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Article

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Determination of Carnosic Acid by a Novel HPLC-UV Method in Human Plasma and Application to a Prototype Pharmacokinetic Study

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An HPLC method with UV detection was developed for the determination of carnosic acid in human plasma and applied to a pharmacokinetic study after oral administration of Rosemary extract to a healthy volunteer. Sample preparation depends on liquid–liquid extraction with hexane. Chromatographic separation was achieved with C18 column (150 mm × 4.6 mm × 5 μm), at 25°C with isocratic elution, mobile phase composed of solution A (methanol), and solution B (2% o-phosphoric acid in water) (90:10, v/v) at flow rate of 1.0 mL/min. The analyte was detected at 230 nm. The retention time is 4.20 ± 0.03 min. The method was validated in terms of accuracy, precision, specificity, robustness and detection and quantification limits, in accordance with European Medicines Agency guidelines. LOD and LOQ were found to be 0.075 and 0.25 ng/mL, respectively. The method was applied to the analysis of carnosic acid in human plasma with good recovery as 91.7%. The plasma concentration-time profile and pharmacokinetic parameters: AUC_{0-t}, $AUC_{0-\infty}$, C_{max}, t_{max}, $t_{1/2}$ were calculated according to the assays. The method can certainly be used for routine analysis of carnosic acid in human plasma after oral administration of Rosemary extract, and for phase I clinical studies and bioavailability-bioequivalance studies as well.

Introduction

Secondary metabolites play a key role in the discovery of novel drugs. Their original forms or semisynthetic derivatives may be used as drug active substances (1). Rosemary (*Rosmarinus officinalis* L.) is one of the most preferred natural sources for this kind of compounds (2–4). *R. officinalis* L. belongs to the Lamiaceae family, which is native to the Mediterranean region (5). Many studies have shown that Rosemary extract has antibacterial, antioxidant, antidiabetic, antitumor, anti-inflammatory and antinociceptive activities (6–13). Carnosic acid (CA) is an important ingredient to provide those therapeutic effects.

Constituents like carnosol, CA and rosmanol are the main active ingredients of this plant. CA with the chemical name (4aR,10aS) - 5,6- Dihydroxy -7- isopropyl -1,1- dimethyl - 1,3,4,9,10,10a – hexahydro - 2H - phenanthrene - 4a carboxylic acid (Figure 1) is located in chloroplasts and intracellular membranes as a phenolic diterpene considered to the most important antioxidant molecule in sage (*Salvia officinalis* L.) and Rosemary (*R. officinalis* L.) extracts (14–17). However, it is a quite labile molecule in hydrophilic media, which is shortly stable in the solvents in which it can be isolated or analysed, and its degradation is enhanced by light or high temperatures (18, 19).

There are only a few analytical techniques for the quantitative determination of CA in different samples (natural sources, *Rosemary* and *Salvia* extracts, food matrices, rat plasma, human plasma) and most of them are based on chromatographic methods and two of them on capillary electrophoresis with various detection combinations. There are

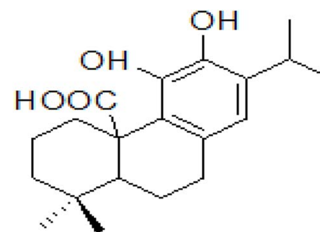

Figure 1. Chemical structure of CA.

only two methods in the literature that describe pharmacokinetics of CA and provide plasma analysis, but these studies were carried out on rat plasma samples (20, 21). The other methods that provide the quantitation of CA in Rosemary extracts used HPLC with electrochemical detection (22, 23): HPLC with evaporative light scattering detection (HPLC-ELSD) (24), UPLC with mass spectrometric detection (25), isoelectric focused adsorptive bubble chromatography (26), CZE with UV detection (27–29) and voltammetry (30). In the literature, there is a method that provides qualitative analysis of CA by using semi-preparative HPLC for isolation and NMR, MS and IR for identification (31). Additionally, an HPLC-PDA method describes determination of CA in food products (32). Although CA is widely preferred by healthcare professionals, patients and the community, there is no method for its determination in human plasma, it is only available for carnosol (33). In this study, it is aimed to develop and validate an HPLC-UV method in order to determine CA in human plasma and apply the method to a prototype pharmacokinetic

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study. The analysis for pharmacokinetic research was carried out, by the approval of the ethic commitee, with the plasma samples of the healthy volunteer after oral administration of Rosemary extract.

Materials and methods

Chemicals and reagents

CA was obtained from Sigma Aldrich (St Louis, USA). Methanol (HPLC grade) and orthophosphoric acid, hexan were supplied from Merck (Darmstadt, Germany). Water was purified by Human (Japan) ultrawater purification system.

Solutions

A stock solution of CA (0.1 μg/mL) was prepared and diluted with water to give standard solutions from 0.25 to 8 ng/mL. Prior to measurements, stock solutions were diluted with ethanol to prepare the working standard solutions in various concentrations. 20-μL aliquots of the working standard solutions were used for HPLC analysis. Depending on the peak areas versus concentration of CA, the chromatograms were annotated.

Instrumentation and chromatographic conditions

The HPLC analyses were performed on a Shimadzu (Japan) liquid chromatograph which consisted of an LC-20AT pump, SIL AT-HT autosampler part, an SPD-20A HT UV spectrophotometric detector, which was set at 230 nm and CTO 10 AC column oven were used. Various mobile phase, column types and stationary phase size combinations were trialed with different flow rates and column temperatures so as to provide the most efficient chromatographic seperation. Chromatographic separation was achieved isocratically at 25°C on a GL Sciences (Japan) C18 (ODS) column with the dimensions; 4.6-mm I.D, 150-mm length and 5-μm particle size. The mobile phase A consists of methanol and mobile phase B is 2% o-phosphoric acid in water, a flow rate of 1.0 mL/min. The prep-HPLC application was carried out with a Waters (USA) 2998 prep-LC system with PDA detection and Zorbax C18 column. LC-ESI-HRMS/MS system was 1290 series, Agilent Technologies, Santa Clara, CA (USA)m including Agilent ZORBAX Eclipse XDB phenyl column.

Sample preparation and general procedure

5-mL venous blood samples were collected from peripheral veins of a volunteer (informed consent from was obtained according to ethical commitee approval) into tubes containing disodium EDTA and centrifuged at 4500 g for 10 min. The resultant plasma samples were stored at –20°C. To extract CA from the human plasma samples different extraction techniques such as liquid–liquid extraction with various extraction solvents (dichloromethane, chloroform, hexane, diethylether) and solid phase extraction with various cartridge type (C8, C18 cartridges) and lenghts with (100-mg bed mass and 3-mL volume) were tested. The extraction with SPE trails were between 72.6 and 88.3%. Liquid–liquid extraction method was applied to perform the extraction of CA in order to prevent possible interferences from plasma. To extract CA from the plasma samples, the 0.5-mL plasma sample was extracted by adding 5 mL of hexane. The contents were mixed by a vortex mixer at moderate speed for 5 min and centrifuged at 4000 g for 5 min. The aqueous layer was discarded. The organic layer was evaporated to dryness under a stream of nitrogen at 40°C. To the residue, 1-mL ethanol solution was added. The solution was mixed with a vortex mixer for 30 s and 20 μL of the derivatized sample was injected to the HPLC system.

Table I. Chromatographic System Suitability Parameters

capacity factor*	resolution*	HETP*	tailing factor*	asymmetry factor*
6.51	2.4	1325.2	1.2	0.8

*mean values of the parameters of all the points in calibration study are mentioned

Results

Chromatographic procedure

Preliminary experiments were carried out to achieve the most efficient chromatographic conditions. Different types of columns were tested at various temperatures. Maximum absorption of CA was observed at 230 nm by UV spectrophotometer and chromatographic separation was performed by isocratic elution at room temperature on a GL Sciences (Japan) C18 (ODS) column with the dimensions (150 mm × 4.6 mm × 5 μm), with methanol and water (2% o-phosphoric acid) (90:10, v/v) containing with a flow rate of 1.0 mL/min. The retention time of CA under these conditions is 4.20 ± 0.03.

Peak areas and resolution values were measured to find the optimal conditions. Representative chromatograms of blank solution, standard solution, spiked plasma and volunteer's plasma sample at $t_{\max}$ are shown in Figure 2(a–e). The system suitability parameters of the method are indicated in Table I, which shows the quality of the chromatographic separation process.

Validation of the method

Validation of the method was carried out according to the following parameters accoring to the principles of the European Medicines Agency (34).

Linearity and sensitivity

The linearity of the method was evaluated by a calibration curve in the range of 0.25–8 ng/mL ($n = 5$). Calibration curves were prepared by the analysis of 5-mL plasma samples spiked with various volumes of each working standard solution. The samples were then submitted to the process of extraction and chromatographic separation described above. Calibration curves were obtained using linear least-squares regression analysis by plotting of peak areas of the derivative, versus the corresponding CA concentrations. The equation of the calibration curve ($n = 5$) obtained from seven points was as follows: $y = 1149.7x + 325.91$ (correlation coefficient = 0.9906), where y shows the peak areas and x indicated the concentrations of CA in ng/mL.

The limit of detection (LOD) and limit of quantitation (LOQ) were determined using the formula: LOD or LOQ = kSDa/b, where $k = 3$ for LOD and 10 for LOQ, SDa is the standard deviation of the intercept, and b is the slope. The parameters for the analytical performance of the proposed method are summarized in Table II.

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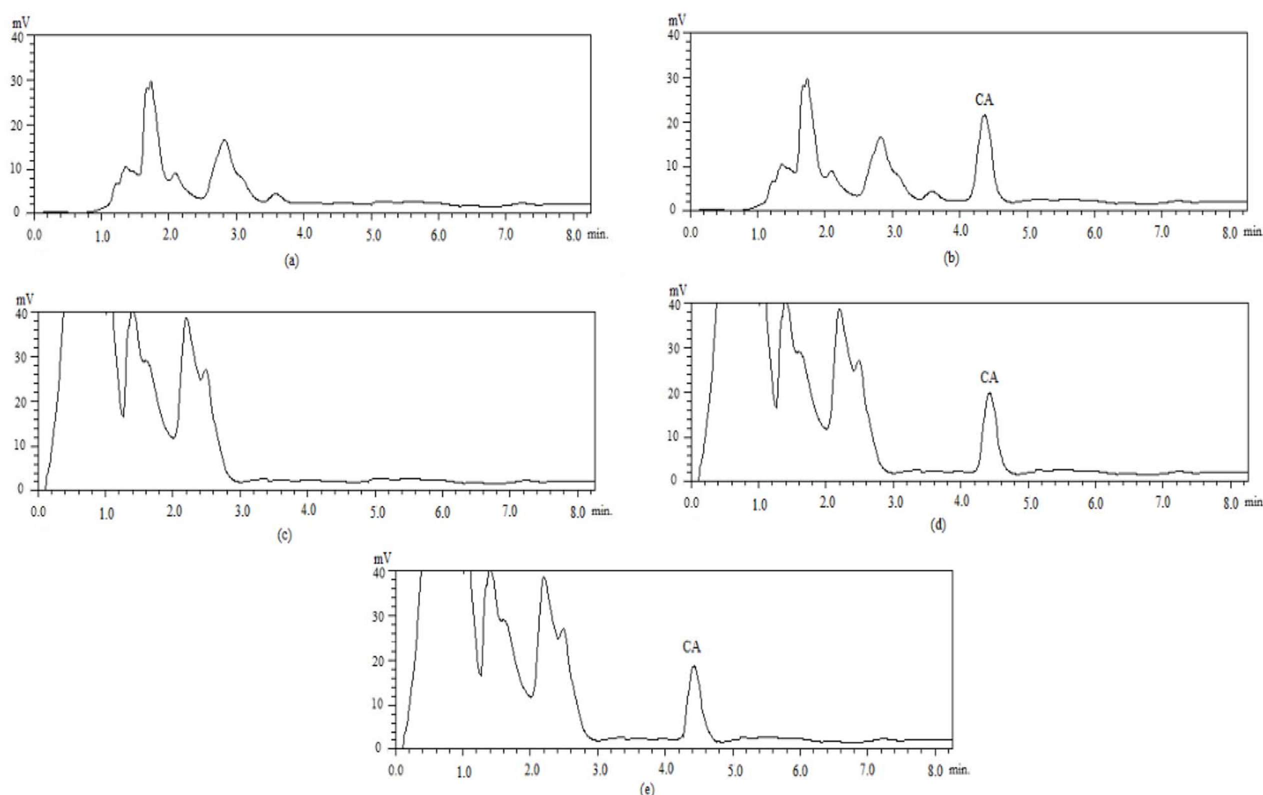

Figure 2. (a) blank (aqueous medium), (b) standard solution (2 ng/mL standard CA solution), (c) blank plasma sample, (d) 2 ng/mL CA spiked to plasma, (e) plasma sample of volunteer after t_{max}.

Table II. Analytical Parameters of the Method

Parameters	Method
Concentration range[a] (ng mL^{-1})	0.25–8
Regression equation[b]	
Intercept± SD	925.91 ± 88.38
Slope ± SD	1149.70 ± 18.05
Correlation coefficient (r^2)	0.9906
LOD (ng mL^{-1})	0.075
LOQ (ng mL^{-1})	0.25

[a] Average of six determinatons [b] $y = xC + b$ where C is the concentration in ng mL^{-1} and y is the peak area

Accuracy, precision and recovery

Accuracy and precision were assessed by determination of the QC samples at three concentration levels. QC samples at three different concentrations (0.75, 2 and 5 ng mL^{-1}) that can be classified as low, medium and high concentrations ($n = 3$) in human plasma were prepared. The accuracy was expressed by recovery values and RME and the precision by RSD. The absolute recovery of CA from human plasma was examined by extraction of spiked human samples and comparison with peak areas obtained the same amounts of aqueous CA solutions. The mean absolute recovery of CA was 95.95%. The relative recovery was calculated as 91.77% by the comparison of the amounts that is added on to spiked and measured by the calibration curve.

Three replicates of samples at each concentration were assayed on the same day for intraday and on three different days for interday precision and accuracy. The RSD values of both intraday and interday assays were all less than 5.13%. According to all these results summarized in Table III, good precision and accuracy were observed.

Robustness

Robustness was assessed by determination of the QC samples at three concentration levels as described at validation section above ($n = 3$). The parameters, that are changed to measure the robustness of the method, are flow-rate, column oven temperature, methanol and aqueous phase contents of the mobile phase. The mobile phase proportions were changed from 90:10 h/h (methanol–water) to 95:5 and 85:15 and flow rate was changed from 1.0 to 0.8 and 1.2 mL/min and the column temperature from 25°C was changed to 20°C and 30°C. These changes had no significant effect on peak areas and resolution values. The recovery values indicate the robustness of the method (Table IV).

Stability

The stability of working standard CA solutions was tested at several storage conditions at QC levels, as three replicates. The trialed storage conditions are keeping at dark and at room temperature for 24 h, keeping in autosampler conditions for 24 h and keeping in refrigerated at 4°C for 1 month. Recovery values for the trialed conditions are 95.7, 98.3 and 96.4%, respectively. The highest RSD % for all these experiments was 5.12%. It is possible to mention under all tested conditions CA were found to be stable.

LC-ESI-HRMS/MS optimization

The LC-ESI-HRMS/MS system (1290 series, Agilent Technologies, Santa Clara, CA, USA) consisted of an automatic

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Table III. Accuracy and Precision of the Method

Existant concentration (ng mL^{-1})	Added concentration (ng mL^{-1})	Found concentration (ng mL^{-1}) (Mean ± SD[1])	Recovery (%)	RSD % of recovery	RSD % of intraday variation	RSD % of interday variation
2	0.75	2.8 ± 0.0577	101.87	2.07	3.9	5.13
	2.00	3.10 ± 0.1069	77.50	3.45	1.75	3.17
	5.00	6.71 ± 0.4000	95.95	5.95	2.75	4.6
	Mean relative recovery = 91.77					

For each concentration n = 3

Table IV. Robustness of the Method

Condition	Value	Recovery %	RSD %
Flow rate mL min^{-1}	0.8	111.46	8.64
	1.2	112	3.50
Mobile phase composition	95:5	97.93	0.97
(methanol:aqueous phase)	85:15	101.2	2.17
Column temperature	20	97	0.36
	30	96.8	0.55

n = 3 for all QC sample levels

degasser, an auto-sampler and a quaternary pump, and was equipped with an column (Agilent ZORBAX Eclipse XDB-Phenyl 4.6 mm × 75 mm, 3.5 μm) with a isocratic system consisting of mobile phase solution A (0.1% formic acid in H_2O) and solution B (0.1% formic acid in ACN) at a flow rate 0.6 mL/min. The column temperature was set at 30°C. Each injection operated for 10 min and the injection volume was 10 μL. The detection was performed on a 6430 triple-quadrupole mass spectrometer (Agilent). The ESI source, negative ionization mode and multiple-reaction monitoring technique were employed. An Agilent Mass Hunter workstation was used to control the equipment and for the data acquisition and analysis. The precursor ion of CA was at *m/z* 331.19144 Da $[M-H]^+$, and the product ion peak at *m/z* 287.20182 Da was attributable to the typical CO_2 loss (44 Da). The precursor ion of CA was at *m/z* 331.19144 Da $[M-H]^+$, and the product ion peak at *m/z* 267.16721 Da was attributable to the typical C_5H_4 loss (64 Da). Product ion mass spectra of CA are exhibited in Figure 3. CA, ESI-HRMS: chemical formula ($C_{20}H_{28}O_4$), exact mass = 330.18311, calculated *m/z* $[M-H]^+$ = 329.17528, experimental *m/z* $[M-H]^+$ = 329.17609.

Application of the method to pharmacokinetic analysis

The proposed method was applied to the determination of CA in plasma for the pharmacokinetic studies. A healthy 27-year-old male volunteer was administered a single oral dose of Rosemary extract containing at least 10% CA (1000 mg). Approximately, 5-mL venous blood samples were collected prior to dosage and 0.25, 0.5, 0.75, 1, 2, 3, 4, 5, 6 h after administration. For the following days, blood samples were collected once a day for 10 days. The blood samples were processed to plasma as described above. Figure 2(e) shows a chromatogram of the plasma sample of the volunteer obtained 1 h after administration single oral dose of 1000-mg Rosemary extract. The samples were stored at −20°C until analysis. Pharmacokinetic parameters were calculated by using the analysis carried out by the proposed method. Area under the plasma concentration-time curves (AUC_{0-6}, $AUC_{0-\infty}$) were calculated using the TOPFIT 2.0 pharmacokinetic and pharmacodynamic data analysis

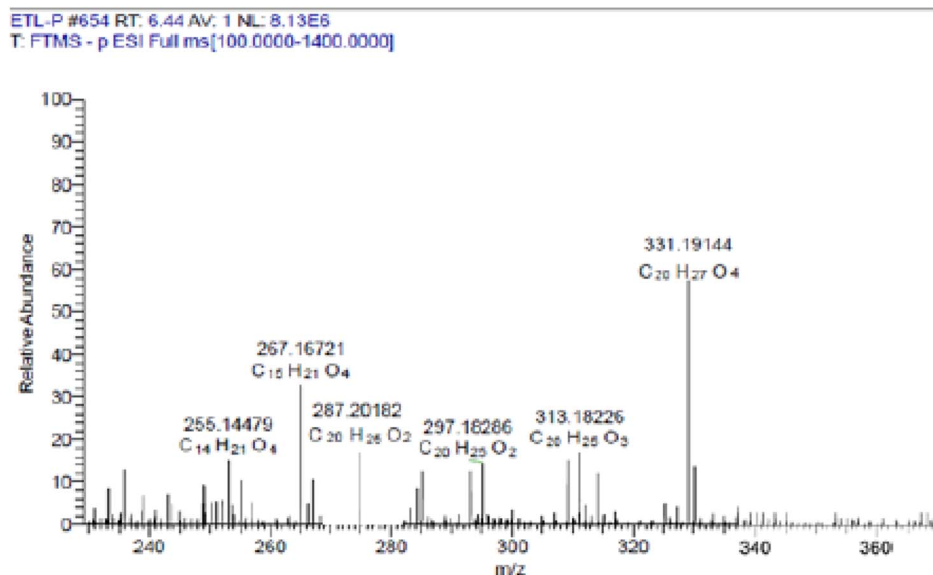

Figure 3. Product ion mass spectra of CA.

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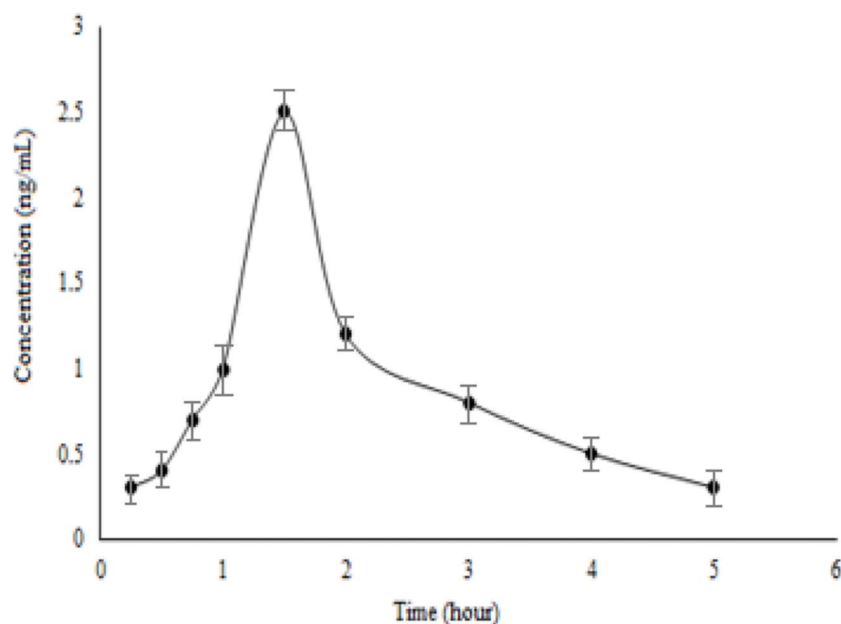

Figure 4. Pharmacokinetic curve of CA between 0 and 6 h after administration of 1000-mg oral dose of Rosemary extract.

Table V. Pharmacokinetic Parameters of CA after Administration of 1000 mg Rosemary Extract

Parameter	Found value
T_{max}[a] (h)	1
C_{max}[b] (ng mL^{-1})	2.27
$t_{1/2}$[c] (h)	6
AUC[d] $_{0\text{–}6}$ (ng h mL^{-1})	96.2
AUC[d] $_{0\text{-}\infty}$ (ng h mL^{-1})	154.6

[a]Time to maximum concentration, [b]Maximum concentration, [c]Elimination half life, [d]Area under the concentration-time curve

system (35). A plasma concentration-time curve of CA after an oral administration of a single dose of 1000 mg of Rosemary extract is shown in Figure 4. Pharmacokinetic parameters are given in Table V.

Discussion

CA is the most preferred diterpenoid found in Rosemary and Salvia extracts due to its therapeutic potential. There are only analytical methods of CA in herbal extracts, food products, nutraceuticals and rat plasma. There is not any published method that provides quantitation of CA in human plasma. The presented method provides sensitive determination of CA and clinical assays for such a widely used secondary metabolite. There is a lack of data for pharmacokinetic parameters of CA in order to evaluate its bioavailability, clinical effects and safety.

Conclusion

This presented study provides fast, simple, sensitive, selective and cost-reduced assay for CA in human plasma. It is applied to a pharmacokinetic study as a prototype research. The aim of this prototype study is to prove that it is possible to use the new analytical method for pharmacokinetic assays for CA by the participation of adequate number of volunteers for a phase I clinical study.

Supplementary Data

Supplementary data are available at *Journal of Chromatographic Science* online.

Funding

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Conflict of Interest statement

None declared.

Ethical approval

All procedure performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Bezmialem Vakıf University approved by the Clinical Trials Ethic Commitee (No: 24/23).

Author Contributions

All authors contributed to the work equally.

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