J Herbmed Pharmacol. 2017; 6(1): 37-42.

Journal of Herbmed Pharmacology

Journal homepage: http://www.herbmedpharmacol.com

Development of a validated HPLC method for determination of an active component in Pycnocycla spinosa and tablets prepared from its extract

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ARTICLE INFO

Article Type:
Original Article

Article History:
Received: 15 August 2016
Accepted: 2 December 2016

Keywords:
Umbelliferae
Pycnocycla spinosa
Formulation
Apigenin
Diarrhea
IBS
HPLC

ABSTRACT

Introduction: *Pycnocycla spinosa*, a native plant of Iran with approved antispasmodic and antidiarrheal activities, could be a suitable candidate and an alternative remedy for the treatment of diarrhea and irritable bowel syndrome (IBS). Therefore, the aim of this study is formulation of an acceptable dosage form and development of a validated high-performance liquid chromatography (HPLC) method for analysis of active ingredients in its extract and pharmaceutical forms.

Methods: Different formulations of *P. spinosa* tablets were prepared by wet granulation method. The prepared tablets were evaluated for hardness, friability, disintegration time and drug assay. HPLC was carried out based on the extract active ingredient: 6-(4-hydroxy-3-methoxyphenyl)-hexanoic acid (HMPHA) determination in *P. spinosa* extract and tablets.

Results: The mean weight, friability, hardness, and disintegration time of selected formulation (tablet 5 mg) were 217.26 mg, 0.69%, 53.6 N and 95.8 seconds, respectively. Similar acceptable results were also found for 10 mg tablets. The assay test showed that the content of HMPHA in each 5 mg and 10 mg tablets were 1.64 µg and 3.59 µg, respectively. The HPLC method showed a good linearity and suitability in its working range: 4.5 to 15 µg/mL.

Conclusion: The data showed that the selected formulation of *P. spinosa* tablets has acceptable physicochemical features.

Implication for health policy/practice/research/medical education:
In the present study, we developed a formulation of an acceptable dosage form and a validated HPLC method for analysis of active ingredients in Pycnocycla spinosa extract and its pharmaceutical forms.

Please cite this paper as: Tavakolli N, Ghanadian M, Asghari Gh, Sadraei H, Asgari-Borjlou1 N,, Tabakhian M. Development of a validated HPLC method for determination of an active component in *Pycnocycla spinosa* and tablets prepared from its extract. J Herbmed Pharmacol. 2017;6(1):37-42.

Introduction

Diarrhea is a common disease of tropical areas responsible for mortality of children and infants. Irritable bowel syndrome (IBS) is another digestive tract disease associated with intestinal movement problems. Anti-motility drugs such as loperamide and anti-muscarinic agents like dicyclomine may relieve diarrhea and reduce intestinal spasm and the pain (1,2). Alverine, mebeverine and peppermint oil are between other antispasmodic agents believed to be direct relaxant of intestinal smooth muscle and may alleviate the pain in IBS and diverticular diseases (3).

Pycnocycla spinosa (Umbelliferae family), a native plant growing wild in Iran (4) has showed also considerable antispasmodic and antidiarrheal activities in mice comparable with loperamide (5-7) and could be a suitable candidate and an alternative remedy for the treatment of diarrhea and IBS. Using chromatography separation techniques, potent fractions have been separated from its crude extract and further researches led to the identification of its bioactive compounds including 3,7,10,14,15-pentaacetyl-5-butanoyl-13,17-

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epoxy-8-myrsinene (PABEM), vanillin, isoacetovanilon (isoapocynine), and 6-(4-hydroxy-3- methoxyphenyl)-hexanoic acid (HMPHA) (8). HMPHA as depicted in figure 1 is a phenyl hexanoid structure which has significantly reduced ileum contraction induced by acetylcholine, serotonine and electrical field stimulation in vitro in a concentration-dependent manner on isolated rat ileum (9). It strongly inhibited gut movements and reduced diarrhea induced by castor oil or sulphate magnesium in rats (9). With regard to mentioned biochemical and pharmacological researches which have done on *P. spinosa* extract (3-9), an acceptable formulation and a reliable high-performance liquid chromatography (HPLC) method for analysis of active ingredients in its pharmaceutical forms is required. To the best of our knowledge, no studies on the quantitative determination of chemical constituents in *P. spinosa* are reported. Therefore, HMPHA as one of the major and bioactive components with high molar UV absorption required for spectrophotometer analysis was selected to be quantified and applied as an important index in the quantity evaluation of the bulk drug or pharmaceutical formulations of *P. spinosa*. In this study a was established as a validated, simple, rapid and accurate method for HMPHA determination in the bulk extract of *P. spinosa* and a tablet dosage form of the plant.

Materials and Methods

Chemicals

Methanol, chloroform, acetone, and hexane were of analytical grade prepared from Merck Company (Germany). Hexane and chloroform HPLC-grade solvents were purchased from Caledon Company (Canada). Cerium ammonium molibydate (CAM) TLC reagent was prepared by adding 0.5 g of ceric ammonium molybdate, 12 g of ammonium molybdate, and 15 mL of sulfuric acid to 235 mL of distilled water in a 250 mL Erlenmeyer flask (10). HMPHA (Figure 1) was isolated form *P. spinosa* extract as described before (8).

Preparation of the Extract

The aerial parts of *P. spinosa* were collected at October from populations grow wild in Isfahan University of Isfahan, Isfahan, Iran. The plant was identified according to the voucher specimen A24 deposited at the herbarium of the Faculty of Pharmacy of Isfahan University of Medical Sciences, Iran. Aerial parts of plant material (400 g) were air-dried in the shade and reduced to fine particles using an electric mill. The powdered plant was extracted exhaustively via percolation using ethanol: water (70:30) as solvent at a flow rate of 200 µL/min for 24 hours. The extract was concentrated under reduced pressure by a rotary evaporator at 40°C to yield 55.5 g gummy extract.

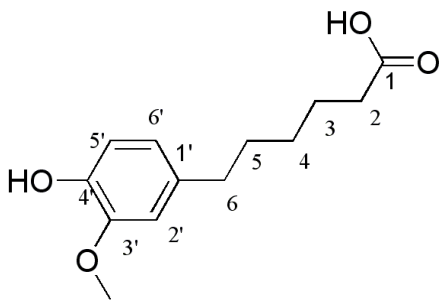

Figure 1. Structure of 6-(4-hydroxy-3-methoxyphenyl)-hexanoic acid (HMPHA) isolated from *Pycnocycla spinosa*.

HPLC method for determination of HMPHA

HMPHA was determined through external standard calibration method on a Waters system, equipped with 515 HPLC pump, 2487 dual wavelength absorbance detector (Waters, Milford, MA, USA). The column was a Nova-Pak Silica, 3.9 × 150 mm (Waters, Milford, MA, USA) and Millennium software was used for the determination of compounds and processing the data.

Base on the knowledge of HMPHA different HPLC methods including reverse phase methods at the initial stage and normal phase chromatographic methods as the second choice were tested.

Preparation of the Standard Solutions

For preparation of the standard solutions, 1000 µg of pure HMPHA powder was diluted in 1 mL of HPLC mobile phase solvent to prepare the stock solution of 1000 µg/mL. Different standard solutions at concentrations of 4.5, 6, 8, 10, 12.5, and 15 µg/mL were prepared by serially diluting the stock solution.

Solvent effect

For evaluation of the solvent effects as one of the affecting parameters on the extraction of the HMPHA from extracts and tablets, chloroform, acetone, and methanol solvents with different strength and selectivity's were selected. Then, two extraction methods consisted of maceration (24 hours) in combination with ultrasonic radiation (120 minutes) or ultra-sonication alone were compared to check the effect of the procedure on the extraction (12).

Selectivity

The ability of the HPLC method to separate all closely eluted compounds and excipients from HMPHA band was calculated by assessing resolution (Rs) and separation factor (α). The tailing factor and column efficiency for HMPHA was also assessed. Column resolution (Rs) and separation factor (α) were calculated by using equation, $Rs=2(Rta-Rtb)/(WA+Wb)$, and $(\alpha)=(Rtb-tm)/(Rta-tm)$, where Rta, Rtb are retention times and Wa, Wb are band width of peak A and B in chromatogram. For a precise and rugged HPLC analysis resolution of 1.5 and symmetry factor less than 1.5 are required (11,14).

Limit of detection and limit of quantification

Limit of detection (LOD) or the minimum amount of analyte which can be detected reliably is a peak which its signal-to-noise ratio is at least 3:1. The limit of quantification (LOQ) or minimum quantifiable amount is the concentration which can be quantified with a

specified level of accuracy and is often calculated with a similar method for LOD whose signal-to-noise ratio is at least 10:1. But in this study LOQ was defined with Relative standard deviation (RSD) less than 3, experimentally, by injecting different concentrations of standard with signal-to-noise ratios of 10, 15, 20, and 25 (13-15).

Linearity and range

The linearity means how well a calibration plot of response versus concentration approximates a straight line. It was done by linear squares regression. Correlation coefficient (r^2) in addition to plot slop and intercept provided desired information on linearity. In HPLC validation methods, a linearity correlation coefficient more than 0.999 is acceptable (14).

Linear range starts from the LOQ of the component of interest as the lower concentration and extend up to its upper concentrations in which the HPLC method have acceptable linearity, accuracy and precision. Therefore, working range in this study was selected within the linear range beyond values expected in the sample at 50, 70, 100, 125, and 150% of HMPHA in 30 mg extract (4.5, 6, 8, 10, 12.5, and 15 µg/mL) (14).

Precision

Precision is the degree of agreement among individual test results. Therefore, precision could be evaluated when a series of concentrations of the same analyte is done repeatedly through repeatability assays like intra-day precision, and intermediate precision assays like inter-day precision. RSD was calculated by using the equation % RSD = (SD/X) * 100, where SD is the standard deviation which is calculated for each level of precision, and X is the mean of responses. When there is an unacceptable RSD, its cause needs to be identified by investigation of the effect of the various factors individually and corrected (14,15). The intra-day precision also termed instrument/injection precision, or injection repeatability was done by the same analyst through repetitive injection of the same sample within the selected range in one day (13,14).

Intermediate precision includes the influence of additional random effects such as operator, instrument, and days in the laboratory was calculated through inter-day precision method by injecting of three replicates of different concentrations of HMPHA (4.5, 6, 8, 10, 12.5 and 15 µg/mL) on three consecutive days in a week (n = 9). Peak area was measured and % RSD was calculated (13,14).

Accuracy

Recovery studies are required to check the accuracy of an analytical method. It ensures that the real quantification of target components is measured. The determination of this parameter was performed by adding the HMPHA as standard beyond the amount in the real sample into a blank matrix of dosage form including calcium phosphate, starch, and magnesium stearate and without *P. spinosa* extract. Pharmaceutical dosage form was made as is described in real tablets containing extract. Obtained tablets (n = 3) were crushed and added to a volumetric flask, separately. They were extracted as is mentioned in the sample preparation method. Then evaporated sample was solubilized into 1 mL of HPLC mobile phase solvent. For each tablet, three determinations were carried out and the recovery percentage was calculated in every case (14).

Tablet preparation

Formulations containing *P. spinosa* extract were prepared by wet granulation method. Five or 10 mg of standardized extract of *P. spinosa*, were mixed with lactose and/or tricalcium phosphate (as filler), and corn starch (as a disintegrating agent), then granulated using starch paste, and dried at 25°C. The granules were lubricated by magnesium stearate and compressed using a single punch press machine (Kilian Co, KS 43373-202, Germany). The resultant tablets were evaluated for their hardness, friability, disintegration time and weight variations. The drug content of tablets was determined according to the USP 27 (11).

Assay of tablets

Drug assay was carried out according to the USP 27 requirements with some modification (11). Since the amounts of the active component (i.e., HMPHA) of tablets containing 5 and 10 mg crude extracts were less than the LOQ obtained by the HPLC method, six tablets of 5 mg and three tablets of 10 mg extract were used for drug assay. Tablets were crushed to a fine powder, then assayed for HMPHA. The powder or the extract was placed in a 100 mL volumetric flask. Twenty milliliters of methanol was added to each sample and extracted overnight (24 hours), followed by ultra-sonication (120 minutes) in a water bath at 45°C. The extract so obtained was filtered and washed with methanol (3X). The filtrates were evaporated to dryness under a stream of nitrogen. The dry residue was resolubilized in 1 mL chloroform: methanol (9:1) and subjected to the HPLC assay, as explained earlier.

Statistical analysis

Data acquisition and analysis were performed using Waters millennium Chromatography Software (Build 1154, Waters Corporation, Milford, USA). The data was reported as mean ± SD.

Results

In selecting the best solvent for the extraction of tablets containing 10 µg HMPHA as standard, methanol, acetone, and chloroform as common solvents extracted 7.55 ± 1.56, 1.90 ± 0.44, and 0.85 ± 0.07 µg of standard, respectively. As cleared methanol showed more selectivity for extraction of HMPHA than acetone and chloroform. The solubility behavior of HMPHA which is a semi polar phenolic compound gives us the key to the choice of the solvent. Theoretically, methanol with more hidelbrand parameter and higher hydrogen bonds with HMPHA have better solubility characteristics than acetone and chloroform for extraction of it. After selecting methanol as the extraction

solvent, for optimizing the extraction method, extraction by ultra-sonication (120 minutes) alone and maceration (24 hours) in combination with ultrasonic radiation (120 minutes) were compared which retrieved 7.50 ± 1.56, and 8.36 ± 0.25 µg standard from 10 µg tablets, respectively. Hence, maceration (24 hours) combined with ultra-sonication of the tablet containing 10 µg HMPHA was selected as the best method for HPLC analysis.

HPLC method for determination of HMPHA

The HPLC method carried out in this study was aimed to develop a chromatographic system, capable of separation, and determination of HMPHA in crude extract and pharmaceutical dosage forms. The preliminary investigations were directed toward evaluating the effect of various factors on the system. An initial HPLC separation, with a standard column Nova-Pack C18 (150 × 3.9 mm, 4 µm), and mobile phases of water: acetonitrile and then phosphate buffer: acetonitrile in different ratios with flow rates of 1-2 mL/min provided badly overlapped peaks with poor separations. Then, normal phase chromatography was selected and affecting factors on resolution like mobile phase composition, temperature, isocratic or gradient criteria were optimized. Finally, in the normal phase, using a Nova-Pack Silica column (150 × 3.9 mm) with 4 µm particle diameter, with chloroform: ethanol (98:2) results in rapid elution of the whole sample in a short time with poor separation. Then decreasing the solvent strength by adding the hexane to 72.5% and then decreasing to 22°C showed sufficient resolution with acceptable run time of 10 minutes. Selectivity factor and resolution for HMPHA with retention time of 6.9 minutes from its closely eluted compound, at retention time of 7.6 minutes was obtained with α = 1.13 and Rs = 1.39. Column efficiency for HPMHA was 3540 theoretical plates and tailing factor was 1.2 using isocratic mobile phase system of hexane (72.5%), and chloroform: ethanol, 98:2 (27.5%) for 10 minutes following with an elution time of 5 minutes. The flow rate was 1 mL/min at 22°C and the injection volume for standards and samples were 20 µL (Figure 2).

Validation

The HPLC method for analysis of HMPHA in *P. spinosa* extract and its pharmaceutical dosage forms were validated through linearity, precision and accuracy tests. Using USP 35-NF 30, the LOD and LOQ were calculated as 0.67 µg/mL and 2.23 µg/mL for HMPHA but experimentally LOQ of 4.50 µg/mL was quantified with acceptable precision. Working range was certified with LOD of 0.67 µg/mL, and LOQ of 4.5 µg/mL beyond values of standard expected in pharmaceutical dosage forms at 4.5, 6, 8, 12.5, and 15 µg/mL. Using the millennium processing software, the calibration curve was determined at 291 nm by linear regression in the range of 4.5-15 µg/mL. The regression equation was y = 17346x + 826, where X is the concentration of HMPHA in sample (µg/mL) with the correlation co-factor (R2) of 0.999. However, HMPHA in the range of 4.5 µg/mL extended up to 250 µg/mL showed also linearity with correlation coefficient of 0.997 and equation formula of y=15041x + 47886.

The instrumental precision by intra-day results and intermediate precision through inter-day results of repetitive HMPHA quantitation in the different concentrations in the selected linear range, 4.5-15 µg/mL, and in extract as is presented in Table 1, were obtained with RSD < 3 which was acceptable.

In this method, accuracy was evaluated as recovery test after adding of 10 µg/mL HMPHA standard into a blank matrix of dosage form including calcium phosphate, starch, magnesium stearate and without *P. spinosa* extract,

Table 1. Repeatability and intermediate precision assays of standards

HMPHA (µg/mL)	Mean ± SD (inter-day n=9)	RSD% (inter-day)	RSD% (intra-day)
4.5	79576 ± 923	1.16	1.51
6	103613 ± 5660	5.46	5.28
8	139187 ± 5168	3.71	0.87
10	171231 ± 2369	1.38	1.50
12.5	220772 ± 9719	4.40	1.86
15	254538 ± 5698	2.24	2.28

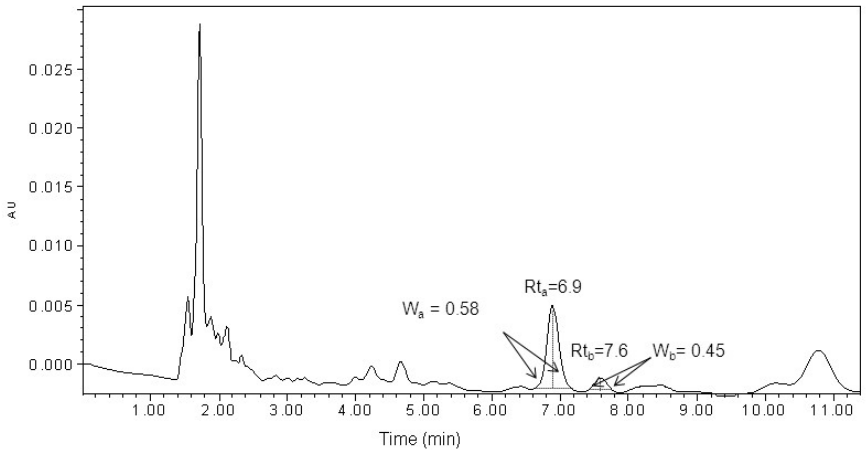

Figure 2. HPLC chromatogram of *Pycnocycla spinosa* extract containing 15 mg extract. HMPHA with retention time of 6.9 min showed column resolution (Rs) of 1.39 using hexane (72.5%), and chloroform: ethanol, 98:2 (27.5%) as mobile phase and flow rate of 1 mL/min at 22°C. Rs was calculated using equation, Rs=2(Rta-Rtb)/(Wa+Wb).

and was found to be 83.6 ± 2.31%.

Tablet preparation

Seven different formulations containing *P. spinosa* extract were prepared by wet granulation method from 5 or 10 mg of standardized extract of *P. spinosa*. Table 2 indicates the amounts of various ingredients used in the formulations.

Physicochemical characteristics of tablets

The results of tablet hardness, friability, weight variations and disintegration time for formulation code: F4, selected formulation (tablet 5 mg and 10 mg) are presented in Table 3. All formulations had desirable physicochemical characteristics within UPS acceptable criteria.

Assay of tablets

The HPLC method developed in this study was applied for quantification of HMPHA in tablets comprising 5 and 10 mg plant extract (formulation F4). Figure 3 demonstrates HPLC chromatograms of extract and tablets each equivalent to 30 mg extract of *P. spinosa*.

Discussion

The present study is the first report of a validated HPLC method for standardization of *P. spinosa* and its pharmaceutical formulations. The developed method showed no interferences with tablet excipients and good resolution between HMPHA and its closely eluted compounds. The method showed a good linearity and suitability in its working range. The retention time for HMPHA was found to be 6.9 minutes. By the aid of the millennium and Excel software, the calibration curve was determined by linear regression in the range of 4.5 to 15 µg/mL. The regression equation was 17346x + 826.06,

Samples	mean ± SD (µg)	RSD%	HMPHA per unit dosage form (µg)
Extract 30 mg	12.21 ± 0.51	4.17	-
Tablet 5 mg (6 units)	10.10 ± 1.07	10.61	1.64 ± 0.36
Tablet 10 mg (3 units)	10.78 ± 0.64	5.89	3.59 ± 0.21

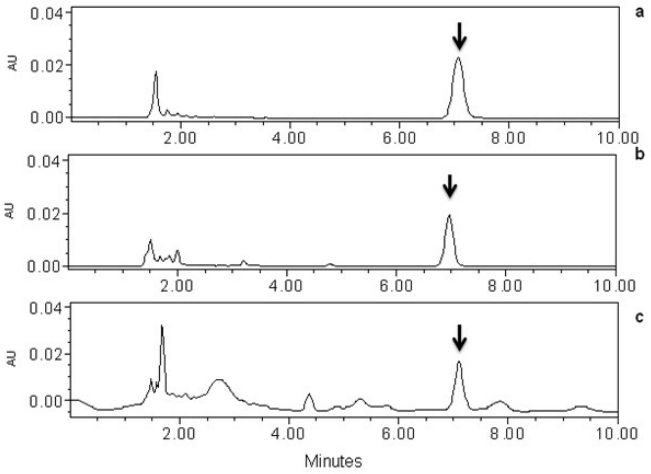

Figure 3. High performance liquid chromatography chromatograms of *Pycnocycla spinosa* extract and its different dosage forms. a: tablet of HMPHA standard (10 µg/mL); b: combined tablets of 5 mg extract of *P. spinosa* (6 units); c: combined tablets of 10 mg extract of *P. spinosa* (3 units). Chromatograms were carried out at 291 nm on a Nova-Pack Silica column (150 × 3.9 mm) using hexane (22.5%) as solvent A and chloroform: ethanol (98:2, 27.5%) as solvent B with isocratic elution at 22ºC and flow rate of 1 mL/min.

Table 2. Formulations of *Pycnocycla spinosa* tablets[a]

Ingredients (mg)	Formulation code						
	F1	F2	F3	F4	F5	F6	F7
Extract[b]	5/10	5/10	5/10	5/10	5/10	5/10	5/10
Lactose	140	50	-	-	95	-	95
Dicalcium Phosphate	50	140	190	95	95	-	-
Starch (filler)	-	-	-	95	-	190	95
Starch (disintegrant)	6	6	6	6	6	6	6
Magnesium Stearate	3	3	3	3	3	3	3
Starch (glue)	10	10	10	10	10	10	10

[a] Total weight of each tablet formulation was adjusted to 214 mg or 219 mg, based on the amount of extract used.
[b] The amount of dried extract used was 5 or 10 mg.

Table 3. Weight variation, hardness, friability and disintegration time of formulation F4 prepared from 5 and 10 mg extract of *Pycnocycla spinosa*

Formulation	Weight variation (mg)	Hardness (N)	Friability (%)	Disintegration time (s)
Tablet 5 mg	217.26 ± 1.01	53.6 ± 1.90	0.69	95.83 ± 12.81
Tablet 10 mg	221.63 ± 0.75	55.0 ± 1.83	0.68	97.0 ± 8.22

where X is the concentration of HMPHA in the sample (µg/mL) with the correlation co-factor R^2 = 0.999 and the percent recovery of 83.6 ± 2.31%. Total extract of *P. spinosa* (13.78% w/w) was standardized to contain 0.041 ± 0.002% of HMPHA which is equivalent to 0.006 ± 0.0003 % of dried powdered plant. LOD was 0.7 µg/mL based on signal to noise ratio, and LOQ was 4.5 µg/mL which was determined experimentally.

The limitation of this study was low sample size of 5 or 10 mg of extract in each tablet unit containing HMPHA less than required for measuring the release profile of formulations.

Conclusion

Our data showed that the selected formulation of *P. spinosa* tablets has acceptable physicochemical features and may be considered as a herbal medication for treatment of diarrhea and IBS.

Acknowledgments

This paper is part of thesis of Asgari Borjlou submitted in partial fulfillment of the requirements for the degree of Pharm D. She is also grateful to the Isfahan Pharmaceutical Sciences Research Center, Isfahan University of Medical Sciences, Isfahan, Iran for their support.

Authors' contributions

NT coordinated the study and participated in most of the experiments. MG carried out the design and contributed in data analysis and writing and finalizing the manuscript. NAB participated in most of the experiments and in manuscript preparation. GA, and HS coordinated the study. MT contributed to finalizing the manuscript.

Conflict of interests

The authors have no conflicts of interest.

Ethical considerations

Ethical consideration has been completely observed by the authors.

Funding/Support

The work was supported by Isfahan Pharmaceutical Sciences Research Center, Isfahan University of Medical Sciences, Iran (No: 393076).

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