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ORIGINAL RESEARCH

High-performance liquid chromatographic assay for glucuronidation activity of 7-ethyl-10-hydroxycamptothecin (SN-38), the active metabolite of irinotecan (CPT-11), in human liver microsomes

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ABSTRACT: A simple and sensitive assay for glucuronidation activity of 7-ethyl-10-hydroxycamptothecin (SN-38), the active metabolite of irinotecan (CPT-11), in human liver microsomes by high-performance liquid chromatography (HPLC) with fluorescence detection is reported. The method was validated for the determination of SN-38 glucuronide (SN-38G) with respect to specificity, linearity, recovery, stability, precision, accuracy, and limits of detection and quantitation. There was no interference from matrix and non-enzymatic reactions. The calibration curve for SN-38G was linear from 5 to 500 nM. Average recoveries ranged from 98 to 100% in spiked human liver microsome samples, and the SN-38G was stable at 4°C for at least 72 h. The newly developed method was found to be more sensitive and selective than previous methods using thin layer chromatography and HPLC. The limit of quantitation for SN-38G was 5 nM (2.5 pmol/assay). The intra- and inter-day precision and accuracy were less than 7 and 4%, respectively. The intra- and inter-day precision of enzyme assay for UDP-glucuronosyltransferase (UGT) activity toward SN-38 in human liver microsomes was less than 4%. With this improved sensitivity, the kinetics of SN-38 glucuronidation in human liver microsomes could be determined more precisely. Therefore, this method is applicable to *in vitro* study on the side effects and drug interactions of CPT-11 using small amounts of biological sample. Copyright © 2001 John Wiley & Sons, Ltd.

INTRODUCTION

Irinotecan (7-ethyl-10-[4-(1-piperidino)-1-piperidino]-carbonyloxycamptothecin, CPT-11) (Fig. 1) is a water-soluble analog of camptothecin used in the second-line treatment of advanced colon cancer (Sawada *et al.*, 1991; Wiseman and Markham, 1996). CPT-11 inhibits topoisomerase I activity through the formation of stable topoisomerase I-DNA cleavable complexes (Creemers *et al.*, 1994; Vassal *et al.*, 1996). CPT-11 is metabolized by carboxylesterases in intestinal mucosa, plasma and liver to an active metabolite, 7-ethyl-10-hydroxycamptothecin (SN-38) (Fig. 1), which has a 100–1000-fold higher antitumor activity than CPT-11 (Kawato *et al.*, 1991a,b). SN-38 is further conjugated by hepatic UDP-glucuronosyltransferases (UGTs, EC 2.4.1.17) to an inactive β-glucuronide derivative (SN-38G) (Fig. 1), and excreted into bile and urine (Atsumi *et al.*, 1991; Rivory and Robert, 1995; Haaz *et al.*, 1997; Iyer *et al.*, 1998).

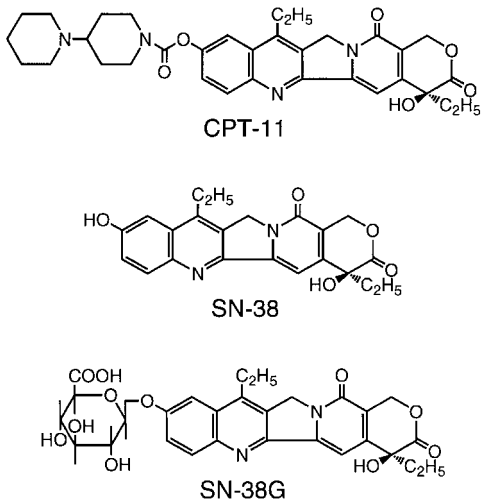

Figure 1. Chemical structures of CPT-11, SN-38 and SN-38G.

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Abbreviations used: CPT-11, 7-ethyl-10-[4-(1-piperidino)-1-piperidino]carbonyloxycamptothecine; SN-38, 7-ethyl-10-hydroxycamptothecin; UGTs, UDP-glucuronosyltransferases.

38G can be deconjugated to form SN-38 by intestinal bacterial β-glucuronidase (Kaneda *et al.*, 1990; Takasuna *et al.*, 1996).

The major side effect of CPT-11 is severe diarrhea, and it has been suggested that the toxicity is due to an accumulation of SN-38 in the intestine (Araki *et al.*, 1993). Furthermore, SN-38 glucuronidation rates closely correlate with the severity of diarrhea in patients treated with increasing doses of CPT-11 (Gupta *et al.*, 1994). These findings indicate that glucuronidation of SN-38 protects against the side effects of CPT-11. Thus, the conjugation of SN-38 to SN-38G by hepatic UGTs is a critical step in the sequential metabolic pathway of CPT-11. UGTs are important detoxication enzymes of drug metabolism that convert a wide variety of xenobiotics and endobiotic lipid-soluble substrates into water-soluble and excretable compounds, and are mainly associated with the endoplasmic reticulum of hepatocytes (Dutton, 1980; Burchell and Coughtrie, 1989). To date, about 40 UGT isoforms have been identified from various mammalian species by protein purification or cDNA cloning, and classified into two families of protein termed UGT1 and UGT2 on the basis of amino acid sequence similarities and gene structure (Mackenzie *et al.*, 1997; Burchell *et al.*, 1998; de Wildt *et al.*, 1999).

Recently, the UGT isoforms involved in SN-38 glucuronidation have been broadly identified using recominant human UGTs expressed in mammalian cell lines (Iyer *et al.*, 1998; Ciotti *et al.*, 1999). Iyer *et al.* (1998) measured SN-38 glucuronidation activity in human liver microsomes and expressed UGTs by HPLC. However, the method requires a concentration step to detect SN-38G, and the estimation of activity is represented as the peak height ratio of SN-38G to internal standard (camptothecin). On the other hand, Ciotti *et al.* (1999) reported a thin layer chromatographic method to measured radioactivity of metabolite from SN-38 in cDNA-expressed human UGTs using [^{14}C]UDP-glucuronic acid as donor; however, the background originating from the radiolabeled chemical interferes with the determination of UGT activity. Since these are indirect methods, they do not allow for the determination of low activity samples. Furthermore, the limit of detection or quantitation is not reported. Recently, a method for measuring CPT-11 and its metabolites in rat plasma (*in vivo* system) by HPLC has been developed, but it is not applicable to *in vitro* systems (Kurita and Kaneda, 1999). Several genetic polymorphisms have been identified in the UGT family (Mackenzie *et al.*, 1997; Burchell *et al.*, 1998; de Wildt *et al.*, 1999), and development of a quantitative assay for UGT activity toward SN-38 in human tissues (*in vitro* system) is an important aspect of clinical research.

The present paper describes a simple and sensitive method for the assay of SN-38 glucuronidation activity in human liver microsomes by HPLC with fluorescence detection. This method was validated with respect to specificity, linearity, recovery, stability, precision, accuracy and limits of detection and quantitation.

EXPERIMENTAL

Chemicals and materials. SN-38 (Lot 970507R) and SN-38G (Lot 970326) were kindly supplied by Yakult Honsha Co. (Tokyo, Japan). The purity (≥99%) of each compound was confirmed by analytical HPLC. UDP-glucuronic acid (trisodium salt) was obtained from Yamasa Shoyu Co. (Choshi, Japan). Sodium 1-octanesulfonate was obtained from Wako Pure Chemical Industries (Osaka, Japan). All other chemicals and HPLC solvents were of the highest quality commercially available. Pooled liver microsomes from 10 human donors (16–73 years old) were purchased from Gentest (Woburn, MA, USA). The microsomal protein content was determined according to Lowry *et al.* (1951) using bovine serum albumin as standard. The microsomal samples were stored at −80°C until use.

Standards. A stock solution (2.5 mM) of SN-38G was prepared by dissolving 7.11 mg of SN-38G in 5 mL of methanol. Stock solutions (0.25–25 μM) for calibration curves and quality control samples were prepared by the serial dilution of the 2.5 mM stock solution with methanol. These solutions were stored at −20°C protected from light and were stable for at least 4 weeks. Working solutions were freshly prepared each day and were obtained by dilution from the stock solutions with 50 mM Tris–HCl buffer (pH 7.4)–10% $HClO_4$ (w/v) (80:20, v/v) or the solution containing liver microsomes (100 μg protein/mL).

Incubations. SN-38 glucuronidation activity was evaluated by quantitation of the SN-38G production in human liver microsomes. Incubations were performed in 5 mL glass test tubes. The standard incubation mixture contained SN-38 (2.5–150 μM) as substrate, human liver microsomes (0–400 μg protein/mL), 10 mM $MgCl_2$ and 5 mM UDP-glucuronic acid in a final volume of 400 μL of 50 mM Tris–HCl buffer (pH 7.4). SN-38 was dissolved in dimethyl sulfoxide/0.05 N NaOH (50:50). The final concentration of dimethyl sulfoxide in the reaction medium was 0.5% (v/v), and the pH was checked for verification. After preincubation at 37°C for 1 min, the reaction was started by the addition of UDP–glucuronic acid. The mixture was incubated at 37°C for 0–80 min and the reaction terminated with 100 μL of 10% (w/v) $HClO_4$ with vortexing. Deproteined samples were placed on ice for 30 min. The contents of the tubes were transferred to 1.5 mL polypropylene test tubes and centrifuged at 12,000 ***g*** for 10 min at 4°C. The supernatant was filtered with a PTFE membrane filter of 0.45 μm pore size (Millipore, Bedford, MA, USA) and analyzed by HPLC within 8 h. Blank samples contained all components except the UDP-glucuronic acid, which was added after termination of the reaction.

Apparatus and HPLC conditions. HPLC analysis was performed using a Shimadzu SCL-10A system controller (Kyoto, Japan) consisting of three LC-10AD pumps, an SIL-10A auto injector with sample cooler, an RF-10A fluorescence detector, a CTO-10A column oven, a DGU-3A degasser and a C-R7A chromatopac integrator. The samples were cooled at 4°C, and

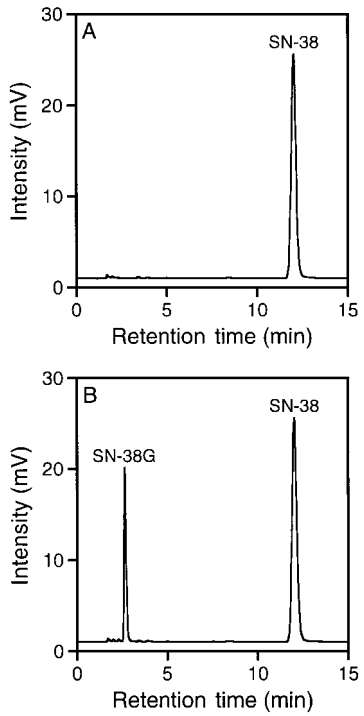

Figure 2. HPLC analysis of SN-38 glucuronidation activity in human liver microsomes. Reactions were performed in the presence of SN-38 (100 μM) and liver microsomes (100 μg protein/mL) in a total volume of 400 μL for 20 min. The method of sample preparation and the HPLC conditions are described in the Experimental section. (A) Blank sample; (B) incubation sample.

10 μL aliquots were injected into an Inertsil ODS-80A column (5 μm, 150 × 4.6 mm i.d., GL Sciences, Tokyo, Japan). The column was kept at 40°C. The product was eluted isocratically with 50 mM KH_2PO_4 containing 3 mM sodium 1-octanesulfonate–acetonitrile–methanol (72:22:6, v/v/v) at a flow rate of 1.0 mL/min. The pH of the aqueous portion was adjusted to 2.5 with 60% $HClO_4$. The excitation and emission wavelengths were fixed at 370 and 425 nm, respectively.

Method validation. A calibration curve was prepared at seven concentrations of SN-38G (5, 10, 20, 50, 100, 200 and 500 nM), and the linear regression line was constructed by plotting peak area of analyte against concentration. The intra- and inter-day precision and accuracy of the method were assessed by performing repeated analyses of SN-38G in quality control samples of three concentrations (5, 50 and 500 nM). Concentrations in quality samples were calculated from the resulting peak areas and the regression equation of the calibration curve. The intra-day precision and accuracy were determined by analyzing five times in triplicate on the same day. The inter-day precision and accuracy were determined by analyzing once in triplicate daily for 5 days. The precision was given by relative standard deviation (RSD), while the accuracy was calculated as relative mean error (RME) of back-calculated concentrations from nominal concentrations. An RSD and RME less than 10% were considered acceptable. The limit of quantitation was defined as the lowest concentration of SN-38G determined with acceptable precision and accuracy under the stated experimental conditions. It corresponds to the sample concentration of SN-38G resulting in a peak area of 10 times the noise level. The intra- and inter-day precisons of the assay for UGT activity toward SN-38 in human liver microsomes were also assessed at three substrate concentrations (5, 30 and 100 μM). Three samples were analyzed five times for the intra-day precision and once in triplicate daily for 5 days for the inter-day precision. The recovery of SN-38G from human liver microsomes was evaluated by comparing the areas of pure standards with those of quality control samples containing the same concentrations of standards (5, 50 and 500 nM) in pentuplicate. The specificity of the method was assessed to evaluate the influence of the matrix and non-enzymatic reactions in blank samples. The stability of SN-38G in quality control samples at concentrations of 5, 50 and 500 nM containing human liver microsomes was assessed after storage in a refrigerator (4°C) and at room temperature (27°C) for various intervals (8, 24 and 72 h) in pentuplicate, by comparing peak areas from initial and subsequent determinations.

Kinetic analysis. Incubation conditions were chosen such that product formation was linear with respect to both microsomal protein concentration and incubation time for the determination of SN-38 glucuronidation activity in human liver microsomes. The microsomal protein concentration and incubation time were 100 μg/mL and 20 min, respectively. Substrate concentration was 2.5–150 μM. Kinetic parameters such as K_m and V_{max} were estimated using the software EnzymeKinetics version 1.4 (Trinity Software, Campton, NH, USA) desined for nonlinear regression analysis of a hyperbolic Michaelis–Menten equation. Three separate experiments were performed in triplicate. Similar results were obtained in all experiments.

RESULTS AND DISCUSSION

Specificity

Human liver microsomes were incubated with SN-38 in the presence of UDP-glucuronic acid and the amount of SN-38G formed was determined by HPLC (Fig. 2). SN-38G was detected with a retention time of 2.7 min. Unconjugated SN-38 was eluted in 12.0 min. The interfering peaks for the determination of SN-38G were not detected in blank samples from human liver microsomes. When the HPLC conditions of Kurita and Kaneda (1999) were employed, the interfering peak originating from matrix or non-enzymatic reactions was found at a retention time of SN-38G (data not shown).

Linearity and limit of quantitation

A calibration curve ($y = ax + b$) was prepared from a solution to which SN-38G had been added in the range of 5–500 nM. A high correlation was found between the amount of SN-38G (x: nM), and the peak area (y: peak area/1000; Table 1). The means of the difference between the true and back-calculated concentration of the standards for intra- and inter-assay were 1.5 and 1.2%

Table 1. Calibration curve for the determination of SN-38G

	Linear regression equation ($y = ax + b$) (mean ± SD)		
	Slope (a)	Intercept (b)	Correlation (r^2)
Intra-day ($n = 5$)	0.7969 ± 0.0040	0.4055 ± 0.2635	0.9999 ± 0.0001
Inter-day ($n = 5$)	0.8043 ± 0.0116	0.7170 ± 0.2923	0.9998 ± 0.0003

SN-38G dissolved in methanol was diluted with 50 mM Tris–HCl buffer (pH 7.4) in a total volume of 400 μL and then 100 μL of 10% $HClO_4$ was added. The HPLC conditions are described in the Experimental section.

(range 0.2–9.5 and 0.1–7.4%), respectively; this indicates the suitability of the calibration model. The criteria used to estimate the limit of quantitation were maximal intra- and inter-day variation in precision and accuracy of 10%. The limit of quantitation for SN-38G was estimated to be 5 nM (approximately 2.8 ng/mL). This value was consistent with that in a previous study on measuring CPT-11 and its metabolites in plasma (Kurita and Kaneda, 1999).

Recovery and stability

The recoveries from human liver microsomes (100 μg protein/mL) spiked with SN-38G at concentrations of 5, 50 and 500 nM were 98.5 ± 1.5, 99.8 ± 1.3 and 98.9 ± 1.8%, respectively. When the enzyme reactions were stopped with 100 μL of 10% (w/v) trichloroacetic acid or 100 μL of 10% (w/v) zinc sulfate in a preliminary study, the recovery of SN-38G from liver microsomes was only 59–72% (data not shown). To check the stability of the biological matrix, human liver microsomes were spiked with SN-38G separately at three different concentrations. The results are shown in Table 2. SN-38G was stable for 72 h at 4°C at all concentrations. After storage for 72 h at 27°C, SN-38G was slightly degraded at all concentrations, although the stability was retained for 24 h.

Reproducibility

The quality control samples were prepared by spiking human liver microsomes with SN-38G at three different concentrations, and were analyzed with the calibration standards as described above. The precision was better than 7% and the accuracy did not exceed 4% at all concentrations of quality control samples (Table 3). As shown in Table 4, the intra- and inter-day precision of the assay for UGT activity toward SN-38 in human liver microsomes was also less than 4%.

Linearity of SN-38G formation

To determine the optimal reaction conditions for the assay of SN-38 glucuronidation activity in human liver microsomes, the dependence of the enzyme activity on incubation time and microsomal protein concentration was studied. Substrate concentrations of 5, 30 and 100 μM were used. Fig. 3 shows the time-dependent formation of SN-38G by human liver microsomes (100 μg protein/mL). The formation was linear for 80 min at all substrate concentrations ($r^2 = 0.996$–0.999). Similarly, in the assay of UGT activity toward SN-38 in human liver microsomes (incubation for 20 min), SN-38G formation was found to be linear up to 400 μg protein/mL at all substrate concentrations ($r^2 = 0.995$–0.999; Fig. 4). The limit of quantitation is 2.5 pmol of SN-38G/assay, and allows for glucuronidation activity as low as 0.20 pmol/min/mg protein to be

Table 2. Stability of SN-38G in human liver microsomes

Concentration (pmol/mL)	Recovery (mean ± SD) (%)		
	8 h	24 h	72 h
4°C ($n = 5$)			
5	98.8 ± 1.4	98.6 ± 1.5	99.7 ± 1.0
50	99.0 ± 0.7	99.0 ± 0.8	99.2 ± 1.1
500	99.7 ± 0.9	98.8 ± 0.3	99.5 ± 0.7
27°C ($n = 5$)			
5	98.1 ± 0.8	97.5 ± 1.5	96.4 ± 1.2
50	98.8 ± 1.6	98.3 ± 1.4	96.9 ± 0.5
500	99.2 ± 1.0	98.8 ± 2.5	97.1 ± 0.6

SN-38G was added to 50 mM Tris–HCl buffer (pH 7.4) containing liver microsomal protein (100 μg/mL) in a total volume of 400 μL and then 100 μL of 10% $HClO_4$ was added. The method of sample preparation and the HPLC conditions are described in the Experimental section.

Table 3. Precision and accuracy of the determination for SN-38G in human liver microsomes

Nominal concentration (pmol/mL)	Analyzed concentration (mean ± SD; pmol/mL)	RSD (%)	RME (%)
Intra-day ($n = 5$)			
5	5.167 ± 0.293	5.5	3.3
50	50.98 ± 1.50	2.9	2.0
500	501.2 ± 4.8	1.0	0.2
Inter-day ($n = 5$)			
5	4.821 ± 0.302	6.3	−3.6
50	50.89 ± 0.95	1.9	1.8
500	497.2 ± 9.4	1.9	−0.6

SN-38G was added to 50 mM Tris–HCl buffer (pH 7.4) containing liver microsomal protein (100 μg/mL) in a total volume of 400 μL and then 100 μL of 10% $HClO_4$ was added. The method of sample preparation and the HPLC conditions are described in the Experimental section.

Table 4. Precision of the assay for SN-38 glucuronidation activity in human liver microsomes

Substrate concentration (μM)	Activity (mean ± SD) (pmol/min/mg protein)	RSD (%)
Intra-day ($n = 5$)		
5	11.3 ± 0.3	2.6
30	57.5 ± 0.8	1.4
100	81.2 ± 1.5	1.8
Inter-day ($n = 5$)		
5	11.8 ± 0.4	3.4
30	55.6 ± 1.2	2.2
100	79.1 ± 1.7	2.1

Reactions were performed in the presence of SN-38, UDP-glucuronic acid and liver microsomes (100 μg protein/mL) in a total volume of 400 μL for 20 min. The method of sample preparation and the HPLC conditions are described in the Experimental section.

determined [incubation of 160 μg protein/incubation mixture (400 μg protein/mL) for 80 min]. These profiles of SN-38 glucuronidation formation were similar to the results in a previous report (Iyer *et al.*, 1998).

Kinetic analysis

To characterize the enzymology of UGTs toward SN-38 in human liver microsomes, kinetic analysis was performed by measuring fifteen concentrations of substrate between 2.5 and 150 μM. The non-linear regression curve is shown in Fig. 5. The K_m and V_{max} for SN-38 glucuronidation in human liver microsomes were 30.6 ± 2.2 μM and 103 ± 2 pmol/min/mg protein, respectively. Further study is required to identify the isoforms and contribution rates of UGT involved in SN-38 glucuronidation in humans.

CONCLUSION

Although CPT-11 is a useful anticancer agent for colon cancer, it can cause severe diarrhea due to an accumulation of SN-38, the active metabolite of CPT-11, in the intestine (Araki *et al.*, 1993; Wiseman and Markham, 1996). Glucuronidation is an important detoxication pathway for SN-38, and information about the activity of hepatic UGT toward SN-38 in humans is beneficial to medicine. However, the range of glucuronidation activities toward drugs in humans is generally broad because of genetic polymorphisms in the UGT family (Mackenzie *et al.*, 1997; Burchell *et al.*, 1998; de Wildt *et al.*, 1999), and high sensitivity is needed to assay SN-38 glucuronidation activity from small amounts of biological samples. With the method described here, the UGT activity toward SN-38 at low and high concentrations in human liver was precisely determined using small amounts of microsomal proteins without extraction and radiolabeled chemical. Therefore, this method for the assay of SN-38 glucuronidation activity is sensitive and efficient, and should be useful for the *in vitro* study of the side effects and drug

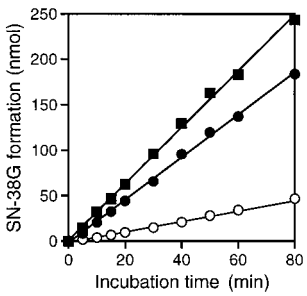

Figure 3. Dependence on incubation time of SN-38 glucuronidation activity in human liver microsomes. Reactions were performed in the presence of SN-38 (5, 30 or 100 μM) and liver microsomes (100 μg protein/mL) in a total volume of 400 μL for 0–80 min. The method of sample preparation and the HPLC conditions are described in the Experimental section. Symbols are: (○) 5 μM SN-38; (●) 30 μM SN-38; and (■) 100 μM SN-38. Each point represents the mean of three separate experiments.

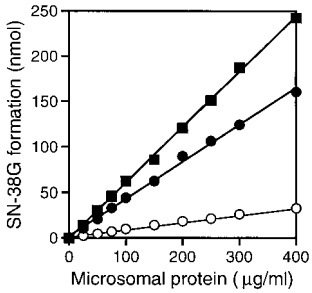

Figure 4. Dependence on protein concentration of SN-38 glucuronidation activity in human liver microsomes. Reactions were performed in the presence of SN-38 (5, 30 or 100 μM) and liver microsomes (0–400 μg protein/mL) in a total volume of 400 μL for 20 min. The method of sample preparation and the HPLC conditions are described in the Experimental section. Symbols are: (○) 5 μM SN-38; (●) 30 μM SN-38; and (■) 100 μM SN-38. Each point represents the mean of three separate experiments.

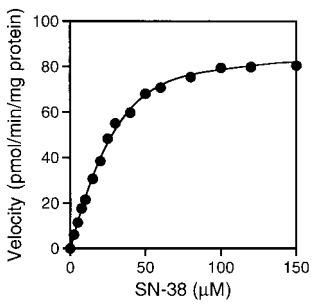

Figure 5. Dependence on substrate concentration of SN-38 glucuronidation activity in human liver microsomes. Reactions were performed in the presence of SN-38 (2.5–150 μM) and liver microsomes (100 μg protein/mL) in a total volume of 400 μL for 20 min. The method of sample preparation and the HPLC conditions are described in the Experimental section. Each point represents the mean of three separate experiments.

interactions of CPT-11 using small amounts of biological sample.

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