Pharmacokinetic study of lappaconitine hydrobromide in mice by LC-MS

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Abstract: A high sensitive and rapid method was developed for the analysis of lappaconitine in mouse plasma using liquid chromatography coupled to mass spectrometry (LC-MS). Detection was performed by positive ion electrospray ionization (ESI) in multiple reaction monitoring (MRM) mode, monitoring the transitions m/z 585 → m/z 535 and m/z 356 → m/z 192, for the quantification of lappaconitine and tetrahydropalmatine (internal standard, IS), respectively. The method was linear over the concentration range of 3.0−2 000.0 ng·mL^{-1}. The lower limit of quantification was 3.0 ng·mL^{-1}. Intra- and inter-run precisions (RSD) were both less than 9.9% and accuracy (RE) within ± 4.8%. After single intravenous injections of lappaconitine hydrobromide at 1.0, 2.0 and 4.0 mg·kg^{-1}, the elimination half-lives ($t_{1/2}$) were 0.47, 0.48 and 0.49 h, and the areas under the curve (AUC$_{0-t}$) were 55.5, 110.5 and 402.9 ng·h·mL^{-1}, separately. The pharmacokinetic profile of lappaconitine was linear at relatively lower dose levels (1.0–2.0 mg·kg^{-1}). When the dose increased farther to 4.0 mg·kg^{-1}, the V_z and CL decreased, and the increase fold of the AUC was much larger than that of the dose.

Key words: lappaconitine hydrobromide; HPLC-MS; pharmacokinetics

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LC-MS 方法研究氢溴酸高乌甲素在小鼠体内的药代动力学

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摘要: 本文建立了一种快速、灵敏的 LC-MS 法用于检测小鼠血浆中的高乌甲素浓度。采用 ESI 源和多反应监测 (MRM) 的方式进行检测, 所选用的高乌甲素和内标延胡索乙素的反应离子对分别为 m/z 585 → 535 和 m/z 356 → m/z 192。该方法在 3.0～2 000.0 ng·mL^{-1} 浓度内线性关系良好, 定量下限为 3.0 ng·mL^{-1}, 日内和日间精密度 (RSD) 均小于 9.9%, 准确度 (RE) 在 ±4.8% 之内。氢溴酸高乌甲素分别以 1.0、2.0 和 4.0 mg·kg^{-1} 单剂量静脉注射给予小鼠后, $t_{1/2}$ 分别为 0.47、0.48 和 0.49 h, AUC$_{0-t}$ 分别为 55.5、110.5 和 402.9 ng·h·mL^{-1}。实验结果表明, 氢溴酸高乌甲素单剂量静脉注射给予小鼠后, 在低剂量 (1.0～2.0 mg·kg^{-1}) 范围内其药动学行为符合线性动力学特征, 当给药剂量 (2.0 mg·kg^{-1}) 增大至 4.0 mg·kg^{-1} 时, AUC 增加至约 4 倍, 而 V_z 和 CL 却显著降低, 呈现非线性动力学特征, 可能与高浓度下药物血浆蛋白结合率的降低有关。

关键词: 氢溴酸高乌甲素; HPLC-MS; 药代动力学

Lappaconitine ((1α, 14α, 16β)-20-ethyl-1, 14, 16-trimethoxyaconitane-4, 8, 9-triol 4-(2-(acetylamino)benzoate)) is a diterpene alkaloid extracted from roots of *Aconitum sinomontanum* (Figure 1). It is non-narcotic central nervous system analgesics, with strong analgesic action[1, 2]. Analgesic activity of lappaconitine is greater than that of indometacin and acetylsalicylic acid, but generally about 2 to 5 times less than that of morphine[2]. Moreover, lappaconitine also has local anesthesia and anti-inflammation effect[3]. So far, the study on pharmacokinetics of lappaconitine is not thorough. HPLC-UV methods were reported for the determination of lappaconitine and its metabolites in plasma or urine[4−6]. Methods reported in literatures were time consuming (about 15 min), and the lower limit of quantification (LLOQ) was 0.5 $\mu g \cdot mL^{-1}$, and it was not sensitive enough for the pharmacokinetic study of lappaconitine. In the present study, a more sensitive and selective LC-MS^n method is established for the pharmacokinetic study of lappaconitine in mice.
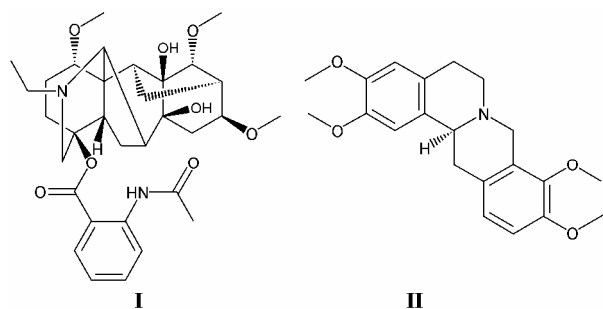
Figure 1   Chemical structure of lappaconitine (I) and tetrahydropalmatine (II, IS)

Materials and methods

Chemicals and reagent   Lappaconitine hydrobromide (99.0% purity) and tetrahydropalmatine (IS, 98.0% purity) were obtained from Qizheng Tibetan-Medicine Group (Lanzhou, China). Methanol (HPLC grade) was purchased from Kangkede Chemical Co. (Tianjin, China). Acetic acid (analytical grade) was purchased from Shenyang Chemical Co. (Shenyang, China). Purified water was purchased from Wahaha Group Co., LTD (Hangzhou, China).

Instruments and methods   Liquid chromatographic separation and mass spectrometric detection were performed using a P230 pump manufactured by Elite Industrial Corporation (Dalian, China) and 1100 Series LC/MSD Trap SL mass spectrometer manufactured by Agilent Technologies (Santa Clara, California, USA), equipped with an ESI source. The chromatographic

separation was achieved on an Agilent Zorbax SB C_{18} (150 mm × 4.6 mm ID, 5 μm) analytical column at a temperature of 20 ℃. The system was run in isocratic mode with mobile phase (methanol-water-acetic acid, 60 : 40 : 0.5, *v/v/v*). Mobile phase was duly filtered through 0.45 μm Millipore filter and delivered at a flow rate of 0.5 $mL \cdot min^{-1}$. Helium was used as collision gas with the collision energy of 0.8 eV for lappaconitine and 1.0 eV for IS. The mass spectrometer was operated at ESI positive ion mode and detection of the ions was performed in multiple reaction monitoring (MRM). The selected mass-to-charge (*m/z*) ratio of lappaconitine and IS used in the MRM were as follows: lappaconitine, *m/z* 585 → *m/z* 535; IS, *m/z* 356 → *m/z* 192.

Standard and working solutions   Stock solution of lappaconitine hydrobromide was prepared in methanol at the concentration of 400.0 $\mu g \cdot mL^{-1}$ (calculated as the free base) by dissolving an appropriate amount of the chemical reference substance. A series of working solutions of lappaconitine were obtained by further diluting the stock solution with purified water. Stock solution of IS was also prepared in methanol at the concentration of 400.0 $\mu g \cdot mL^{-1}$ and diluted to 10.0 $\mu g \cdot mL^{-1}$ with purified water. All solutions were stored at 4 ℃.

Calibration curves were prepared by spiking 100 μL of the appropriate standard solution to 100 μL of blank plasma. Effective concentrations in plasma samples were 3.0, 10.0, 20.0, 80.0, 400.0, 1 000.0, and 2 000.0 $ng \cdot mL^{-1}$ for lappaconitine. The quality control (QC) samples were separately prepared in blank plasma at the concentrations of 10.0, 80.0 and 1 800.0 $ng \cdot mL^{-1}$. The QC samples were used for the assessment of the accuracy and precision of the assay method. All the calibration and QC samples were then extracted by the method described in the subsequent section and analyzed.

Sample preparation   Frozen plasma samples were put in room temperature water bath to thaw quickly and treated immediately. A mixture consisting of 100 μL of plasma sample, 50 μL of IS solution (10.0 $\mu g \cdot mL^{-1}$ tetrahydropalmatine), 100 μL of purified water and 60 μL of sodium hydroxide solution (1.0 $mol \cdot L^{-1}$) was pooled in a test tube for processing. Then 1 mL of extraction solvent (*n*-hexane-isopropanol, 95 : 5) was added, and the mixture was vortexed for 1 min, shook for 10 min and then centrifuged at 3 500 g for 5 min. After centrifugation, the upper organic layer was removed and evaporated to dryness at 40 ℃ under a gentle

stream of nitrogen. The resulting residue was then reconstituted in 100 μL mobile phase and 20 μL of the solution obtained was injected into the LC-MS system.

Method validation The validation of the LC-MS method included selectivity, linearity, inter- and intra-run precision and accuracy, matrix effects, stability and recovery studies.

Selectivity Six pre-dose plasma samples from different mice were used to evaluate the specificity. Blank samples were extracted and analyzed to assure that it is free of interfering response values. The MRM chromatograms of blank plasma samples were compared with those at LLOQ. Peak areas of endogenous compounds co-eluting with the analyte need to be less than 20% of the peak area of the LLOQ standard.

Linearity and LLOQ Calibration standards were prepared and analyzed in duplicate on three consecutive days. The calibration curves (peak area ratios of analyte over IS versus the nominal analyte concentration) were fitted by weighted ($1/x^2$) least squares linear regression. To establish linearity, the coefficient of correlation (r) should be more than 0.99 and deviations of the calculated concentrations should be within ±15% from nominal concentrations except for the LLOQ level, at which a deviation of ±20% is permitted. The LLOQ was determined by quantifying blank plasma spiked with decreasing concentrations of drugs and defined as the lowest concentration for which the deviation from the nominal concentration and the imprecision (CV) did not exceed 20%.

Accuracy and precision Intra-run precision and accuracy were evaluated by analysis of QC samples at three levels (10.0, 80.0 and 1 800.0 ng·mL^{-1}) with six determinations per concentration in the same day. The inter-run precision was measured over three consecutive days. The accuracy was expressed as the relative error (RE), and the precision was evaluated by the relative standard deviation (RSD). The RSD should not exceed 15% and the accuracy should be within ±15% of the actual value for QC samples.

Extraction recovery and matrix effects Extraction recovery (ER) presents the extraction efficiency of a method, which is calculated as the ratio of the peak area of a processed QC sample on that of a processed blank plasma sample spiked with analyte after extraction. These analyses were performed at three concentration levels of 10.0, 80.0 and 1 800.0 ng·mL^{-1} in triplicate. The matrix effect of mouse plasma was examined by comparing the MS response of the analyte at three concentration levels spiked post-extraction into plasma to the MS response of the same analyte present in the neat mobile phase[7].

Stability Freeze and thaw stability: plasma samples at two concentration levels (10.0, 1 000.0 ng·mL^{-1}) were stored at −20 ℃ for 12 h and thawed unassisted at room temperature. When completely thawed, the samples were refrozen for 12 h under the same conditions. The freeze-thaw cycles were repeated twice and the samples were analyzed after one and three freeze (−20 ℃) -thaw (room temperature) cycles, separately.

Short-term stability: plasma samples at two concentration levels (10.0, 1 000.0 ng·mL^{-1}) were kept at room temperature and the samples were analyzed after 15, 30, 60 and 120 min, separately.

Long-term stability: plasma samples at two concentration levels (10.0, 1 000.0 ng·mL^{-1}) were kept at −20 ℃ for one month, and then analyzed.

Stability after extraction: the processed plasma samples at two concentration levels (10.0, 1 000.0 ng·mL^{-1}) were placed at ambient temperature for 24 h, and then analyzed.

Application of the method to pharmacokinetic study All animal studies were performed in accordance with the experimental protocols approved by the Animal Care Committee of Dalian Medical University. Male KM mice (20 − 25 g) used in the present study were supplied by Dalian Medical University (Dalian, China). Mice were housed in standard cages and allowed free movement and access to water and standard laboratory diet throughout the experiments. After a single intravenous injection of lappaconitine hydrobromide at 1.0, 2.0 and 4.0 mg·kg^{-1} (dissolved in saline, injection volume of 0.5 mL·kg^{-1}), serial blood samples (0.5 mL) were collected into heparinized tubes from the ophthalmic vein at 1, 5, 10, 15, 30 min and 1, 1.5, 2 h post-dose. At every point-in-time, five mice were treated. Plasma was immediately separated by centrifugation at 12 000 ×g for 5 min and stored frozen at −20 ℃ until analysis. Pharmacokinetic parameters were calculated with non-compartmental model of Topfit program (Version 2.0, Gustav Fischer Verlag, Stuttgart, Germany) on a personal computer.

Results

1 Method validation

1.1 Selectivity The typical chromatograms of a blank, a spiked plasma sample with lappaconitine

(3.0 ng·mL^{-1}) and IS (10.0 μg·mL^{-1}) and extracted unknown plasma samples are shown in Figure 2. No endogenous peak interference was found with the quantification of lappaconitine and IS.

1.2　Accuracy and precision　The results of the accuracy and precision for lappaconitine, determined by replicate of QC samples, are presented in Table 1. The accuracy was within ± 4.8% in terms of RE and the precision was found to be less than 9.9% in terms of RSD.
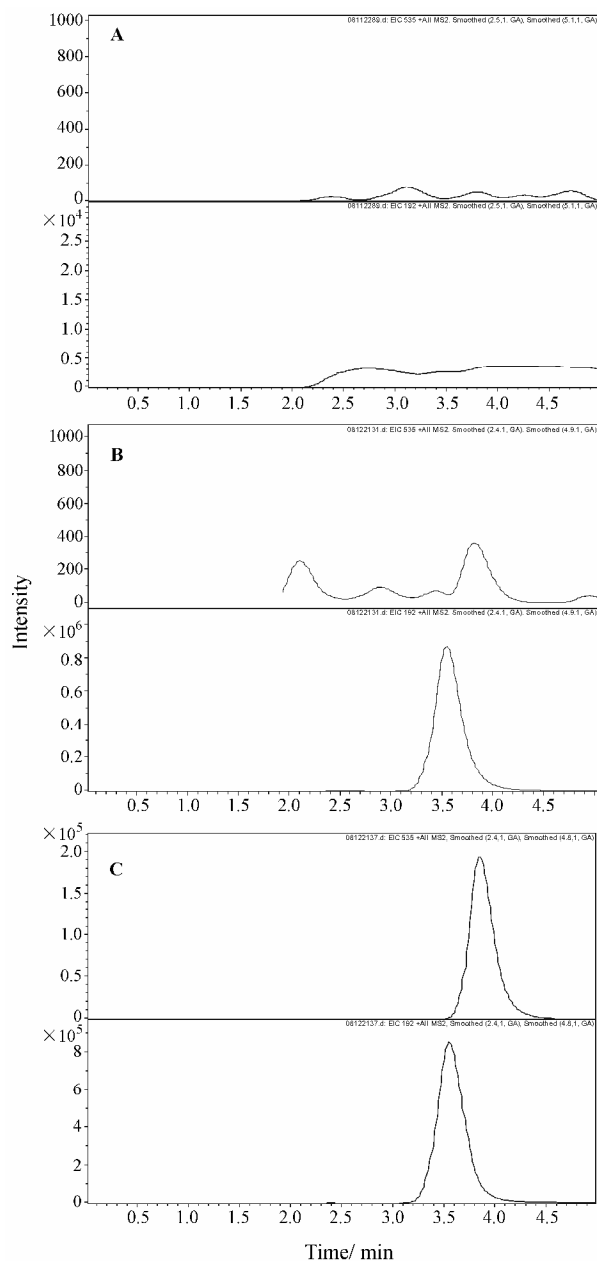
Figure 2　Representative MRM chromatograms of lappaconitine and IS in mouse plasma samples.　A: A blank plasma sample; B: A blank plasma sample spiked with lappaconitine at the LLOQ of 3.0 ng·mL^{-1} and IS (10.0 μg·mL^{-1}); C: Plasma sample from a mouse at 5 min after an iv injection of 2.0 mg·kg^{-1} lappaconitine

1.3　Extraction recovery and matrix effects　The extraction recovery of lappaconitine was calculated by analyzing three replicates at 10.0, 80.0 and 1 800.0 ng·mL^{-1}. The extraction recoveries of the assay were 91.4% ± 4.1%, 94.3% ± 4.8% and 97.7% ± 5.4% for the low, middle and high concentration levels, respectively.

Table 1　Precisions and accuracy of the HPLC-MS method for the determination of lappaconitine in mouse plasma.　$n = 6$

Added C /ng·mL^{-1}	Found C /ng·mL^{-1}	Intra-run RSD/%	Inter-run RSD/%	Relative error/%
10.0	9.52	7.1	8.8	−4.8
80.0	86.3	9.9	9.0	7.9
1 800.0	1 812.2	8.2	5.3	0.68

Matrix effect was defined as the direct or indirect alteration or interference in response due to the presence of unintended analytes or other interfering substances in sample. In our experiment, the matrix effect of the studied plasma was evaluated by the ratio of the response of the analyte spiked into post-extraction plasma over that of the analyte in neat mobile phase. Results are shown in Table 2, and no significant matrix effect was found.

Table 2　The matrix effect of the studied plasma on the response of the analytes.　$n = 6$

| Analyte	Added C/ng·mL^{-1}	Mean peak area		Absolute matrix effect /%	RSD/%
		Plasma	Mobile phase		
Lappaconitine	10.0	63 389	65 028	102.6	5.2
	80.0	539 293	508 746	94.3	4.8
	1 800.0	11 841 889	11 524 098	97.3	7.1
IS	10 000	91 045 902	90 798 453	99.7	3.5

1.4　Stability　The stability of lappaconitine in mouse plasma was studied, and the results indicated that lappaconitine was stable for 15 min at room temperature, for at least 30 days at −20 ℃, and for only one freeze-thaw cycle. By keeping processed plasma samples under ambient conditions, lappaconitine was found to be stable after preparation for 24 h.

1.5　Application to the pharmacokinetic study　The established LC-MS method was successfully applied to the determination of lappaconitine in mouse plasma after a single intravenous injection dose of 1.0, 2.0 and 4.0 mg·kg^{-1}, separately. Before analysis, all the unknown samples were thawed to room temperature and processed within 15 min. Noncompartment model was used to calculate the pharmacokinetic parameters with Topfit 2.0 program. The corresponding pharma-

cokinetic parameters are shown in Table 3. The concentration of lappaconitine decreased quickly after intravenous injection as shown in Figure 3. The half lives of lappaconitine were both about 0.5 h, and the areas under plasma concentration-time curve (AUC_{0-t}) were 55.5, 110.5 and 402.9 ng·h·mL^{-1} at the injection dose of 1.0, 2.0 and 4.0 mg·kg^{-1} in mice, respectively.

Table 3 Pharmacokinetic parameters for lappaconitin after a single intravenous injection of 1.0, 2.0 and 4.0 mg·kg^{-1} to mice, respectively

Parameter	Dose for mice /mg·kg^{-1}		
	1.0	2.0	4.0
k_e /h^{-1}	1.47	1.45	1.43
$t_{1/2}$ /h	0.47	0.48	0.49
AUC_{0-t} /ng·h·mL^{-1}	55.5	110.5	402.9
$AUC_{0-\infty}$ /ng·h·mL^{-1}	56.3	112.6	413.5
MRT /h	0.24	0.25	0.34
CL /mL·min^{-1}·kg^{-1}	296	296	161
V_z /L·kg^{-1}	12.1	12.3	6.76
V_{ss} /L·kg^{-1}	4.75	5.32	3.91
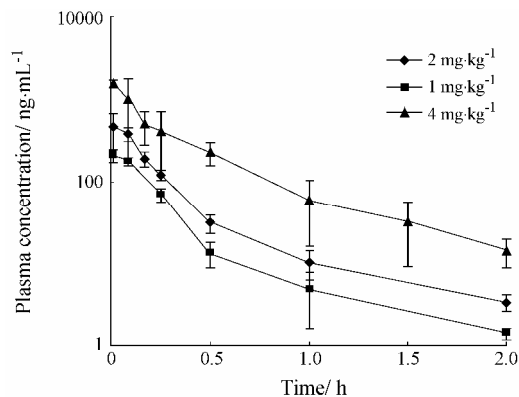
Figure 3 Mean plasma concentration-time profiles of lappaconitine after a single intravenous injection of 1.0, 2.0 and 4.0 mg·kg^{-1} lappaconitine hydrobromide to mice, respectively. $n = 5$, mean ± SD

Discussion

Mass spectrometric conditions were optimized so as to achieve the maximum stable response of the precursor ions and the major product ions of the analytes. MRM was set for the detection of lappaconitine and IS in this study. MRM afforded by tandem mass spectrometry has great advantages in reducing interference and enhancing sensitivity over selected ion monitoring (SIM). In positive ion mode, both lappaconitine and IS gave protonated molecular ions, [M+H]$^+$, as the major species. The product ion mass spectra showed that the greatest intensity was presented at m/z 535 for lappaconitine, and m/z 192 for

the IS. To optimize the response of lappaconitine, two predominant product ions of m/z 535 and m/z 567 were both selected firstly. With the addition of m/z 567, the absolute response of lappconitine was increased, but the signal to noise ratio of lappaconitine was decreased, it indicated that the specificity of m/z 585 to m/z 567 (loss of H$_2$O) was poor. So m/z 535 (loss of H$_2$O and CH$_3$OH) was selected as the only product ion in the MRM for lappaconitine. The mass parameters such as spray voltage, capillary temperature, nitrogen sheath gas and the collision gas pressure and energy were tuned to observe the maximal response of the product ion m/z 535.

LLE (liquid–liquid extraction) is one of the commonly used methods for sample preparation. When using LLE, the most important factor is to choose an appropriate organic solvent, which can give good selectivity and extraction recovery. In this study, different extraction conditions were evaluated including organic extraction solvents and pH. Lappaconitine is an alkaloid, so 0.1 mol·L^{-1} NaOH was used as the pH modifier to improve the extraction recovery. With the presence of pH modifier, the recovery of lappaconitine from mouse plasma was proved high and repeatable. In addition, three organic extraction solvent systems were screened: n-hexane-dichloromethane-2-propanol (20 : 10 : 1), n-hexane-dichloromethane (2 : 1), n-hexane-isopropanol (95 : 5). The latter clearly yielded the highest recovery and no significant interferences. Only 1.0 mL organic solvents was used per sample, which greatly reduced the drying time and environmental pollution, thus simplifying the whole sample preparation procedure which is essential for high throughput bioanalysis.

Although, lappaconitine is being used in clinical practice, its pharmacokinetic efficacy and safety data are limited. This study reports the pharmacokinetics of lappaconitine in mouse plasma after intravenous administration at three different doses. Based on the LD$_{50}$ of lappaconitine (iv, 8.59 mg·kg^{-1}) in mouse reported in the literature[8], the study was designed initially with the highest dose of 5.0 mg·kg^{-1}. However, it was observed that intravenous administration of lappaconitine hydrobromide at the dose of 5.0 mg·kg^{-1} caused mouse death immediately, which was consistent with the results of Guan et al[4]. Consequently, the intravenous administration levels were set at 1.0, 2.0 and 4.0 mg·kg^{-1}.

The disposition of lappaconitine hydrobromide

in mice has been previously reported after oral and i.v. administration at a single dose[4, 9]. The pharmacokinetic parameters of lappaconitine in the present study were different from those reported by Guan et al. In the present study, the $t_{1/2}$ was shorter (0.47, 0.48 and 0.49 h vs. 4.72 h) and $AUC_{0-\infty}$ was lower (56.3, 112.6 and 413.5 ng·h·mL^{-1} vs. 10.5 μg·h·mL^{-1}). Differences could be related to the age or sex of mice used. Also factors such as the exposure to certain drugs or environmental pollutants can affect the rate of biotransformation and excretion of animals.

The pharmacokinetic data showed that lappaconitine had a short half-life and a moderate clearance in mice. The two parameters of k_e and $t_{1/2}$ didn't vary within the dose range of 1.0 to 4.0 mg·kg^{-1}, which showed that the elimination rate of lappaconitine was unchanged. Other pharmacokinetic parameters given in this study, such as AUC (divided by dose), CL and V_z (the apparent distribution volume), were also unchanged over the dose range of 1.0−2.0 mg·kg^{-1}, but varied significantly at the dose of 4.0 mg·kg^{-1}. When the dose increased to 4.0 mg·kg^{-1}, V_z decreased from 12.3 to 6.76 L·kg^{-1}. It might be due to the decrease of the protein binding rate and the lowered tissue affinity, which made the plasma concentration increase. Then the unchanged k_e and decreased V_z led to the lower CL (161 vs. 296 mL·min^{-1}·kg^{-1}).

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