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Liquid chromatographic/mass spectrometry assay of triptolide in dog plasma and its application to pharmacokinetic study

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Abstract

A rapid and sensitive method for the determination of triptolide in dog plasma was developed and validated, using high-performance liquid chromatography/atmospheric pressure chemical ionization mass spectrometry (LC/APCI/MS). Sample preparation consisted of liquid–liquid extraction with ethyl acetate from dog plasma. The analytes and internal standard prednisolone were well separated on a Zorbax Extend-C_{18} analytical column. Detection was performed on a triple quadrupole mass spectrometer using selected-ion monitoring (SIM) mode on the deprotonated ions $[M-H]^-$ at *m/z* 359. Calibration curves were linear over the concentration range of 0.5–200 ng/mL of triptolide with the intra- and inter-day precision (the relative standard deviation values) were being less than 7%. Triptolide was stable under different conditions. The intra-day and inter-day accuracy were 99.3–105.2% and 101.3–107.0%, respectively. The lower limit of quantification was 0.5 ng/mL. The method was successfully applied to a pharmacokinetic study after an intragastric administration (i.g.) of triptolide to dogs with a dose of 0.05 mg/kg. The results confirm that the assay is suitable for the pharmacokinetic study of triptolide.

Keywords: Triptolide; LC/APCI/MS; Pharmacokinetics

1. Introduction

Triptolide (Fig. 1), a diterpenoid triepoxide compound, is isolated from the roots of *Tripterygium wilfordii* Hook. f.(Celastraceae, TWHF). This plant is popularly known in Chinese as "Lei-gong-teng" (Thunder God Vine) and is one Chinese herbal medicine used to treat some autoimmune disorders such as rheumatoid arthritis, systemic lupus erythematosus, and skin diseases [1,2]. The ethanol, ethyl acetate and other extracts of the roots have been found to have potent immunosuppressive, anti-cancer and anti-inflammatory properties and are used widely in China for treatment of rheumatoid arthritis [3]. Triptolide, a major active component of TWHF, is deemed to account for the immunosuppressive [4], anti-cancer [5,6] and anti-fertility activities [7] of the extracts. Triptolide has also been reported to be effective in the treatment of rheumatoid arthritis [8] and leukaemia [9]. Beside its effect, it is well known that triptolide has a small margin between the therapeutic and toxic doses and serious toxicity on digestive, urogenital and blood circulatory system [10]. Although plenty of work on the pharmacology has been reported, pharmacokinetic and toxicokinetic properties of triptolide have seldom been studied, which is essential for its further research as a novel drug.

To date, a variety of analytical methods including UV spectrophotometry [11], thin layer chromatography (TLC) [12], LC with UV detection [13–17], and capillary electrophoresis (CE) [18] have been reported. Most of them, however, were developed for quantitative determination of triptolide in plants or medical preparations. However, there were fewer methods reported to be used in biological fluids. Yang et al. [19] and Wang et al. [20] developed a GC and LC method, respectively, for the determination of triptolide in human plasma. Although the published GC [19] method was sensitive (LLOQ: 1 ng/mL), it required derivatization for triptolide with trifluoroacetic anhydride, which required laborious sample pretreatment and was not convenient for large numbers of samples in pharmacokinetic and toxicokinetic research. The LC [20] method for determina-

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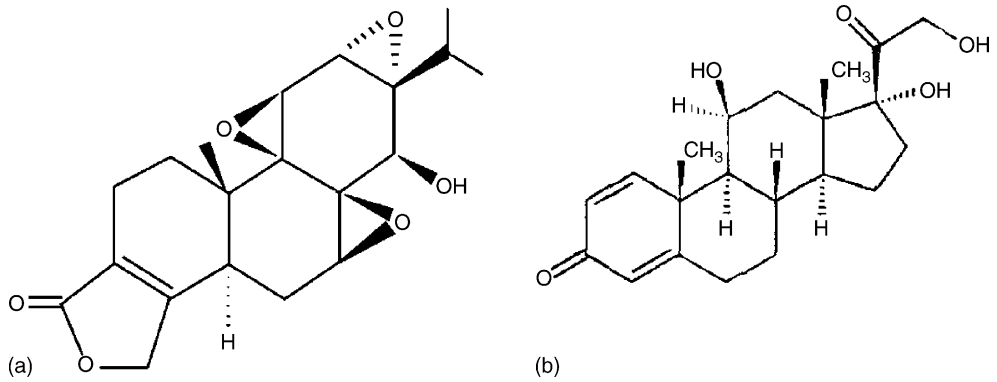

Fig. 1. Structures of triptolide molecular mass = 360.4036 (a) and prednisolone (I.S.), molecular mass = 360.4472 (b).

tion of triptolide in plasma was prone to interference from other substances and was not sensitive enough (LLOQ: 10 ng/mL) to support the pharmacokinetic and toxicokinetic studies in dog. Furthermore, both GC [19] and LC [20] experienced a tediously long analysis time requiring 20 and 22 min, respectively.

The aim of this study was to develop and validate a rapid (run time: 5 min) and sensitive (LLOQ: 0.5 ng/mL) LC–APCI–MS method for the determination of triptolide using prednisolone as an internal standard (I.S.) in dog plasma. The present method was successfully applied to monitoring the plasma concentration of triptolide after i.g. administration to dogs.

2. Experimental

2.1. Chemicals and reagents

Triptolide was kindly provided by Prof. Chen Yun (Chinese Academy of Medical Sciences & Peking Union Medical College, Beijing, China). Prednisolone was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). LC grade acetonitrile and methanol were obtained from Fisher Scientific (Toronto, Canada). Deionized water was purified using a Milli-Q system (Millipore, Milford, MA, USA). Ethyl acetate and other chemicals and solvents used were of analytical grade.

2.2. Instrument and analytical conditions

The LC system consisted of a LC-10AD pump, a DGU-14AM degasser, a Shimadzu 10ATvp autosampler, and a CTO-10 Avp column oven (Shimadzu, Kyoto, Japan). A Shimadzu 2010A liquid chromatograph–mass spectrometer (Shimadzu, Kyoto, Japan) equipped with atmosphere pressure chemical ionization (APCI) probe was used in the study. Analyses were performed on a Zorbax Extend-C_{18} analytical column (4.6 mm × 150 mm i.d., particle size 5 μm, Agilent Technologies, U.S.A.) protected by a ODS guard column (Security Guard, Phenomenex®, U.S.A.) at 40 °C. The mobile phase was composed of acetonitrile/methanol/0.05% triethylamine aqueous solution (30:50:20, v/v/v) at a flow rate of 0.75 mL/min. Under these conditions, triptolide eluted at approximately 3.3 min and the internal standard at 4.0 min (Fig. 3).

2.3. Mass spectrometric conditions

All measurements were carried out using the negative ion APCI mode. Mass spectrometer conditions were optimized to obtain maximum sensitivity. The curve dissolution line (CDL) temperature was 200 °C, the APCI probe temperature was 400 °C and the heat block temperature was 200 °C. A detector voltage of 1.6 kV and a probe voltage of 4.5 kV were fixed as in the tuning method. Liquid nitrogen (99.995%, from Gas Supplier Center of Nanjing University, China) was used as the nebulizer gas and curtain gas source at 2.5 and 2.0 L/min, respectively. Mass spectra were obtained at a dwell time of 0.2 s in SIM mode and 1 s in scan mode. LC/MS solution Version 2.04 working on Windows 2000 operating system was used for data processing. $[M-H]^-$ at *m/z* 359 was for both triptolide and the I.S. (Fig. 2). Peak area ratios of the analytes to I.S. were calculated and the calibration curve was established by fitting these ratios to the corresponding nominal concentrations.

2.4. Preparation of stock solutions and quality control samples

The standard stock solutions of 1 mg/mL of triptolide was prepared in methanol. A series of standard working solutions were obtained by further dilution of the standard stock solutions with methanol. Internal standard (I.S.) working solution (2 μg/mL) was prepared by diluting I.S. stock solution with methanol. All solutions were stored at 4 °C. Appropriate amounts of working solution were diluted with drug-free plasma to obtain a calibration standard range of 0.5–200 ng/mL. Quality control (QC) samples (1, 10, 100 ng/mL) were prepared in the same way.

2.5. Sample preparation

A portion (1 mL) of the plasma, spiked with I.S. working solution (50 μL), was vortex-mixed for 30 s and extracted with ethyl acetate (6 mL) after 2 min vortex mixing. The tubes were then centrifuged at 4000 rpm for 5 min. The upper organic phase (4 mL) was transferred into a 10 mL glass tube and evaporated to dryness in a SpeedVac System (Thermo

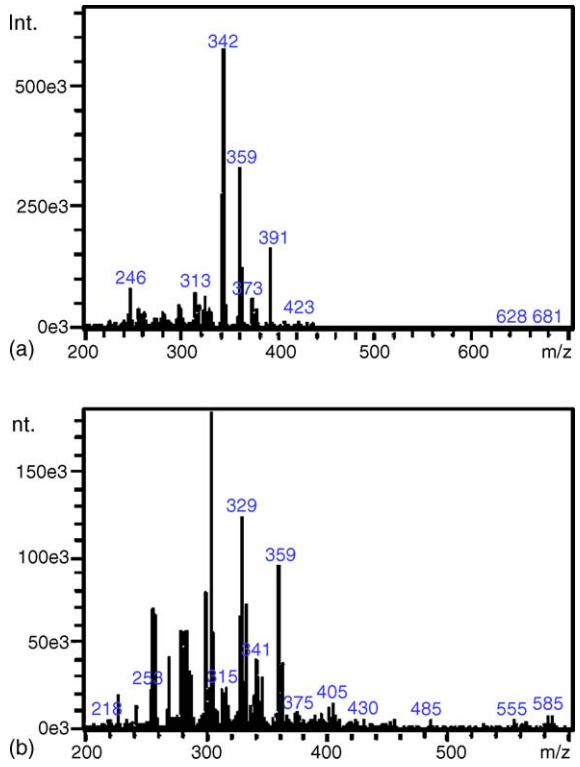

Fig. 2. Negative ion mass spectra of triptolide and prednisolone (I.S.): (a) triptolide, *m/z* 359; (b) I.S., *m/z* 359.

Savant SPD 2010, Thermo Electron Corporation, U.S.A.). The residues were then reconstituted with 100 μL methanol and centrifuged at 14,000 rpm for 10 min after vortex mixing, and 10 μL supernatant was injected onto the column for analysis.

2.6. Calibration curves and assay validation

The calibration curve consisted of nine concentration levels (0.5, 1, 2, 5, 10, 20, 50, 100, 200 ng/mL of triptolide in dog plasma). The calibration curve was constructed by adding 50 μL of I.S. and varying the concentrations of triptolide in blank rat plasma. Plasma samples were quantified using the ratio of the peak area of triptolide to that of I.S. Peak area ratios were plotted against concentrations and triptolide concentrations were calculated using a least squares linear regression. Linear regression analysis was performed using Microsoft Excel 2002.

The method was fully validated for its specificity, linearity, lower limit of quantification (LLOQ), accuracy and precision. The precision was calculated as the relative standard deviation (R.S.D.) and the accuracy was evaluated as analytical recovery. Intra-day precision and accuracy were evaluated at three different plasma concentrations (1.0, 10.0, 100.0 ng/mL) by replicate analyses of five spiked plasma samples on the same day. The inter-day precision and accuracy determinations were carried out on five different days. Recovery experiments were performed by comparing the analytical results of extracted samples at three concentrations with pure standards without extraction. The LLOQ was considered as the concentration that produced a signal-to-noise (S/N) ratio greater over 10. The sample solution stability was assessed at three concentration levels (1.0, 10.0, 100.0 ng/mL). The freeze and thaw stability study samples at three concentrations were stored at −20 °C and subjected to three freeze–thaw cycles. The short-term stability of triptolide during storage in the autosampler at 4 °C, was performed by repeated injection every 2 h for a period of 10 h. The long-term stability of triptolide in plasma was assessed in three concentration levels after storage at −20 °C for 4 weeks.

2.7. Pharmacokinetic study

Six beagle dogs (9–10 kg) were purchased from the Laboratory Animal Center at China Pharmaceutical University. After an overnight fast (12 h), the dogs were given a single dose of triptolide solution formulated in 1% ethanol aqueous solution (v/v). The dogs were further fasted for 4 h after i.g. administration with free access to water. About 2.5 mL of blood samples were collected from the foreleg vein into heparinized tubes before (0 h) and at 0.08, 0.17, 0.25, 0.5, 0.75, 1, 1.5, 2, 4, 6, 8, 10 and 12 h after dosing. Plasma was separated by centrifugation at 4000 rpm for 5 min and kept at −20 °C until analysis.

3. Results and discussion

3.1. LC/APCI/MS method

Initially we found that the intensity of the signal of triptolide was weak under ESI conditions. There was, however, abundant ionization observed under APCI conditions when monitoring the signal for the deprotonated molecule, $[M-H]^-$ (see Fig. 2). Triptolide possesses few polar function groups, making it a favorable candidate for APCI, since this technique performs well in ionizing analyte molecules of weak or moderate polarity. In addition to the choice of APCI as the ionization method for the assay, the sensitivity of triptolide was enhanced by adding different amounts of basic compounds (triethylamine, diethylamine as well as ammonia) to the mobile phase. Addition of 0.05% triethylamine to the mobile phase was found to be an important factor for acquiring higher sensitivity. We found that the lower limit of quantitation (LLOQ) could be further increased to 0.5 ng/mL by utilizing the acetonitrile–methanol–0.05% triethylamine solution (30:50:20, v/v/v) as the mobile phase.

In order to ensure the accuracy of the analytical method, an internal standard having similar ionization efficiency as the analyte is preferred. Tripchlorolide and tripdiolide, two homologs of the triptolide, were firstly evaluated as the internal standards. However, neither of them was suitable for the intended purpose, since tripchlorolide could be transformed into triptolide in vitro and tripdiolide could be the metabolite of triptolide in vivo. Prednisolone, a molecular of similar molecular mass, structure and polarity (Fig. 1), was chosen as internal standard. Under these conditions, triptolide and I.S. were well separated and eluted

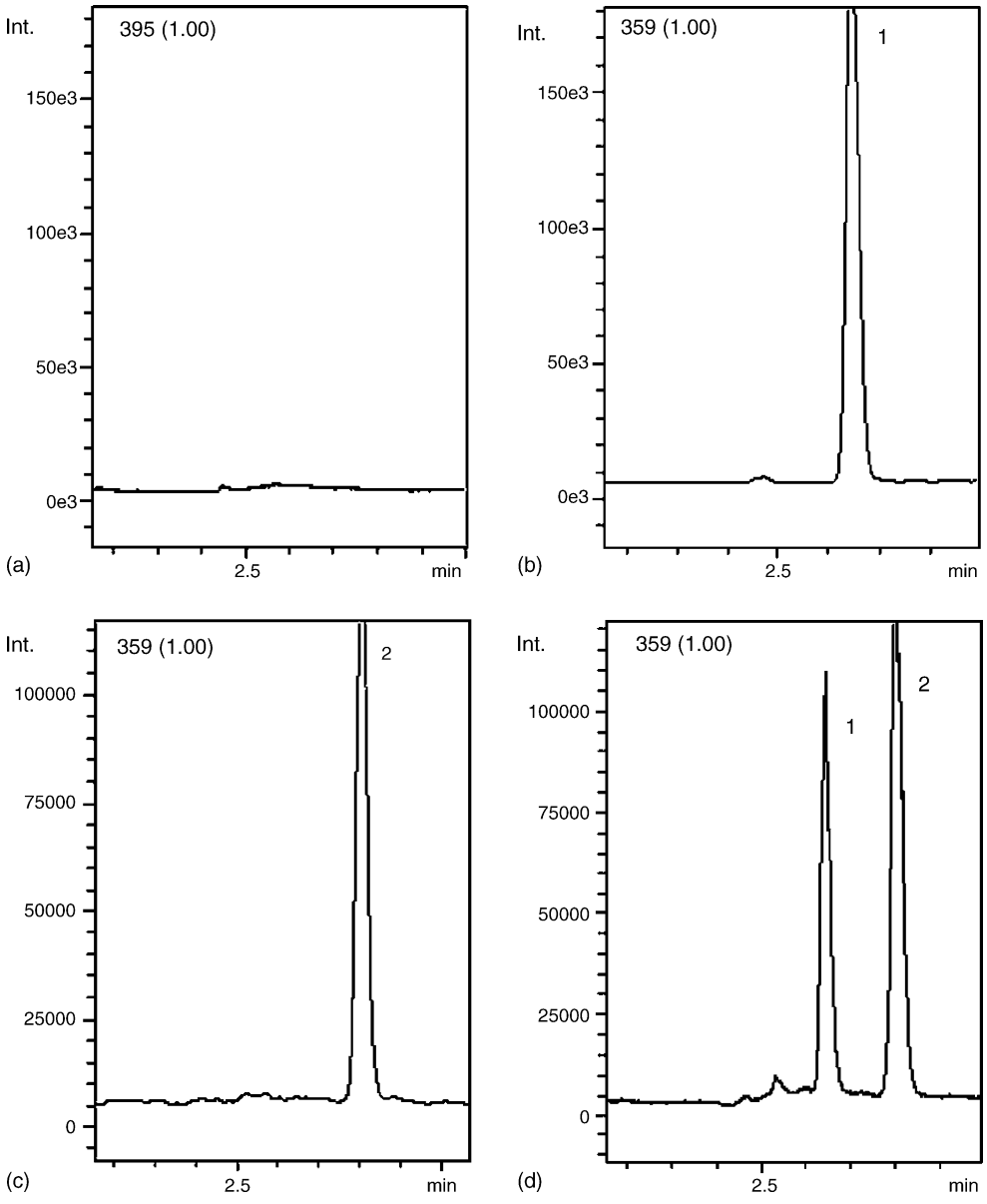

Fig. 3. Typical SIM chromatograms of: (a) blank dog plasma; (b) plasma spiked with triptolide; (c) plasma spiked with I.S.; (d) plasma sample obtained from a dog at 0.5 h after i.g. administration of 0.05 mg/kg triptolide, (1) triptolide and (2) prednisolone (I.S.).

at approximately 3.3 and 4.0 min respectively (see Fig. 3) with stable signal.

3.2. Method validation

3.2.1. Linearity and LLOQ

Calibration curves were plotted as the peak area ratio (triptolide/I.S.) versus triptolide concentrations. Results for the calibration curve ($n = 5$) showed good linearity ($r^2 > 0.999$) over the concentration range of 0.5–200 ng/mL, with a linear regression equation of $y = 0.0106x + 0.0005$, where x = triptolide concentration in ng/mL and y = triptolide area/I.S. area. The LLOQ was established at a concentration of 0.5 ng/mL for triptolide (R.S.D. < 20%) and is sufficient to support its pharmacokinetic and toxicokinetic studies.

3.2.2. Specificity and sensitivity

Typical chromatograms of the blank and spiked plasma are given in Fig. 3, showing the retention times of 3.3 and 4.0 min for triptolide and the I.S., respectively. There were no co-eluting interfering peaks observed in the matrix.

3.2.3. Accuracy and precision

Table 1 shows a summary of intra- and inter-day precision and accuracy at low, medium and high concentrations of triptolide in plasma. The intra-day precision (expressed as percent relative standard deviation, R.S.D.%) ranged from 5.2 to 7.0% and the intra-day accuracy (expressed as percent of nominal values) ranged from 99.3 to 105.2%. The method showed reproducibility with inter-day precision ranging from 5.8 to 7.1%. The inter-day accuracy ranged from 101.3 to 107.0%. These results indicated

Table 1
Accuracy and precision of LC–MS method for determining triptolide in plasma samples

Nominal concentration (ng/mL)	Intra-day ($n=5$)			Inter-day ($n=3$)		
	Concentration found (mean ± S.D.) (ng/mL)	Precision[a] (%)	Accuracy[b] (%)	Concentration found (mean ± S.D.) (ng/mL)	Precision[a] (%)	Accuracy[b] (%)
1.0	1.1 ± 0.1	7.0	105.2	1.1 ± 0.1	7.1	107.0
10.0	10.3 ± 0.7	6.9	103.1	10.2 ± 0.6	6.3	102.1
100.0	99.3 ± 5.2	5.2	99.3	101.3 ± 5.9	5.8	101.3

[a] Expressed as R.S.D.%: (S.D./mean) × 100.
[b] Calculated as (%): (mean concentration found/nominal concentration) × 100.

that the present method has a satisfactory accuracy, precision and reproducibility.

3.2.4. Recovery

The extraction recovery of triptolide was 80.2 ± 4.9% on average, and the dependence on concentration was negligible. The recovery of I.S. was 62.3% at the concentration used in the assay (2.0 μg/mL). Recovery of I.S. was low, but it was consistent, precise and reproducible.

3.2.5. Stability

Table 2 displays stability for triptolide under the following conditions has been shown: (1) stability of triptolide in dog plasma through at least three freeze–thaw cycles, (2) stability of triptolide in dog plasma at 4 °C in the autosampler for at least 10 h, (3) stability of triptolide for at least 4 weeks at −20 °C stored. As a result, triptolide showed very good stability at the three conditions. The concentration accuracy was found to range from 99.8 to 104.6%, 98.9 to 104.4% and 101.9 to 109.4%, respectively after three freeze–thaw cycles, stored at 4 °C for10 h and at −20 °C for 4 weeks.

3.2.6. Matrix effect

The reliability of quantitative LC/MS data obtained from the determination of drugs in biological matrices can be adversely affected by endogenous components in those biological fluids, resulting in ion suppression of the analyte signal. The results showed that no matrix effect or interferences from endogenous compounds were detected for assays of plasma from six different sources of dogs.

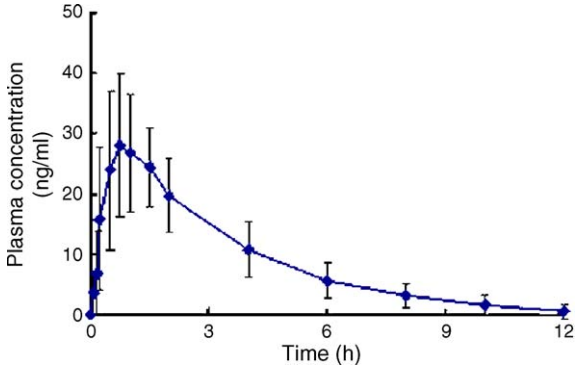

Fig. 4. Mean plasma concentration–time profile of triptolide after i.g. administration of 0.05 mg/kg triptolide to six beagle dogs.

3.3. Pharmacokinetic study

The LC/APCI/MS method developed yielded satisfactory results for the determination of triptolide in dog plasma and has been successfully used for the pharmacokinetic study of six beagle dogs following i.g. administration at a single dose of 0.05 mg/kg triptolide solution. The mean plasma concentration–time profiles for triptolide are shown in Fig. 4. The pharmacokinetic parameters of triptolide were determined by DAS (Drug and Statistics) software. The maximum plasma concentrations (C_{max}) were 28.03 ± 11.76 ng/mL, The T_{max} values were 0.96 ± 0.30 h, The MRT_{0-t_n} were 3.00 ± 0.54 h, and the $T_{1/2k_a}$ were 0.50 ± 0.38 h.

Table 2
Stability of triptolide in dog plasma under different storage conditions

Storage conditions	Concentration added (ng/mL)	Concentration found (mean ± S.D., ng/mL)	Remaining percentage[a] (%)
Freeze–thaw	1.0	1.0 ± 0.1	99.8
	10.0	10.5 ± 1.0	104.6
	100.0	100.7 ± 5.3	100.7
At 4 °C, 10 h	1.0	1.0 ± 0.1	104.4
	10.0	9.9 ± 0.7	99.4
	100.0	98.9 ± 2.9	98.9
At −20 °C, 4 weeks	1.0	1.1 ± 0.10	109.4
	10.0	10.3 ± 0.8	102.6
	100.0	101.9 ± 5.6	101.9

[a] Remaining percentage (%) = (concentration found)/(concentration added) × 100; $n=5$.

4. Conclusion

A rapid, sensitive and accurate liquid chromatography with atmospheric pressure chemical ionization mass spectrometry was developed for the determination of triptolide in dog plasma. The method offers high sensitivity with a lower limit of quantitation of 0.5 ng/mL, wide linearity, and specificity without interferences from endogenous substances. In addition, the simplicity of sample preparation facilitates its application in the pharmacokinetic and toxicokinetic studies of triptolide.

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