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SIMULTANEOUS ANALYSIS OF FOUR ANALGESIC AND ANTI-INFLAMMATORY COMPOUNDS ILLEGALLY MIXED IN HERBAL MEDICINES BY HPLC – PDA METHOD

Thach Thi Bo Pha[1*], Lam Thi Thuy Kieu[2]

Abstract – *The illegal mixing of modern pharmaceuticals into herbal medicines to increase their effects can cause many serious complications due to uncontrolled dosage and duration of drug action. This constitutes a legal violation and fraudulent practices in production and needs to be controlled. Therefore, this study aimed to develop quality control methods for the simultaneous analysis of active ingredients illegally mixed into marketed herbal medicines. Specifically, a high-performance liquid chromatography method was developed for the simultaneous quantification of four compounds, paracetamol, diclofenac, dexamethasone, and methylprednisolone, in traditional herbal preparations. The method demonstrated high specificity, accuracy, precision, and linearity, with a linear range of 1.0–20.0 µg/mL for paracetamol and 1.5–30.0 µg/mL for the other three compounds. The analytical method meets the regular requirements of a quantitative process according to the International Conference on Harmonization (ICH) guidelines and the Association of Official Analytical Collaboration (AOAC). This method can be recommended for the routine quality control of herbal preparations available on the market.*

Keywords: herbal medicines, HPLC method, simultaneous analysis.

I. INTRODUCTION

Bone and joint pain are common symptoms in the elderly. These days, more and more people are interested in using herbal medicines because it is commonly believed that medicines from herbs often have no side effects and can be used for a long time. However, the treatment effectiveness of herbal medicines is often slow, and it is difficult for patients to persist in taking medication long term. To increase treatment effects and deceive patients, some pharmaceutical manufacturers have mixed illegal pharmaceutical active ingredients into traditional medicine preparations. This action can cause serious and unfortunate consequences because of uncontrolled dosage and duration of drug action [1–3].

Glucocorticoids (GCs) and non-steroidal anti-inflammatory drugs (NSAIDs) are often combined in traditional medicine preparations to enhance their analgesic and anti-inflammatory effects [2]. Among them, paracetamol, diclofenac, dexamethason, and methyl prednisolone are four commonly used active ingredients known for their strong anti-inflammatory effects, as well as their accessibility and low cost. When patients use traditional medicine preparations illegally mixed with anti-inflammatory pain relievers for a long time, they will experience gastric bleeding, Cushing's syndrome, osteoporosis, adrenal dysfunction, and others [1, 2].

Detecting the presence of banned substances in oriental medicine preparations is difficult due to low detection levels and complex sample composition. Several analytical methods have been applied to identify pharmaceutical active ingredients blended in oriental medicinal preparations such as thin-layer chromatography (TLC) [1, 4], high performance liquid chromatography with PDA probe connection (HPLC-PDA) [4–6], liquid chromatography with mass spectrometry probe (LC-MS) [2, 7], mass spectrometry method with direct sample injection mode [3] and liquid chromatography coupled to tandem mass spec-

[1,2]Tra Vinh University, Vietnam
*Corresponding author: ttbpha@tvu.edu.vn
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trometry (LC-MS/MS) [8]. HPLC-PDA, LC-MS, and LC-MS/MS are modern analytical methods widely chosen in analytical processes with the advantages of fast analysis, high accuracy, and sensitivity. The developed method can be used to analyze drugs containing single or multiple ingredients. The solvents used for the process are, readily available and easy to buy, and easy to use. However, the LC-MS and LC-MS/MS methods are chromatographic methods using mass spectrometry detectors, a type of detector with very high sensitivity but quite expensive, so not all drug quality control agencies can equip them. Meanwhile, the HPLC-PDA method uses a PDA detector, a highly sensitive and very popular detector, which is equipped in most testing facilities to serve the analysis of products circulating on the market.

For the above reasons, an HPLC-PDA method was developed to simultaneously analyze four illegally adulterated analgesic and anti-inflammatory compounds in herbal medicines. This method provides a scientific basis for quality control and contributes to ensuring the safety of consumers using herbal medicine products on the market.

II. LITERATURE REVIEW

GCs and NSAIDs are groups of drugs used in the treatment of pain and inflammation. To increase the pain-relieving and anti-inflammatory effectiveness of herbal medicines, drug manufacturers have illegally mixed these compounds in oriental medicine preparations, causing several public health problems such as osteoporosis, muscle atrophy, adrenal insufficiency, and the like [1, 2]. The situation has caught the interest of scholars in developing different methods for analyzing prohibited substances in herbal medicines. Specifically, Nguyen Thi Huynh et al. [4] successfully developed a qualitative and quantitative method for dexamethasone acetate using high performance liquid chromatography (HPLC) technique according to Association of Official Analytical Collaboration (AOAC) guidelines. In 2011, Le Dao Khanh Long et al. [1] used the thin layer chromatography (TLC) method to detect the presence of dexamethason, betamethason, prednisolone, prednisone, and hydrocortisone in traditional medicines. Their study found that 7/22 samples collected from the market contained dexamethasone or betamethasone. By using the LC-MS/MS method, Dao Thi Cam Minh [2] and Lawati et al. [8] identified a large number of samples containing active ingredients in traditional medicines, 25/184 and 7/33 samples, respectively. Klinsunthorn et al. [7] used the HPLC method to identify nine compounds belonging to GCs in herbal medicines. The developed process has been applied to identify illegally mixed glucocorticoid compounds in traditional medicine products on the market. The results detected that 3/6 samples on the market contained illegal glucocorticoids. In general, the processes for quantifying GC and NSAID compounds in traditional herbal preparations commonly use the reversed-phase HPLC technique, with the probes of PDA, MS, and MS/MS. Additionally, the quantitative procedures for two or more compounds used a gradient elution program, with the flow rate used in the procedures usually being 1.0 mL/min, which is similar to the results in this study. Moreover, the quantitative processes mainly use the HPLC method combined with MS or MS/MS probes due to their highly sensitive probes in detecting analytes with minor sample volumes. However, high-cost equipment is the main drawback of using types of MS detectors, making it difficult to apply in most facilities. Besides, the mobile phase solvent used for these detectors is less popular than the mobile phase used for UV-Vis and PDA detectors. Because of the HPLC-MS method's disadvantages, the HPLC-PDA method is chosen in this study. The PDA detector is also highly sensitive and suitable for analyzing simultaneously banned substances in oriental medicines. Furthermore, HPLC-PDA equipment is now available at most drug quality control agencies. Therefore, the developed quantitative process can be widely applied in quality control of traditional preparations, which makes this research significantly different from

published works.

III. METHODOLOGY

A. Material

Reference substances: Paracetamol (PR) is 100% pure. Diclofenac (DC) has a purity of 99.76%. Dexamethasone acetate (DX) and Methylprednisolone (MP) are 99.5% and 98.71% pure, respectively. All of these substances are provided by the Institute of Drug Quality Control, Ho Chi Minh City.

Solvent: Distilled water is used for HPLC. Methanol (MeOH) and Acetonitrile (ACN) meet standards for liquid chromatography. Phosphoric acid meets analytical standards.

Equipment: In the study, an Ultimate 3000 liquid chromatography (UHPLC) system (Thermo Scientific – USA) connected to a photodiode array (PDA) detector is used for quantitative processes. Phenomenex C8 and C18 columns (150 x 4.6 mm; 5 μm) are chosen for chromatography. Besides, analytical balances, ultrasonic baths, and laboratory glassware of appropriate precision are required for each test.

B. Preparation

Blank: The mobile phase solvent is used as a blank.

Stock standard solution: The standards are dissolved respectively in acetonitrile to obtain stock standard solutions with concentrations of 250 μg/mL for paracetamol (PR) and 375 μg/mL for three standards: MP, DX, and DC. After that, these stock solutions are diluted with mobile phase solvent to obtain standards with concentrations suitable for each test of the qualification process.

Placebo: To create a representative sample matrix for traditional medicine preparations, the study mixed six types of traditional medicines representing common dosage forms used for the treatment of bone and joint diseases that have been granted registration numbers and were determined to be free of analytes.

Spike sample: An amount of standard solution is added to the placebo sample to achieve the specified concentration corresponding to each evaluation criterion.

Sample: 0.5 g of the spike sample is accurately weighed into a glass vial with a lid, and 10 ml of ACN is added to dissolve the sample. The sample solution is sonicated for 20 minutes and then centrifuged at 4,000 rpm for 15 minutes. The extract is collected into an appropriate test tube. The solvent is evaporated. Then, the residues are dissolved in exactly 5 ml of MeOH and then filtered through a 0.45 μm membrane filter before being injected into the liquid chromatography system.

C. Investigation of extraction solvent

Expected sample process: 0.5 g of placebo sample (well mixed) is weighed into a glass vial with a lid. The required amount of the standard solution at a given concentration is exactly added. The sample is homogenized with a vortex mixer. 10 ml of extraction solvent (*) is added and sonicated for 10 minutes and then centrifuged at 4,000 rpm for 15 minutes. The extract is collected in a suitable test tube. The solvent is evaporated. The residue is dissolved in exactly 5 ml of extraction solvent (**) and injected into the liquid chromatographic system after being filtered through a 0.45 μm filter.

The active ingredients contained in the medicinal herbs, as well as the properties of the analytes, acetonitrile and methanol, were selected to investigate the extraction solvent. The standard solution at the given concentration is added to the placebo samples and then extracted according to the expected sample processing procedure. The extraction solvent (*)/(**) is replaced with the investigation solvents DMC 1, DMC 2, and DMC 3, respectively, as presented in the extraction solvent investigation results section. Each test is performed six times.

Evaluation: The extract was evaluated by visual observation, and the extraction efficiency of the analytes was evaluated by the peak area obtained from the chromatogram.

By visually observing, the extraction solvent must be selected to dissolve the original sample

and be able to remove the most unwanted sample matrix. The expected results that are extracted tend to be colorless, transparent, or homogeneous. The extraction efficiency is evaluated by the peak area of the analytes obtained from chromatography, and the solvents should give the highest and most stable peak area.

D. Investigation of extraction time

After selecting the extraction solvent, the ultrasonic extraction time is investigated. According to the sample processing procedure, the spike sample extraction is carried out with different ultrasonic times of 10 minutes, 20 minutes, and 30 minutes, respectively. Each test is carried out six times.

Evaluation: The shortest extraction time is selected for its highest and most stable efficiency. The extraction efficiency of the analytes was evaluated by the peak area obtained from the chromatogram.

Method validation: After investigating the appropriate sample processing conditions and chromatographic conditions, the analytical method is evaluated according to the guidelines of the International Conference on Harmonization (ICH) [9] and AOAC [10] including the system suitability testing, specificity, limits of detection (LOD), limits of quantification (LOQ), linearity and determination range, precision, and accuracy.

IV. RESULTS AND DISCUSSION

A. Research results

Survey results of sample processing conditions

Extraction solvent

From the results of the chromatographic condition survey in the research of Thach Thi Bo Pha et al. [11] and the expected sample processing conditions, an extraction solvent (*)/(**) survey is conducted according to the sample treatment process with three extraction solvent systems as follows: DMC 1 extraction solvent system includes ACN/MeOH; DMC 2 extraction solvent system includes ACN/ACN and DMC 3 extraction solvent system consists of MeOH/MeOH with a fixed extraction time of 10 minutes (Table 1).

The results of the extraction solvent survey in Table 1 show that the DMC 1 solvent system has the highest recovery rate with a rate of over 50% substances and an RSD% value of < 13.9% compared to the two extraction solvent systems DMC 2 and DMC 3 with a recovery value of < 50% and an RSD > 16.7%. It was found that the DMC 1 solvent system with ACN for ultrasonic extraction and MeOH for residue dissolution is suitable for extracting analytes from the generated placebo sample matrix. Therefore, the DMC 1 extraction solvent system was chosen to continue the survey of the extraction time.

Extraction time

Using the selected extraction solvent, a survey of ultrasonic extraction time was conducted at 10 minutes, 20 minutes, and 30 minutes (Table 2).

Table 2 shows that with the same sample processing conditions and chromatographic conditions, the extraction time at 20 minutes and 30 minutes give a good recovery rate with an average recovery value of six samples in the range of 80.0–110.0% and RSD < 7.3%, except for MP with an extraction time at 30 minutes with a recovery value of 128.64 % for the 4th extraction and value RSD % = 12.5. Generally, the extraction times of 20 minutes and 30 minutes are suitable for extracting the analyzed samples. However, with the extraction time at 20 minutes, the results show that the dispersion of the recovery rate between extractions is lower. Thus, to shorten the sample analysis time, the extraction time of 20 minutes is selected for sample processing.

Chromatographic conditions for analyzing the studied substances include: phenomenex C8 column (150 x 4.6 mm; 5 μm); mobile phase: ACN–1% phosphoric acid water; flow rate: 1.0 mL/min; detection wavelength: 245 nm; injection volume: 10μL [11]. In addition, from the above survey results, the following sample processing conditions are proposed: 0.5 g of the sample is accurately weighed into a stoppered glass vial; 10 mL of ACN is added and sonicated for 20

Table 1: Results of the extraction solvent survey

No.	Recovery (%)											
	DMC 1				DMC 2				DMC 3			
	PR	MP	DX	DC	PR	MP	DX	DC	PR	MP	DX	DC
1	81.2	75.5	81.2	79.3	4.6	29.2	44.2	55.2	43.6	47.0	44.2	55.2
2	80.9	80.5	87.6	78.7	2.1	21.7	79.9	77.4	60.2	70.6	79.9	77.4
3	78.0	80.3	87.7	78.4	3.7	21.0	59.7	63.2	52.4	55.6	59.7	63.2
4	76.9	77.4	89.7	77.3	3.7	19.0	25.7	61.4	55.9	64.0	25.7	61.4
5	75.2	75.3	83.9	71.3	5.5	28.1	26.7	50.1	40.6	50.0	26.7	50.1
6	53.8	61.1	72.3	65.1	5.3	27.6	23.0	52.2	40.1	46.6	23.0	52.2
Average	74.3	75.0	83.7	75.0	4.2	24.4	43.2	59.9	48.8	55.6	43.2	59.9
RSD%	13.9	9.6	7.6	7.5	30.2	17.8	52.9	16.7	17.5	17.7	52. 9	16.7

Table 2: Results of the extraction time survey

No.	Recovery (%)											
	10 minutes				20 minutes				30 minutes			
	PR	MP	DX	DC	PR	MP	DX	DC	PR	MP	DX	DC
1	81.2	75.5	81.2	79.3	96.6	99.7	94.8	103.9	98.1	95.0	89.8	97.7
2	80.9	80.5	87.6	78.7	97.1	99.8	96.0	100.4	102.1	96.8	93.8	98.7
3	78.0	80.3	87.7	78.4	100.6	101.5	97.0	103.8	101.4	96.2	93.6	98.1
4	76.9	77.4	89.7	77.3	96.5	99.6	96.2	101.3	111.4	128.6	99.1	105.1
5	75.2	75.3	83.9	71.3	99.3	101.1	97.3	102.4	105.9	100.6	96.7	102.7
6	53.8	61.1	72.3	65.1	98.4	100.8	96.2	101.6	106.1	99.9	96.5	100.0
Average	74.3	75.0	83.7	75.0	98.1	100.4	96.2	102.3	104.2	102.9	94.9	100.4
RSD%	13.9	9.6	7.6	7.5	1.7	0.8	0.9	1.4	4.5	12.5	3.4	2.9

minutes, and then centrifuged at 4,000 rpm for 15 minutes. The extract is collected in a suitable test tube. After the evaporation of solvent, the residue is dissolved in exactly 5 mL of MeOH and filtered through a 0.45 μm membrane filter before injection into the chromatographic system.

Results of method validation

System suitability

Experiments were conducted to investigate the system suitability on a reference solution at a concentration of 10 μg/mL (Table 3).

The results of the system suitability survey on the standard solution at a concentration of 10 μg/mL on the C8 column in Table 3 show that the RSD% values of the retention time and peak area of the studied substances do not exceed 1.9%. The asymmetry ranges from $1.1 \leq As \leq 1.5$, and the resolution between analyzed peaks and impurity peaks is ≥ 1.5.

Specificity

The research performed chromatographic analysis of the blank sample, the placebo sample, the standard solution sample, and the spike sample (Figure 1).

The results of the specificity survey in Figure 1 show that the chromatograms of the blank sample and placebo sample do not show peaks at the retention time corresponding to the chromatogram of the standard solution sample, under the same analysis conditions. The chromatogram of the spike sample shows a peak with a retention time corresponding to the main peak in the chromatogram of the standard solution. The peaks of the analytes in the spike sample chromatogram are completely separated from the impurity peaks in the sample matrix. The spike sample and reference samples have the same purity as measured by peak area. The UV-Vis spectrum of the spike sample corresponds to the UV-Vis spectrum of the reference sample. Thus, the procedure is specific.

Calibration plot, determination range, accuracy

Table 3: Results of the system suitability survey on a standard solution of 10 μg/mL (n = 6)

Analyte	No.	Retention time (min)	Peak Area (mAU)	Asymmetry (A_s)	Resolution (Rs)
PR	Average	2.969	9.303	1.1	20.0
	RSD %	0.1	1.1		
MP	Average	9.815	6.214	1.4	1.6
	RSD %	0.1	1.0		
Impurity	-	-	-	-	16.0
DX	Average	13.650	6.077	1.3	1.5
	RSD %	0.04	1.5		
Impurity	-	-	-	-	2.5
DC	Average	14.520	3.808	1.5	-
	RSD %	0.03	1.9		

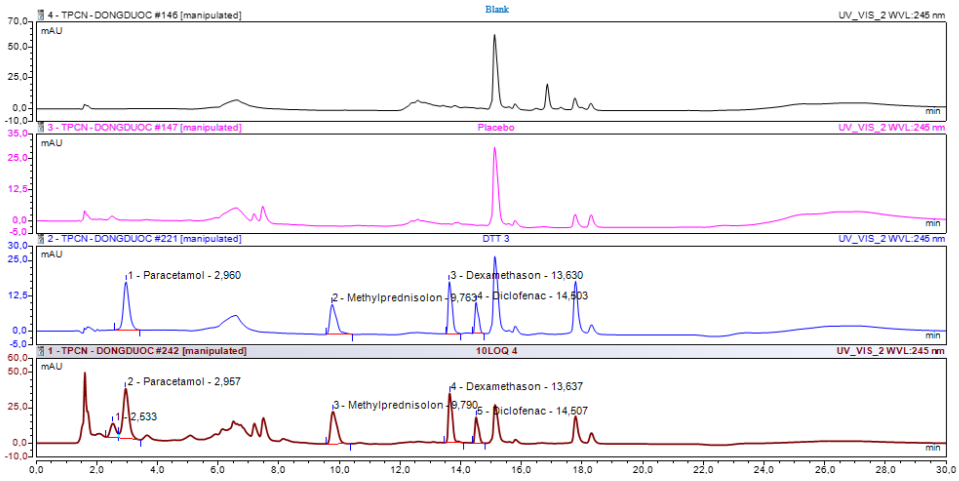

Fig. 1: Results of the extraction solvent survey

and precision, limits of detection and limits of quantification

The linearity and determination range of the analytes are investigated using standard solutions, including two concentration intervals depending on the response signal of each analyte (1.0–20.0 μg/mL for PR and 1.5–30.0 μg/mL for the other three substances: MP, DX, and DC). The LOQ of each analyte is determined experimentally on a spike sample diluted to concentrations at which accuracy and precision are achieved. The LOD of each substance is determined from 1/3 of the corresponding LOQ value and verified by experimentally diluting the spike sample to concentrations at which the analyte is still detectable. The accuracy and precision of the method are evaluated on a placebo sample by adding the standard solution at three concentration levels: LOQ, five times LOQ, and 10 times LOQ. Each concentration is performed on six independent samples (Table 4).

The survey results in Table 4 show that the analytical method achieved the linearity with an R^2 value > 0.9995. The recoveries of four analytes with a concentration range of 1.0–15.0 μg/mL are 80.0–110.0%, except for methylprednisolone, which has a recovery of 101.3–111.3%. The RSD% values of all analytes are not more than 7.3%. The above results show that this method has high accuracy and reliability.

In short, the developed HPLC-PDA method for the quality control of traditional medicine meets the requirements of the suitability of the system, with high specificity, accuracy, and precision.

Table 4: Results of linearity, determination range, LOD, LOQ, accuracy, and precision survey

Analytical Substances	Con-centration (µg/mL)	Accuracy and precision (n = 6)			Equation regression	R^2	Concentration range (µg/mL)	LOQ (µg/mL)	LOD (µg/mL)
		Recovery (%)	*RSD (%)*	Determination range (µg/mL)					
PR	1.0	93.0–101.8	3.1	1.0 – 10.0	y = 0.7274x - 0.111	0.9996	1.0 – 20.0	1.0	0.25
	5.0	98.8–105.0	2.1						
	10.0	99.7–106.0	2.7						
MP	1.5	101.3–111.3	3.7	1.5 – 15.0	y = 0.3305x - 0.1567	0.9999	1.5 – 30.0	1.5	0.5
	7.5	102.5–110.4	2.5						
	15.0	101.3–109.0	3.0						
DX	1.5	99.8–109.7	3.5	1.5 – 15.0	y = 0.3004x - 0.0022	0.9995	1.5 – 30.0	1.5	0.5
	7.5	101.3–105.8	1.7						
	15.0	103.0–109.1	2.3						
DC	1.5	89.1–100.0	4.2	1.5 – 15.0	y = 0.1493x - 0.0678	0.9999	1.5 – 30.0	1.5	0.5
	7.5	93.7–103.2	3.9						
	15.0	102.5–109.6	2.7						

The method can be applied to both qualitative and quantitative analysis of herbal preparations available on the market.

B. Discussion

The results indicate that a simultaneous quantification process of four analgesic and anti-inflammatory compounds illegally mixed in herbal preparations by HPLC-PDA method has been successfully developed and may be recommended for regular quality control of herbal preparations available in the market. The study contributes to clarifying the situation of illegal mixing of modern pharmaceuticals into traditional herbal medicines to create quick and obvious effects and deceive patients. Thus, the development of analytical methods for the presence of active ingredients in herbal drugs is necessary.

Traditional medicine is a preparation form of medicinal herbs, and the sample composition is often complex. Therefore, the sample processing process needs to examine factors such as extraction time and extraction solvent to ensure accurate determination of the analyte content in herbal medicines. Typically, the presence of active ingredients in herbal drugs is not allowed, and the dosage, if any, will vary. Hence, to determine the analyte content in these samples, a linear curve with a concentration range of 1.0–30.0 μg/mL is constructed based on the response of each analyte. In addition, the accuracy and precision are achieved by adding a precise amount of standard solution to the placebo samples, using three levels of concentration: LOQ, 5x LOQ, and 10x LOQ, and then performing the extraction and analysis process on the HPLC system.

The results suggest the developed quantitative procedure has a short analysis time (30 minutes) and relatively simple sample analysis and processing procedures. Moreover, the method is successfully validated according to the guidelines of ICH and AOAC with a wide linear range, high accuracy, and precision.

The results of this study also show similarities with those of a study by Huetos et al. [4] in terms of the detection limits and quantification limits of the studied substances. Specifically, the definition range of studied substances from 10 to 160 μg/mL is wider than the range defined in this study, which is 1.0–30.0 μg/mL [4]. Otherwise, this study holds the lower limit of the definition range of 1.0 μg/mL compared to Huetos et al.'s study [4], which is an advantage in the analysis of impurities. Furthermore, the results also demonstrate a correlation in terms of recovery rate, accuracy, and repeatability compared with the study of Lawati et al. [8].

V. CONCLUSION

The study has successfully developed a quantitative process for the simultaneous analysis of four analgesic and anti-inflammatory compounds illegally mixed in herbal medicines by the HPLC-PDA method. With simple sample processing and common solvents, a quantitative method has been developed with high specificity, accuracy, and

linearity with a linear range of 1.0–20.0 μg/mL for paracetamol and 1.5–30.0 μg/mL for all three substances: methyl prednisolon, dexamethasone, and diclofenac. This method can be applied to routinely test the quality of herbal preparations available in the market. The generalizability of the results is limited because this research focuses on solid-form herbal medicines; liquid-form herbal medicines are beyond the scope of this study. Furthermore, other pain-relieving and anti-inflammatory compounds belonging to the corticoid and NSAID groups are often mixed into herbal medicines by facilities. Due to a lack of data on corticoid and NSAID groups, the results cannot confirm the presence of these substances in the selected samples. Therefore, future research avenues should focus on developing a procedure for the simultaneous quantification of more substances, representing the entire herbal medicinal preparations, including both solid and liquid forms.

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