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Simultaneous determination of NK-104 and its lactone in biological samples by column-switching high-performance liquid chromatography with ultraviolet detection

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Abstract

A simple and sensitive column-switching HPLC method has been developed for the simultaneous determination of NK-104 (HMG–CoA reductase inhibitor) and its lactone in human and dog plasma. Plasma sample was extracted with methyl *tert*-butyl ether and then the extract was subjected to methylation with diazomethane to prevent the mutual conversion between NK-104 and its lactone. The extract was injected into the column-switching HPLC system. The calibration curves of NK-104 and NK-104 lactone were linear over the ranges 0.5 to 100 ng/ml for human plasma samples and 0.5 to 500 ng/ml for dog plasma, respectively. The intra-day and inter-day C.V. values of these analytes were less than 13.3%. The intra-day and inter-day accuracies of these analytes were between −14.0 and 6.5%. The proposed method has been applied to plasma samples obtained after oral administration of a single 2 mg dose of NK-104 to volunteers.

Keywords: NK-104

1. Introduction

NK-104, monocalcium bis[(3R,5S,6E)-7-[2-cyclopropyl-4-(4-fluorophenyl)-3-quinolyl]-3,5-dihydroxy-6-heptenoate], is a new synthetic drug being a potent and long acting inhibitor of 3-hydroxy-3-methylglutaryl coenzyme A (HMG–CoA) reductase [1]. This agent has been reported to lower total cholesterol and total triglyceride in serum of patient with hypercholesterolemia [2].

Lactone form of NK-104 (NK-104 lactone), which can be reversibly converted to the parent drug of active form, is found as a metabolite in plasma following oral administration of NK-104 to animals [3]. This suggests that NK-104 and NK-104 lactone coexist at equilibrium in blood after administration of the drug. For pharmacokinetic studies in dog and human, a sensitive and selective method is required for the quantification of both NK-104 and NK-104 lactone in plasma. NK-104 lactone is hydrolyzed and converted to NK-104 in various conditions. NK-104 is also dehydrated forming NK-104 lactone. This phenomenon indicates that the mutual conversion may occur through operations such as extraction and purification. To avoid these potential problems, methylation of NK-104 was attempted with diazo-

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methane. A heart-cut technique using column-switching HPLC eliminates the intermediate off-line steps such as further purification of the extracting procedure [4,5]. Therefore we have evaluated the usefulness of HPLC using a column-switching technique and developed a simultaneous method of determining NK-104 and NK-104 lactone in plasma.

2. Experimental

2.1. Reagents and materials

NK-104 (purity: >99% HPLC), NK-104 lactone (purity: >99% HPLC) and internal standard, (6E)-(±)-7-[2-isopropyl-4-(4-fluorophenyl)-3-quinolyl]-3,5-dihydroxy-6-heptenoate (I-1938), were supplied by Nissan Chemical Industries (Saitama, Japan) (see Fig. 1). NK-104 concentration was expressed as an anhydrous salt, because NK-104 contains about 10% adsorbed water. Analytical reagent grade acetonitrile, potassium dihydrogenphosphate and methyl *tert*-butyl ether (MTBE) were purchased from Tokyo Kasei Kogyo Co. (Tokyo, Japan). Diazomethane–ether solution was made as follows: 100 ml of 5% *N*-nitroso-*N*-methyl urethane (>90%: Tokyo Kasei Kogyo Co)–diethyl ether was dropped into a mixture of 6 ml of 20% (w/v) potassium hydroxide–methanol solution and 50 ml of diethyl ether, and the solution was distilled at about 50°C in the draft chamber. This diazomethane–ether solution could be stored at −20°C over a month without deteriorating the methyl reaction.

2.2. Instruments

Analyses were performed on an HPLC system consisting of two LC-6A pumps, an SIL-6A auto-sampler, a CTO-6A column oven, an SPD-6A UV detector, an FCV-3AL solvent changing valve and an FCV-2AH six-port switching valve, all of which were controlled by an SCL-6A system controller (all from Shimadzu, Kyoto, Japan). A CDS data system (LA Soft, Tokyo, Japan) was used for the peak-area measurements. The column switching HPLC was performed with two Cosmosil-5C18 columns (150× 4.6 mm I.D., Nacalai Tesque, Kyoto, Japan) for the pre-separation and analytical separation. Two mobile phases, 0.2 *M* ammonium acetate buffer (pH 4)–acetonitrile (5:5, v/v) for the pre-separation and 0.2 *M* acetic acid–acetonitrile (5:5, v/v) for the analytical separation, were maintained at a flow-rate of 1 ml/min. The detection was carried out at 250 nm with a UV detector. The column temperature was maintained at 40°C.

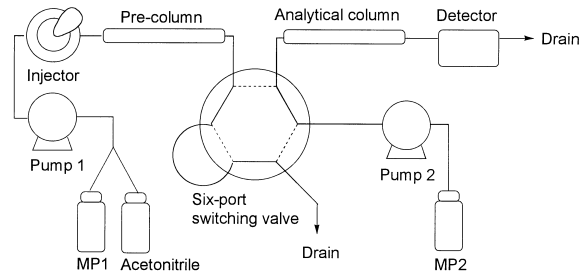
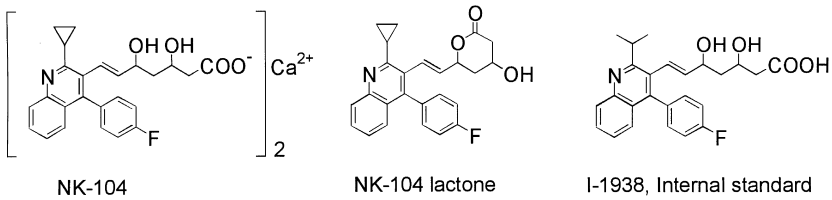


Fig. 2. Schematic diagram of column-switching system. MP1 and MP2; mobile phase 1 and 2. Solid line; equilibration and injection, dotted line heart-cut period.

2.3. Analytical systems and operating conditions

A schematic diagram of the column-switching HPLC system is shown in Fig. 2. The injected sample was first separated on the pre-column. From



NK-104 NK-104 lactone I-1938, Internal standard

Fig. 1. Chemical structures of NK-104, NK-104 lactone and internal standard.

11 to 13.6 min, the flow path was changed to the analytical column by valve switching. Consequently, the heart-cut fraction containing NK-104 methyl ester, NK-104 lactone, and I-1938 methyl ester were transferred from the pre-column to the analytical column. After the valve position was switched back to the initial position, the heart-cut fraction was further separated on the analytical column. Meanwhile the pre-column was washed with acetonitrile for 5 min to remove late-eluting substances, and then equilibrated with initial conditions for 10 min. The valve operations were carried out automatically by the SCL-6A controller according to a predetermined time program. The total analysis time was about 30 min.

2.4. Preparation of standard solution

NK-104, NK-104 lactone and I-1938 are sensitive to sunlight. Care should be taken to minimize exposure to sunlight while handling the drug substances and samples. Stock solutions of NK-104 were prepared in water, and further diluted with water to appropriate concentrations for standard preparation. Stock solutions of NK-104 lactone were prepared in MTBE, and further diluted with MTBE to appropriate concentrations. Stock solution of internal standard was prepared in water, and diluted with 1 *M* potassium dihydrogenphosphate to 0.2 μg/ml for the human plasma determination and 1 μg/ml for the dog plasma. These stock solutions could be stored at 5°C for about a month.

Plasma standards were prepared by adding 0.2 ml of NK-104 standard solution and 5 ml of NK-104 lactone standard solution to control plasma (1 ml). The final concentration ranges of both NK-104 and NK-104 lactone were from 0.5 to 100 ng/ml of human plasma and from 0.5 to 500 ng/ml of dog plasma.

2.5. Sample preparation

Internal standard solutions (0.2 ml) were spiked into plasma samples (1.0 ml) in each colored tube, and then 1 *M* potassium dihydrogenphosphate (0.3 ml) and water (0.2 ml) were added. The sample mixture was extracted with 6 ml of MTBE by shaking for 10 min on a horizontal shaker at 90 rpm, and by centrifuging for 5 min at approximately $700\times g$. The organic layer was transferred to another colored tube and subsequently diazomethane–ether solution (0.5 ml) was added. The reaction mixture was kept at room temperature for 30 min. The excessive diazomethane was degraded by adding 1 *M* potassium dihydrogenphosphate (2 ml), and by shaking for 5 min. The organic layer was evaporated to dryness under a gentle stream of nitrogen at 40°C. The residue was reconstituted in 150 μl of 0.2 *M* ammonium acetate buffer (pH 4)–acetonitrile (5:5, v/v), and an aliquot of 80 μl was injected into the HPLC system.

2.6. Assay validation

Samples for evaluating stability were prepared separately by adding appropriate concentrations of NK-104 as saline solution (0.1 ml) or NK-104 lactone as methanol–saline (2:98, v/v) solution to pooled plasma (10 ml). Each sample was stored as follows: 1 to 4 h at 0 or 37°C with a concentration of 100 ng/ml ($n=1$); 1 to 13 weeks at −20°C with two concentrations (30 and 450 ng/ml, $n=2$); 0 to 2 freeze/thaw cycles with two concentrations ($n=2$). The calibration curves for NK-104 and NK-104 lactone were generated by plotting peak area ratio of these analytes versus internal standard, and by applying the weighted linear least-square regression procedure using $1/Y^0$ as the weight factor.

Stability of NK-104 and NK-104 lactone in plasma was evaluated by estimating these residual contents under the storage conditions investigated. Extraction rates of NK-104 and NK-104 lactone were calculated by comparing peak-areas obtained for the spiked samples versus direct injections.

The intra-day coefficient of variation (C.V.) and accuracy of the quantification were assayed for the determination of NK-104 and NK-104 lactone by analysis of plasma samples ($n=5$ or 6) spiked with the analytes at three or four different concentrations. The C.V. and accuracy for inter-day assay were evaluated by analysis of samples ($n=3$ or 5) at the same concentrations, repeated for three or four different days.

3. Results and discussion

3.1. Derivatization

NK-104 and NK-104 lactone under various assay conditions are mutually converted by dehydration and hydrolysis, respectively. These phenomena are undesirable for a selective assay of NK-104 and NK-104 lactone. We attempted methylation of NK-104 with diazomethane. This NK-104 methyl ester is not dehydrated. Therefore, NK-104 methyl ester is clearly distinguishable even though some of the NK-104 lactone is hydrolyzed to NK-104. In this study, the mutual influence between NK-104 and NK-104 lactone that depends on the process of assay could be reduced remarkably.

The corresponding methyl esters of NK-104 and internal standard (I-1938) reacted immediately with diazomethane in MTBE solvent, and gave almost quantitative yields. In addition, NK-104 lactone neither gave its derivative nor changed to NK-104 in this reaction.

3.2. Column-switching condition

NK-104 methyl ester, NK-104 lactone and I-1938 methyl ester have a quinoline ring in their structures, and show the retention behavior of weak basic compound on a reversed-phase column (Fig. 3). These analytes showed similar retention time on the mobile phase ranging in pH from 3.5 to 5, and were co-eluted from column. Although the retention time of these analytes was reduced in the acidic mobile phase (pH 2.7), the peaks were clearly separated. Therefore, initially, pre-separation was achieved using a mobile phase of pH 4 and subsequently the heart-cut fraction was separated using a mobile phase of pH 2.7. A combination of two mobile phases having different pH values produced not only sharp peaks of these analytes, but also good separation from endogenous peaks. The carryover of late-eluting substances was eliminated completely by washing pre-column with acetonitrile before next analysis.

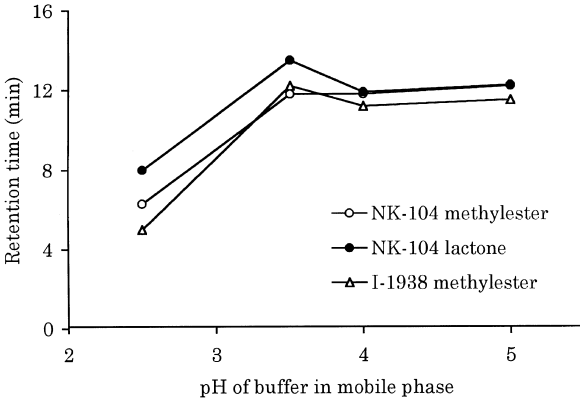


Fig. 3. Effect of the buffer pH in the mobile phase on the retention times of NK-104 methylester, NK-104 lactone and I-1938 methylester. Mobile phases; 0.2 *M* ammonium acetate buffer (pH 2.7–5)–acetonitrile (5:5, v/v).

3.3. Stability in biological fluids

The stability of NK-104 or NK-104 lactone under storage condition at 37, 0 or −20°C was evaluated separately in dog and human plasma, respectively. NK-104 was very stable in human and dog plasma under this storage conditions (data not shown). On the other hand, some of the NK-104 lactone in plasma changed gradually to NK-104 under storage at 37°C because it is somewhat unstable (Fig. 4). The stability of NK-104 lactone is much improved if the plasma samples are stored at 0 or −20°C (Figs. 4 and 5). This indicates that the plasma collected should be immediately kept on ice and stored at −20°C to minimize the hydrolysis of NK-104 lactone. The quantification should be finished within 3 months for human plasma samples and 3 weeks for dog plasma samples. Also the stability of NK-104 or

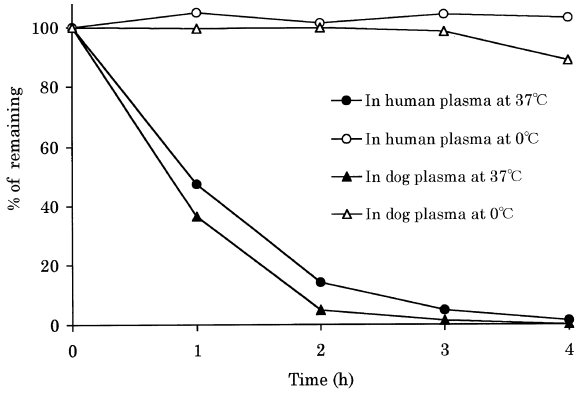


Fig. 4. Stability of NK-104 lactone in human and dog plasma under storage at 0 or 37°C.

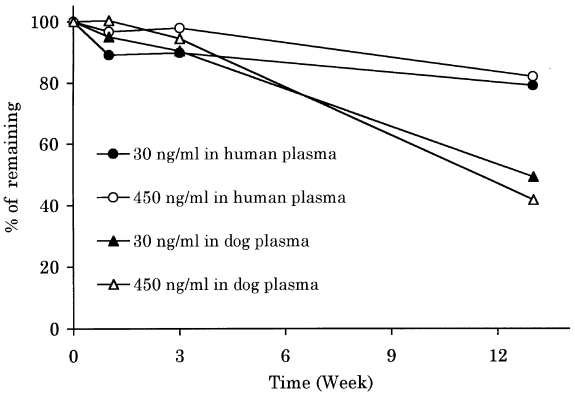


Fig. 5. Stability of NK-104 lactone in human and dog plasma under storage at −20°C.

NK-104 lactone through 2 freeze/thaw cycles was evaluated separately in dog and human plasma, respectively. Both analytes were stable in these plasmas. These things indicated that freeze/thaw operation does not affect the stability of NK-104 and NK-104 lactone until 2 cycles (Table 1).

3.4. Extraction rate

The extraction rates of NK-104 added to human plasma at concentrations of 20 and 100 ng/ml were 73 ($n=3$) and 75% ($n=3$), respectively, whereas those for NK-104 lactone were 94 and 101% at the same concentrations. The extraction rate of NK-104 added to dog plasma at 100 ng/ml was 105% ($n=3$), while that for NK-104 lactone was 95% ($n=3$) at the same concentration. Acceptable recoveries were obtained for both analytes, and no emulsion was formed during this solvent extraction.

3.5. Precision and accuracy

Linearity of NK-104 and NK-104 lactone was evaluated over the concentration ranges 0.5 to 100 ng/ml of human plasma and 0.5 to 500 ng/ml of dog plasma. The calibration curves of NK-104 and NK-104 lactone for human plasma were $Y=0.0158X+0.0077$ ($r=0.999$) and $Y=0.0200\ X-0.0033$ ($r=0.999$), respectively, and these relationship for dog plasma were $Y=0.00279\ X+0.00387$ ($r=0.999$) and $Y=0.00321X-0.00442$ ($r=0.999$),

The intra-day and inter-day variability of NK-104 and NK-104 lactone are summarized in Tables 2 and 3. In human plasma, the intra-day C.V. values were less than 13.3%, and the intra-day accuracies were between −14.0 and 6.2%, within the concentration range of the calibration curves for both analytes. Also the inter-day C.V. values were less than 5.3%, and the inter-day accuracies were between −2.3 and 4.7%. In dog plasma, the intra-day C.V. values was less than 13.3%, and the intra-day accuracies were between −14.0 and 2.4% for both analytes. The inter-day C.V. values were less than 4.6% and the inter-day accuracies were between −0.5 and 6.5%.

The limits of quantification for both NK-104 and NK-104 lactone in human plasma were set to 0.5

Table 1
Freeze–thaw stability of NK-104 and NK-104 lactone in plasma. The samples were thawed in ice water and frozen within 2 h of thawing in each cycle

Matrix	Compound	Nominal concentration added[a]	Concentration found (ng/ml)[b]		
			Freeze–thaw cycle		
			Initial	First	Second
Human plasma	NK-104	Low conc.	29.2 (100.0)	28.7 (98.1)	26.9 (92.0)
		High conc.	445 (100.0)	446 (100.2)	447 (100.4)
	NK-104 lactone	Low conc.	26.5 (100.0)	25.9 (97.7)	25.8 (97.4)
		High conc.	440 (100.0)	413 (93.8)	408 (92.7)
Dog plasma	NK-104	Low conc.	28.2 (100.0)	29.0 (102.7)	28.3 (100.4)
		High conc.	448 (100.0)	443 (98.9)	437 (97.5)
	NK-104 lactone	Low conc.	26.4 (100.0)	25.7 (97.2)	24.7 (93.4)
		High conc.	368 (100.0)	392 (106.7)	363 (98.6)

[a] Low conc., 30 ng/ml; high conc., 450 ng/ml.
[b] $n=2$ for each concentration. Parenthesis represents the percentage against the initial value.

Table 2
Intra-day precision and accuracy for NK-104 and NK-104 lactone determination

Matrix	Compound	Concentration added (ng/ml)	Concentration found (ng/ml)	C.V. (%)	Accuracy (%)
Human plasma (n=5)	NK-104	0.5[a]	0.50	0.0	0.0
		10	10.6	2.0	6.2
		100	101.6	0.8	1.6
	NK-104 lactone	0.5[a]	0.50	0.0	0.0
		10	10.2	2.3	2.0
		100	98.5	0.8	−1.5
Dog Plasma (n=6)	NK−104	0.5[a]	0.43	13.3	−14.0
		2	2.01	4.5	0.6
		30	30.7	1.2	2.4
		450	453.7	1.2	0.8
	NK−104 lactone	0.5[a]	0.48	11.7	−5.0
		2	1.96	3.7	−2.3
		30	30.2	1.2	0.6
		450	442.3	6.3	−1.7

[a] Limit of quantification.

ng/ml, which is the lowest concentration that could be measured with acceptable C.V. (<20%) and accuracy (<20%) (Table 2).

3.6. Application to biological samples

The described method was applied to the determination of plasma levels of NK-104 and NK-104 lactone after oral administration of 2 mg of NK-104 to six human volunteers. NK-104 and NK-104 lactone were quantifiable in plasma up to 24 and 32 h, respectively, after dosing of NK-104 (Fig. 6). The mean Cmax values for NK-104 and NK-104 lactone were 26.1 and 16.7 ng/ml, respectively (Table 4). Fig. 7 shows chromatograms obtained from 2 h post-dose plasma of the volunteers. The peaks that appeared at around 23 min are due to endogenous substances co-eluted with the analytes from the pre-

Table 3
Inter-day precision and accuracy for NK-104 and NK-104 lactone determination. Inter-day assay for human plasma was evaluated by analyzing samples at three different concentrations (n=5) for three different days. Dog plasma was examined at three different concentrations (n=3) for four different days

Matrix	Compound	Concentration added (ng/ml)	Concentration found (ng/ml)	C.V. (%)	Accuracy (%)
Human plasma	NK-104	0.5	0.50	0.0	0.0
		10	10.5	1.4	4.7
		100	101.2	0.4	1.2
	NK-104 lactone	0.5	0.50	0.0	0.0
		10	9.8	5.3	−2.3
		100	99.0	0.6	−1.0
Dog Plasma	NK-104	2	2.13	1.9	6.5
		30	31.0	4.6	3.4
		450	447.8	1.0	−0.5
	NK-104 lactone	2	2.04	2.2	2.0
		30	31.1	3.2	3.6
		450	451.1	0.6	0.2

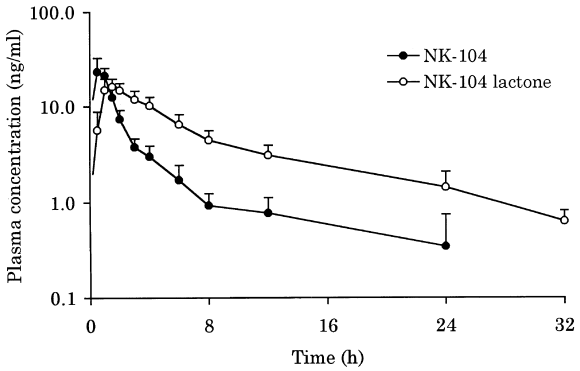


Fig. 6. Plasma concentration–time curves of NK-104 and NK-104 lactone after oral administration of 2 mg of NK-104 to fasting human volunteers. Each point represents the mean and S.D. of six volunteers.

column. This method provided sufficient sensitivity to measure the low plasma concentration, and was applicable to various samples such as urine, feces and tissue homogenate (data not shown).

4. Conclusion

A selective and highly sensitive column-switching HPLC method for the quantification of NK-104 and NK-104 lactone was developed incorporating pre-column derivatization with diazomethane. It was established that the accuracy and precision of the assay are satisfied with the proposed method. Furthermore, this method has adequate quantification limits (0.5 ng/ml) for both analytes in plasma after oral administration of NK-104 to humans.

Table 4
Pharmacokinetic parameters of NK-104 and NK-104 lactone after oral administration of 2 mg of NK-104 to fasting human volunteers

Parameter (unit)	NK-104		NK-104 lactone	
	Mean (n=6)	SD	Mean (n=6)	SD
Cmax (ng/ml)	26.11	6.91	16.72	4.18
Tmax (h)	0.8	0.3	1.5	0.3
AUC(t) (ng.h/ml)	58.8	15.3	124.5	28.4
t1/2 (h)[a]	2.5	0.3	8.9	1.4

[a] t1/2 of NK-104 was calculated using the log–linear portion of the plasma concentration-curve from 3 to 8 h after dosing, and t1/2 of NK-104 lactone was from 8 to 32 h.

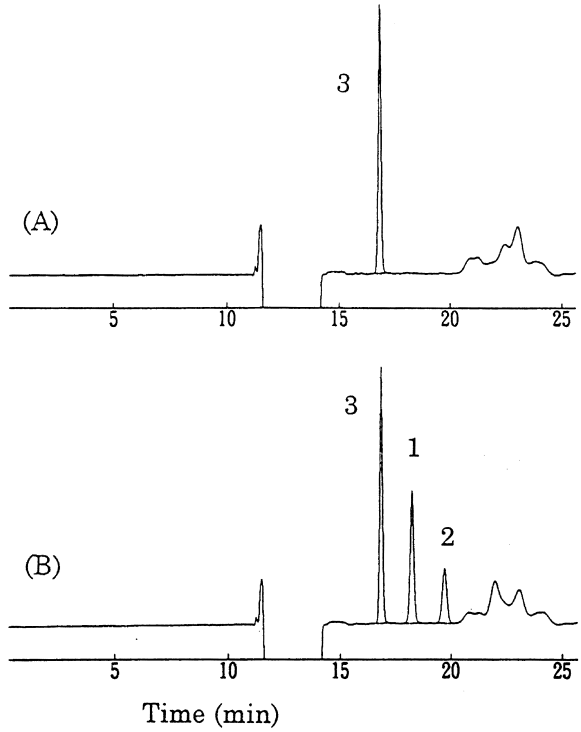


Fig. 7. Typical chromatograms of NK-104 methyl ester (1), NK-104 lactone (2) and I.S. methyl ester (3) by column-switching HPLC. (A) drug-free human plasma, (B) plasma collected at 2 h after oral administration of NK-104 (2 mg per man).

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