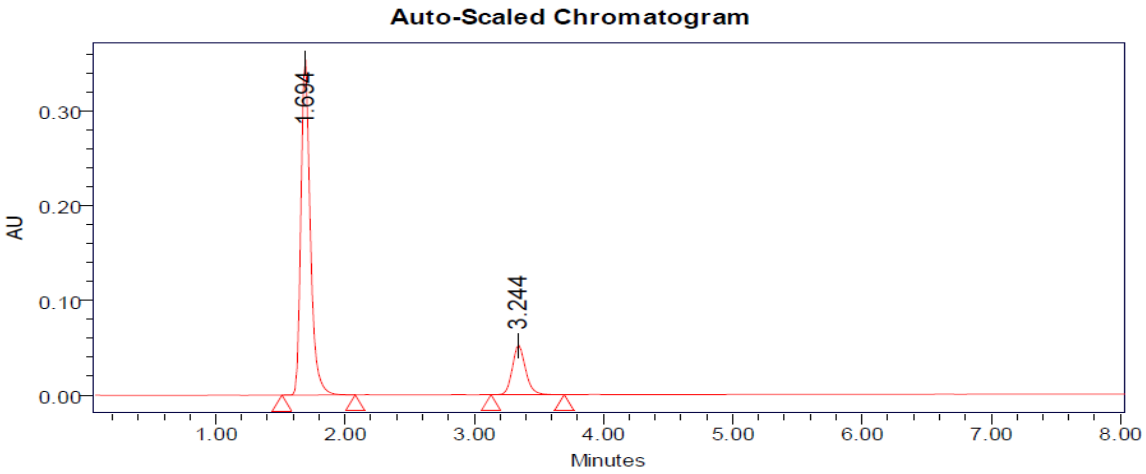

Figure 3: Standard chromatogram

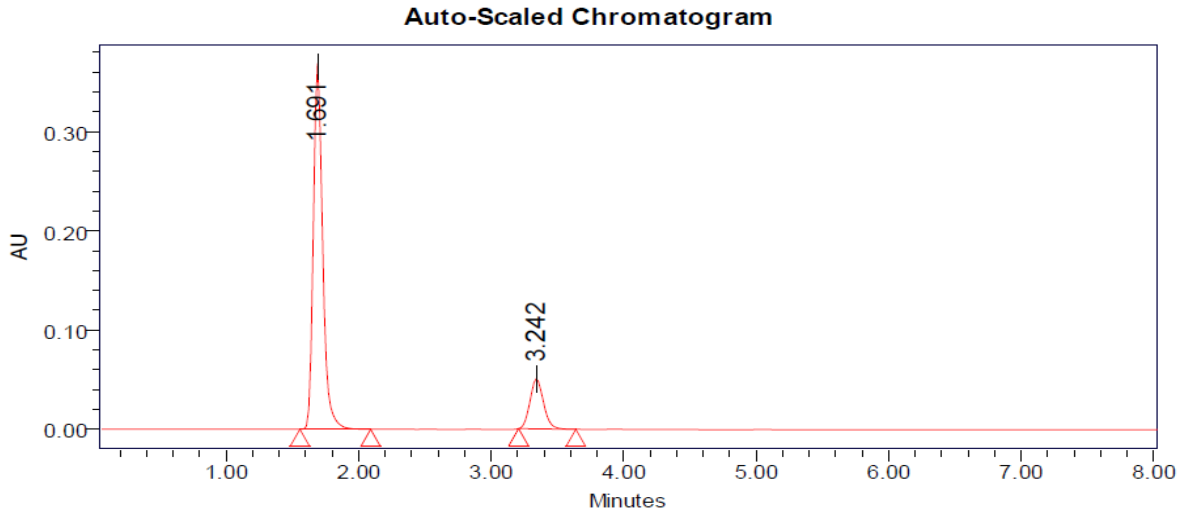

Figure 4: Sample chromatogram

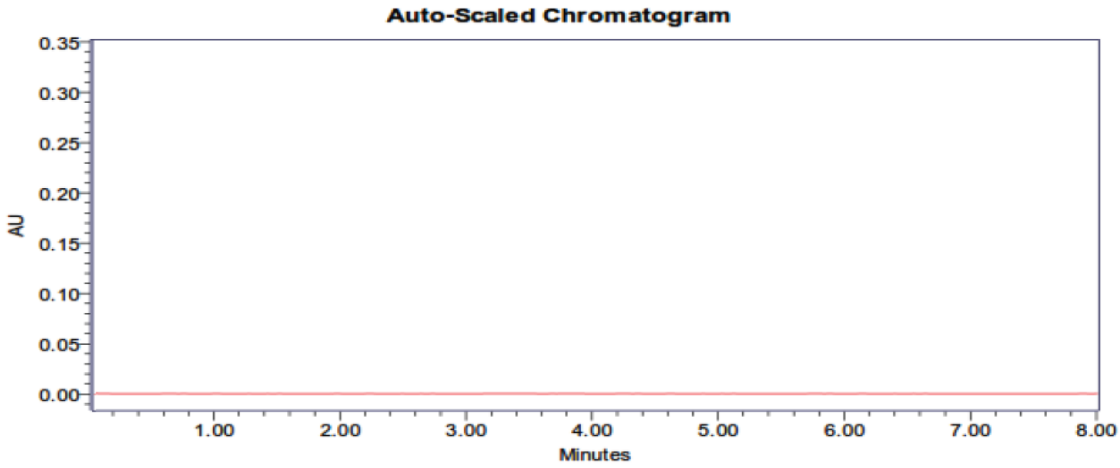

Figure 5: Blank chromatogram

Validation of Analytical method:

Linearity: The linearity study was performed for the concentration of 100 **µg/ml** to 500 **µg/ml** and 30 **µg/ml** to 70 **µg/ml** level. Each level was injected into chromatographic system. The area of each level was used for calculation of correlation coefficient. Inject each level into the chromatographic system and measure the peak area. Plot a graph of peak area versus concentration (on X-axis concentration and on Y-axis Peak area) and calculate the correlation coefficient. The results are shown in table 3,4.

Table 3: Linearity results of Azelnidipine

S. No	Concentration Level (%)	Concentration µg/ml	Average Peak Area
1.	I	100	585985
2.	II	200	1182468
3.	III	300	1768785
4.	IV	400	2326852
5.	V	500	2856874
Correlation coefficient			0.999

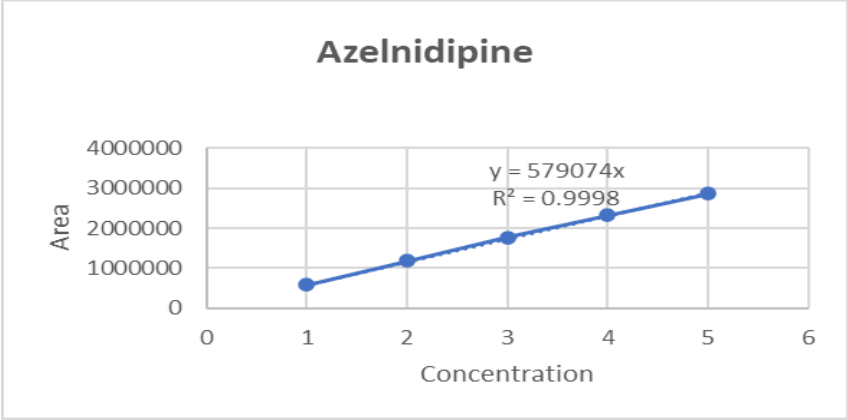

Figure 6: Linearity graph for Azelnidipine

Table 4: Linearity results of Chlorthalidone

S. No	Concentration Level (%)	Concentration µg/ml	Average Peak Area
1	I	30	268764
2	II	40	356958
3	III	50	445631
4	IV	60	535186
5	V	70	624698
Correlation coefficient			0.999

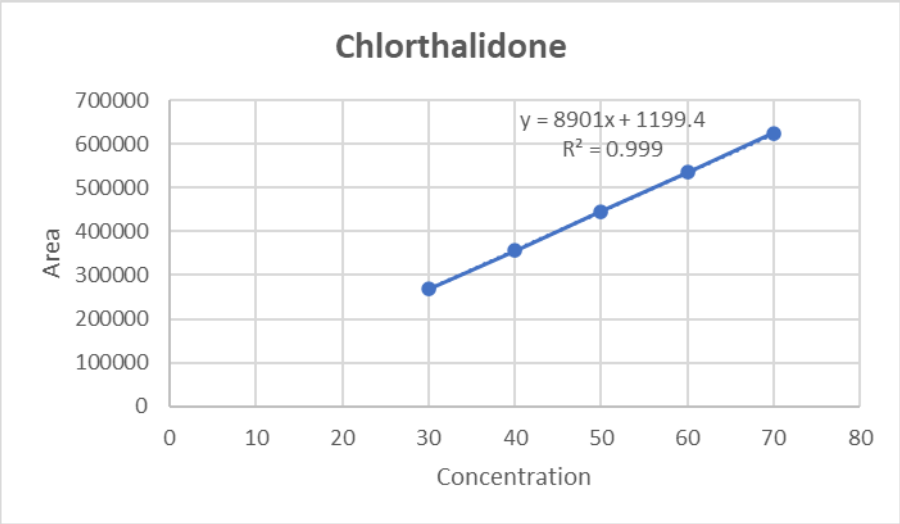

Figure 6: Linearity graph for Chlorthalidone

Accuracy studies: The accuracy was determined by help of recovery study. The recovery method carried out at three level 50%, 100%, 150% and 50%, 100%, 150% Inject the standard solutions into chromatographic system. Calculate the Amount found and Amount added for Azelnidipine and Chlorthalidone and calculate the individual recovery and mean recovery values. The results are shown in table 5,6.

Table 5: Showing accuracy results for Azelnidipine

%Concentration (at specification Level)	Average Area	Amount Added (ppm)	Amount Found (ppm)	% Recovery	Mean Recovery
50%	879537	150	150.048	100.032	100.112%
100%	1743252	300	300.521	100.172	
150%	2609693	450	450.598	100.132	

Table 6: Showing accuracy results for Chlorthalidone

%Concentration (at specification Level)	Average Area	Amount Added (ppm)	Amount Found (ppm)	% Recovery	Mean Recovery
50%	224271	25	25.114	100.456%	100.16%
100%	445748.3	50	49.952	99.904%	
150%	670006.3	75	75.101	100.134%	

Precision Studies: precision was calculated from Coefficient of variance for five replicate injections of the standard. The standard solution was injected for five times and measured the area for all five Injections in HPLC. The %RSD for the area of five replicate injections was found. The results are shown in table 7.

Table 7: Precision results for Azelnidipine and Chlorthalidone

S. No	Sample Area 1	Sample Area 2
1	1658254	426598
2	1658952	426589
3	1654857	426985
4	1659854	426587
5	1653298	426515
Mean	**1657043**	**426654.8**
Std.dev	**2820.29**	**187.5692**
%RSD	**0.1702**	**0.043963**

Ruggedness: To evaluate the intermediate precision of the method, Precision was performed on different day. The standard solution was injected for six times and measured the area for all six injections in HPLC. The %RSD for the area of six replicate injections was found. The results are shown in table 8.

Table 8: Ruggedness results of Azelnidipine and Chlorthalidone

S. No	Sample Area 1	Sample Area 2
1	1665985	436598
2	1662598	436855
3	1668484	436598
4	1664598	436587
5	1663579	436741
6	1664587	432659
Mean	**1664972**	**436006.3**
Std. Dev.	**2060.327**	**1643.285**
% RSD	**0.123745**	**0.376895**

Robustness: As part of the Robustness, deliberate change in the Flow rate, Mobile Phase composition, Temperature Variation was made to evaluate the impact on the method. The flow rate was varied at 0.8 ml/min to 1.2 ml/min. The results are shown in table 9,10,11,12.

Table 9: Flow variation results for Azelnidipine

Flow Rate (ml/min)		System suitability Results		
		USP Plate Count	USP Tailing	Retention Time (min)
Less Flow rate	0.8	7365	1.62	1.868
Actual Flow rate	1	7586	1.69	1.688
More Flow rate	1.2	7254	1.61	1.544

Table 10: Flow variation results for Chlorthalidone

Flow Rate (ml/min)		System suitability Results		
		USP Plate Count	USP Tailing	Retention Time (min)
Less Flow rate	0.8	6284	1.51	3.621
Actual Flow rate	1	6235	1.58	3.282
More Flow rate	1.2	6168	1.56	2.998

Table 11: Change in Organic composition for Azelnidipine

Organic phase		System suitability Results		
		USP Plate	USP Tailing	Retention Time (min)
Less organic phase	50:50	7269	1.61	1.868
Actual organic phase	55:45	7586	1.69	1.688
More organic phase	60:40	7496	1.64	1.675

Table 12: Change in Organic composition for Chlorthalidone

Organic phase		System suitability Results		
		USP Plate Count	USP Tailing	Retention Time (min)
Less organic phase	50:50	6182	1.54	3.621
Actual organic phase	55:45	6235	1.58	3.282
More organic phase	60:40	6322	1.56	2.302

LOD and LOQ: The sensitivity of RP-HPLC was determined from LOD and LOQ. Which were calculated from the calibration curve using the following equations as per ICH guidelines. The results are shown in table 13.

LOD = 3.3σ/S and
LOQ = 10 σ/S, where
σ= Standard deviation of y intercept of regression line,
S = Slope of the calibration curve

Table 13: LOD, LOQ of Azelnidipine and Chlorthalidone

Drug	LOD	LOQ
Azelnidipine	2.1	6.3
Chlorthalidone	1.28	3.84

CONCLUSION:

The Developed HPLC method was validated and it was found to be simple, precise, accurate and sensitive for the simultaneous estimation of Azelnidipine and Chlorthalidone in its pure and pharmaceutical dosage form. Hence, this method can easily and conveniently adopt for routine quality control analysis of Chlorthalidone and Azelnidipine in its pure and pharmaceutical dosage form.

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