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Stereospecific high-performance liquid chromatographic analysis of naringenin in urine

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Abstract

A method of analysis of naringenin [(+/−)-4′,5,7-trihydroxyflavanone] in biological fluids is necessary to study the kinetics of in vitro and in vivo metabolism, tissue distribution in fruits and humans. A simple high-performance liquid chromatographic method was developed for simultaneous determination of naringenin enantiomers in rat and human urine. Urine (0.1 ml) was precipitated with cold acetonitrile after addition of the internal standard, daidzein. Separation was achieved on a Chiralcel OD-RH column with UV detection at 292 nm. The calibration curves were linear ranging from 0.5 to 100 μg/ml for each enantiomer. The mean extraction efficiency was >99%. Precision of the assay was <9.4% (CV), and was within 5.4% at the limit of quantitation (0.5 μg/ml). Bias of the assay was lower than 16%, and was within 15% at the limit of quantitation. The assay was applied successfully to the urinary excretion of naringenin in rats and humans.

Keywords: Reversed-phase HPLC; UV-detection; Stereospecific; Enantiomer; Flavonoid

1. Introduction

Naringin [(+/−)-4′,5,7-trihydroxyflavanone 7-rhamnoglucoside] (Fig. 1a) is a chiral flavanone-7-*O*-glycoside present in citrus fruits, tomatoes, cherries, oregano, beans, and cocoa [1–6]. After consumption, the neohesperidose sugar moiety is rapidly cleaved off the parent compound in the gastrointestinal tract and liver to leave the aglycone bioflavonoid naringenin [(+/−)-4′,5,7-trihydroxyflavanone] (Fig. 1b). The ratio between the amount of naringenin and naringin varies among different food products. For instance, citrus fruits contain higher amounts of the glycoside naringin, while tomatoes have higher amounts of the aglycone naringenin [3]. The proposed metabolism of naringenin in the gastrointestinal tract and liver is presented in Fig. 1.

Naringenin has been previously quantified utilizing a variety of methods including high-performance liquid chromatography with UV and photodiode-array detection [7–12], liquid chromatography coupled with mass spectrometry [13–16], gas chromatography coupled with mass spectrometry [17]. All of these methods have overlooked the fact that naringenin is a chiral compound. There are, however, a couple of reports demonstrating that micellar electrokinetic chromatography [18], and multidimensional liquid chromatography coupled with mass spectroscopy [15] can separate naringenin enantiomers. However, baseline resolution and separation was not evident [18], and quantification was not validated in biological matrices [15,18]. There is also a recent report showing the separation of the (−)-naringenin enantiomer using circular dichroism [19], however, the separated enantiomers were not applied to biological systems. There was a report by Geiser et al. in the Pittcon 2000 that a Chiralpak AD-RH under supercritical fluid chromatography (SFC) could separate the enantiomers of naringenin. In our laboratory using a Chiralpak AD-RH column with HPLC we failed to demonstrate baseline resolution for the analysis of naringenin in biological matrices.

Interestingly, the results from various scientific and epidemiological studies have suggested that tomato consump-

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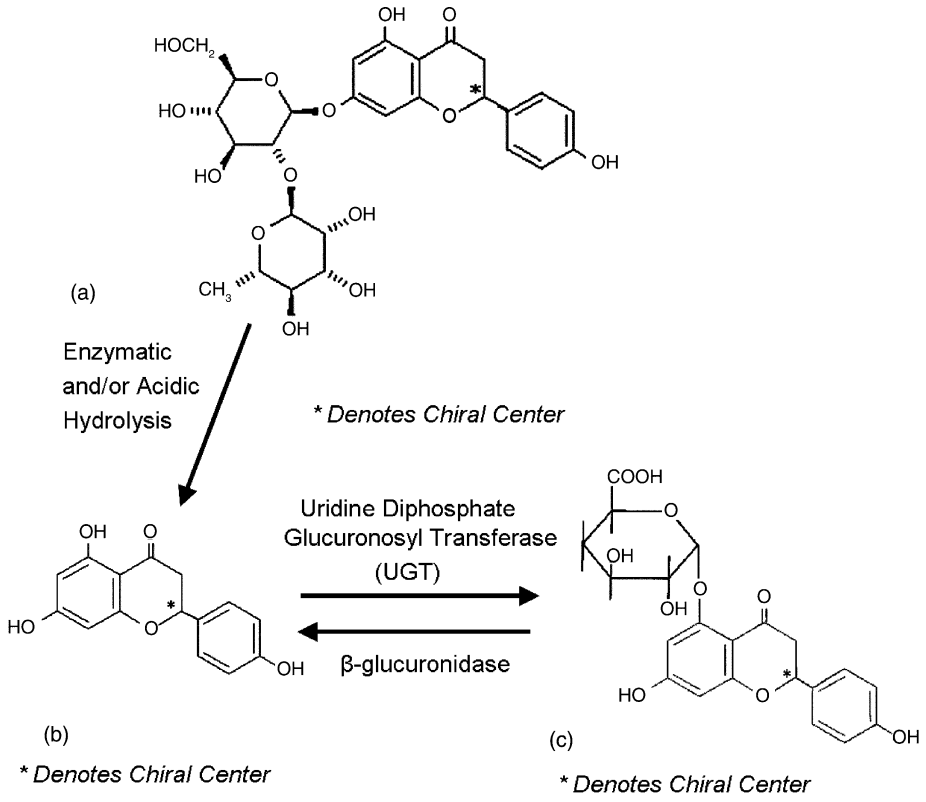

Fig. 1. Naringin metabolism. Structure of naringin (a), structure of naringenin (b), and structure of 5-*O*-naringenin glucoronidate (c). Asterisk (*) denotes Chiral Centre.

tion may prevent some chronic degenerative diseases, and have demonstrated that tomato and tomato-based product consumption may reduce the risk of different types of cancers [20]. Tomatoes are considered one of the most important sources of lycopene and it is generally thought that their potential anti-cancer properties are attributable to lycopene's antioxidant activity [20,21]. Although, plant phenols may contribute to the health protection ascribed to fruit and vegetable consumption, no studies of disposition of the enantiomers of naringenin in tomatoes or after tomato-based product intake have been published. Furthermore, a recent study showed that the co-administration of polyphenols enhances the antioxidants properties of lycopene. Therefore, it is possible that the tomato benefits could be attributed to a positive synergistic action between lycopene and other bioavailable tomato constituents, such as naringenin, rather than only lycopene [20]. Based on this, it is also possible that the tomato benefits could be attributed to one or both of the enantiomers of naringenin.

To our knowledge, no study has been published characterizing the separation of naringenin enantiomers in pharmacokinetic studies as there are no validated direct methods of stereospecific analysis of naringenin in the literature. The Chiralcel OD-RH column is a commercially available column, which can be utilized in the reverse phase. The present study describes a simple stereoselective, isocratic, reversed-phase high-performance liquid chromatography (HPLC) method for the determination of the enantiomers of naringenin and its application to in vivo kinetic studies.

2. Experimental

2.1. Chemicals and reagents

Racemic naringin, naringenin, daidzein, β-glucuronidase Type IX A and *H. pomatia* type-HP-2 were purchased from Sigma Chemicals (St. Louis, MO, USA). HPLC grade acetonitrile and water were purchased from J.T. Baker (Phillipsburg, NJ, USA). Phosphoric acid was from Aldrich Chemical Co. Inc. (Milwaukee, WI, USA). Campbell's Tomato Juice® was purchased from a local grocery. Rats were obtained from Charles River Laboratories. Ethics approval for animal experiments was obtained from Washington State University. Human experiments were conducted with written informed consent according to the principles of the Declaration of Helsinki.

2.2. Chromatographic system and conditions

The HPLC system used was a Shimadzu HPLC (Kyoto, Japan), consisting of an LC-10AT VP pump, a SIL-10AF auto

injector, a SPD-M10A VP spectrophotometric diodearray detector, and a SCL-10A VP system controller. Data collection and integration were accomplished using Shimadzu EZ Start 7.1.1 SP1 software (Kyoto, Japan). The analytical column used was Chiralcel OD-RH column (150 mm × 4.6 mm i.d., 5-μm particle size, Chiral Technologies Inc., Exton, PA, USA). The mobile phase consisted of acetonitrile, water and phosphoric acid (30:70:0.04, v/v/v), filtered and degassed under reduced pressure, prior to use. Separation was carried out isocratically at ambient temperature (25 ± 1 °C), and a flow rate of 0.4 ml/min, with ultraviolet (UV) detection at 292 nm.

2.3. Stock and working standard solutions

Twenty-five milligram of racemic naringenin was accurately weighed on an analytical balance (AG245, Mettler) and dissolved with methanol in a 25 ml volumetric flask to make a stock standard solution with a racemic concentration of 1 mg/ml. A methanolic stock solution of the internal standard (daidzein) was prepared similarly with a concentration of 1 mg/ml. This solution was diluted with methanol to make a working internal standard solution of 25 μg/ml. These solutions were protected from light and stored at −20 °C between uses, for no longer than 3 months. Calibration standards in urine were prepared daily from the stock solution of naringenin by sequential dilution with blank rat or human urine, yielding a series of concentrations namely, 0.5, 1.0, 5.0, 10.0, 50.0 and 100.0 μg/ml of each enantiomer in three replicates.

Quality control (QC) samples were prepared from the stock solution of naringenin by dilution with blank biological fluid to yield target concentrations of 0.5, 1.0, 5.0, 10.0, 50.0 and 100.0 μg/ml. The QC samples were divided into 0.1 ml aliquots in micro centrifuge tubes and stored at −70 °C before use.

2.4. Sample preparation

To the working standards or samples (0.1 ml), 25 μl of internal standard solution (25 μg/ml) and 1 ml of cold acetonitrile were added in 2.0 ml Eppendorf tubes. The mixture was vortexed for 1 min (Vortex Genie-2, VWR Scientific, West Chester, PA, USA), and centrifuged at 15000 rpm for 5 min (Beckman Microfuge centrifuge, Beckman Coulter Inc., Fullerton, CA, USA). The organic phase supernatant was collected into culture tubes (10 mm × 75 mm) and evaporated to dryness using a HetoVac concentrator (Heto-Holten, DK-3450 Allerød, Denmark). The residue was reconstituted with 200 μl of mobile phase, vortexed for 1 min and centrifuged at 5000 rpm for 5 min the supernatant was transferred to HPLC vials and 150 μl of the injected into the HPLC system.

2.5. Precision and accuracy

The within-run precision and accuracy of the replicate assays ($n = 6$) were tested by using six different concentrations of naringenin enantiomers, namely 0.5, 1.0, 5.0, 10.0, 50.0 and 100.0 μg/ml. The between-run precision and accuracy of the assays were estimated from the results of six replicate assays of QC samples on six different days within one week. The precision was evaluated by the relative standard deviation (R.S.D.). The accuracy was estimated based on the mean percentage error of measured concentration to the actual concentration [22].

2.6. Recovery

Recovery for naringenin enantiomers from biological fluids was assessed ($n = 6$) at 0.5, 1.0, 5.0, 10.0, 50.0 and 100 μg/ml and the recovery of the internal standard was evaluated at the concentration used in sample analysis (25 μg/ml). A known amount of naringenin or daidzein was spiked into 0.1 ml biological fluid to give the above concentrations. The samples were treated as described under Section 2.4 and analyzed by HPLC. The extraction efficiency was determined by comparing the peak areas of naringenin or daidzein to those of naringenin or daidzein solutions of corresponding concentration injected directly in the HPLC system without extraction.

2.7. Freeze-thaw and bench-top stability of naringenin samples

The freeze-thaw stability of naringenin enantiomers was evaluated at three concentrations 1.0, 5.0 and 50 μg/ml, using QC samples. These samples were analyzed in triplicate without being frozen at first, and then stored at −70 °C and thawed at room temperature (25 ± 1 °C) for three cycles.

The stability of naringenin in reconstituted extracts during run-time in the HPLC auto-injector was investigated using pooled extracts from QC samples of three concentration levels 1.0, 5.0, and 50.0 μg/ml. Samples were kept in the sample rack of the auto-injector and injected into HPLC system every 4 h, from 0 to 24 h at the temperature of auto-injector (26 ± 1 °C).

2.8. Urinary excretion of naringenin in human and rat

After three days of a citrus and tomato free diet and an overnight fast a healthy 22-year-old male subject (73 kg) drank two cans of Campbell Tomato Juice® (340 ml each). The naringin and naringenin enantiomer content in the tomato juice was quantified and it was determined that the total ingested dose by the healthy volunteer was 16.26 mg of *R*-naringin, 16.67 mg of *S*-naringin, 2.64 mg of *R*-naringenin and 2.68 mg *S*-naringenin. Urine was collected in acid washed container at intervals between 0 and 24 h post-dose and stored at −70 °C until analyzed. Naringin enantiomers were indirectly quantified by taking a 150 μl aliquot of tomato juice and subjecting it to 200 μl of *H. pomatia* Type-HP-2 enzyme in order for complete hydrolysis to the naringenin aglycone as previously described [23].

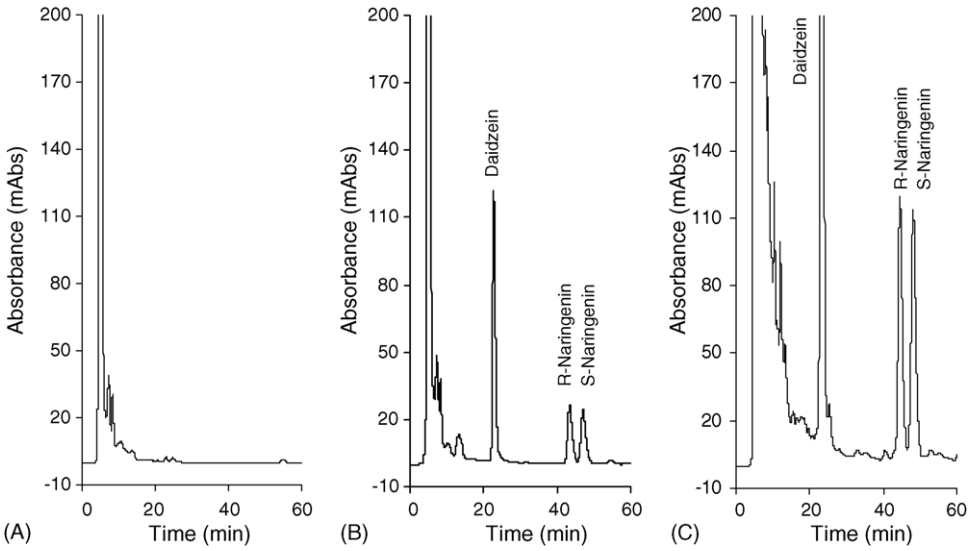

Fig. 2. Representative chromatograms, of (A) drug-free urine, (B) urine containing naringenin (N) enantiomers each with concentration of 10 μg/ml and the internal standard (IS), and (C) 8 h human urine sample containing naringenin enantiomers and the IS.

A male Sprague–Dawley rat (200 g) was placed in a metabolic cage, and fasted for 12 h before dosing. On the day of experiment, the rat was dosed orally with intravenous 20 mg/kg racemic naringenin in polyethylene glycol 400 via jugular cannulation. This dose of naringenin has previously been demonstrated in pharmacokinetic studies in rats [24,25] Urine was collected 0–4, 4–8, 8–24 h and stored at −70 °C until analysis. Urine samples (0.1 ml) were run in duplicate with or without the addition of 40 μl of 500 U/ml β-glucuronidase IX-A and incubated in a shaking water bath at 37 °C for 2 h to liberate any glucuronide conjugates [26].

2.9. Data analysis

Quantification was based on calibration curves constructed using peak area ratio (PAR) of naringenin to internal standard, against naringenin concentrations using unweighted least squares linear regression.

3. Results and discussion

3.1. Chromatography

Separation of naringenin enantiomers and the internal standard in biological fluids was achieved successfully. There were no interfering peaks co-eluted with the compounds of interest (Fig. 2A and C). The order of elution was determined by taking a 150 μl aliquot of grapefruit juice and subjecting it to 200 μl of *H. pomatia* type-HP-2 enzyme [23]. The predominant enantiomer of naringin in grapefruit juice is in the *S* configuration [15,27]. The retention times of *R*- and *S*-naringenin were approximately 43 and 47 min, respectively. The internal standard eluted at approximately 23 min (Fig. 2B).

The performance of the HPLC assay was assessed using the following parameters, namely peak shape and purity, interference from endogenous substances in biological fluid, linearity, limit of quantitation (LOQ), freeze-thaw stability, stability of reconstituted extracts, precision, accuracy and recovery. Various compositions of mobile phase were tested to achieve the best resolution between naringenin enantiomers.

The final mobile phase constitution is acetonitrile, water and phosphoric acid (30:70:0.04, v/v/v), this concentration was chosen because it was possible to attain a good separation. At the beginning the amounts of phosphoric acid and the ratio between acetonitrile and water were tested at different concentration ratios. For instance, increasing the amount of phosphoric acid up to 0.1% reduces the sharpness and resolution of the peaks, thus 0.04% was found to be ideal. It was also observed that increasing the polarity of the mobile phase (water) increases the retention time. Thus, by increasing the amount of acetonitrile the retention times were reduced. However, taken acetonitrile to higher concentrations than 30% will bring the peaks of the naringenin enantiomers too close to each other, and the internal standard too close to the front. The retention times of the analytes were very sensitive to small changes in mobile phase composition on the Chiralcel OD-RH column.

The present assay is practical to use in pre-clinical and clinical applications of naringenin where small sample volumes are obtained.

3.2. Linearity and LOQ

Excellent linear relationships ($r^2 = 0.999$) were demonstrated between PAR of *R*- and *S*-naringenin to the internal standard and the corresponding urine concentrations of naringenin enantiomers over a range of 0.5–100 μg/ml. The mean regression lines from the validation runs were described by

Table 1
Within- and between-day precision and accuracy of the assay for naringenin (N) enantiomers in rat urine ($n = 6$, mean, R.S.D., and bias)

Added	Observed				R.S.D. (%)				Bias (%)			
	Within-day		Between-day		Within-day		Between-day		Within-day		Between-day	
	R-N	*S*-N	*R*-N	*S*-N	*R*-N	*S*-N	*R*-N	*S*-N	*R*-N	*S*-N	*R*-N	*S*-N
Enantiomer concentration (μg/ml)												
0.5	0.54	0.56	0.57	0.52	6.13	0.52	2.27	14.92	8.87	12.74	13.27	4.03
1.0	1.06	1.09	1.07	1.03	6.26	7.53	0.68	9.35	6.35	9.35	6.86	2.57
5.0	5.08	5.12	4.81	4.88	2.64	3.19	4.67	5.28	1.55	2.33	−3.77	−2.42
10	10.31	10.35	10.18	10.27	1.69	1.97	1.60	1.46	3.14	3.55	1.81	2.75
50	48.95	48.63	50.97	50.81	0.52	0.64	2.91	2.87	−2.10	−2.74	1.93	1.63
100	100.53	100.62	99.49	99.65	0.07	0.16	0.67	0.66	0.53	0.62	−0.51	−0.35

R-naringenin (μg/ml) = 0.0439*x* + 0.0146 and *S*-naringenin (μg/ml) = 0.0432*x* + 0.0129.

The LOQ of this assay was 0.5 μg/ml in biological fluids with the corresponding between day relative standard deviation of 2.27 and 14.92% for *R*- and *S*-naringenin, respectively and bias of 13.27 and 4.03% for *R*- and *S*-naringenin, respectively. The back-calculated concentration of QC samples was within the acceptance criteria.

3.3. Precision, accuracy and recovery

The within- and between-run precision (R.S.D.) calculated during replicate assays ($n = 6$) of naringenin enantiomers in human urine was <15% over a wide range of concentrations (Table 1). The intra- and inter-run bias assessed during the replicate assays for naringenin enantiomers varied between −3.77 and 13.27% (Table 1). These data indicated that the developed HPLC method is reproducible and accurate. The mean extraction efficiency for naringenin enantiomers from biological fluids varied from 102.78 to 118.05% (Table 2). In addition, the recovery of daidzein was 98.5% at its concentration used in the assay. High recovery from biological fluids suggested that there was negligible loss of naringenin enantiomers and during the protein precipitation process. Additionally the efficiencies of extraction of naringenin enantiomers and daidzein were comparable.

3.4. Stability of naringenin samples

No significant degradation was detected after the samples of racemic naringenin in biological fluids following three freeze-thaw circles. The recoveries of *R*- and *S*-naringenin were respectively from 99.52 to 108.35% and 84.83 to 107.71% following three freeze-thaw cycles for naringenin QC samples of naringenin or daidzein. There was no significant decomposition observed after the reconstituted extracts of racemic naringenin were stored in the auto-injector at room temperature for 24 h. The measurements were from 98.49 to 98.86% of the initial value for extracts of racemic naringenin in biological fluids of 0.5, 1.0, 5.0, 10.0, 50.0 and 100.0 μg/ml, respectively, during the storage in the auto injector at room temperature for 24 h.

Table 2
Recovery of naringenin enantiomers from rat urine ($n = 6$)

Concentration (μg/ml)	Recovery (%) (mean ± S.D.)	
	R-naringenin	*S*-naringenin
0.5	117.4 ± 5.3	122.4 ± 3.8
1.0	118.1 ± 0.1	121.0 ± 0.1
5.0	106.8 ± 0.6	106.4 ± 0.5
10	107.8 ± 2.1	108.0 ± 2.3
50	109.9 ± 0.1	110.0 ± 0.1
100	103.3 ± 2.4	102.8 ± 2.4

3.5. Urinary excretion of naringenin in human

The HPLC method has been applied to the determination of naringenin enantiomers in the urinary excretion study in a human and rat. Naringenin has previously been demonstrated to be excreted into urine after consumption of tomato, orange and grapefruit in human and rat studies [23,28–30]. Following oral administration of tomato juice to a human and racemic naringenin to a rat, apparent enantioselective renal excretion was observed for naringenin (Figs. 3 and 4). Our laboratory has recently conducted pharmacokinetics studies in three different species after administration of naringenin, naringin, different citrus fruit juices and tomatoes. We have also examined the stereospecific concentrations of

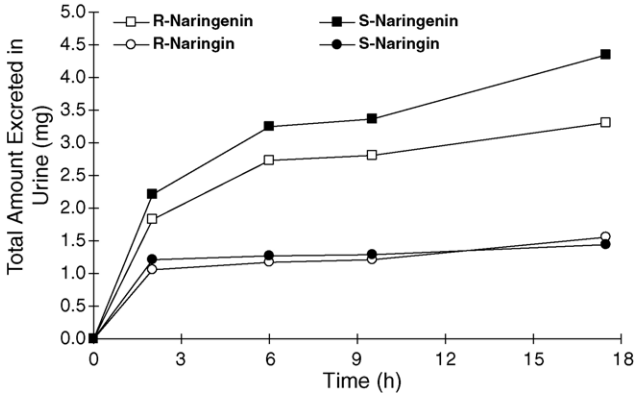

Fig. 3. Cumulative urinary excretion profile of naringenin and naringin enantiomers following the administration of tomato juice orally to a healthy volunteer.

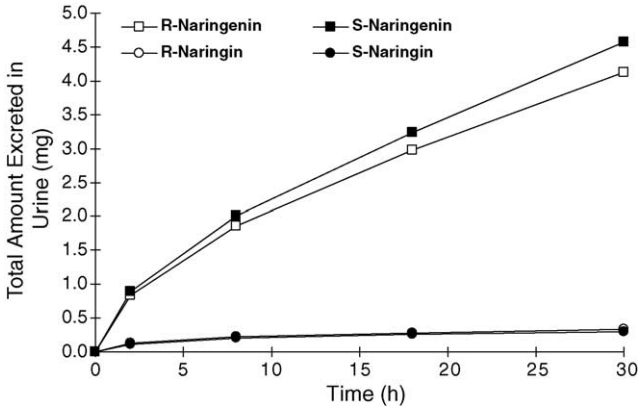

Fig. 4. Cumulative urinary excretion profile of naringenin and naringin enantiomers following the administration of racemic naringenin 20 mg/kg intravenously (i.v.) to a rat.

these flavonoids in various citrus fruit juices, and different genotypes of tomato (unpublished observations). Interestingly, it appears that just like the chiral non-steroidal anti-inflammatory drugs no two chiral flavonoids demonstrate similar stereoselective disposition patters and unique stereospecific methods need to be developed for all the members of this class [31–33].

In summary, the developed HPLC assay is stereospecific, reproducible and accurate. It has been successfully applied to a urinary excretion study of naringenin enantiomers in humans and rats.

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