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Short communication

Simultaneous determination of nitrendipine and one of its metabolites in plasma samples by gas chromatography with electron-capture detection

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Abstract

A sensitive method for GC-ECD simultaneous determination of nitrendipine and its pyridine metabolite M1 in human plasma is described. Felodipine was used as the internal standard. The plasma samples were extracted with toluene. One microlitre of the extract was injected onto the capillary column (polymethylsiloxane) and measured with electron-capture detector. The developed method showed to be linear over the range 0.25–70 for nitrendipine and 0.3–61 ng/ml for its metabolite M1 with an inter-day and intra-day precision in terms of R.S.D. lower than 8% except the concentrations near lowest limit of quantification (LLOQ) (<11% R.S.D.). The LLOQ for nitrendipine was 0.25 and 0.3 ng/ml for its metabolite, respectively. The analytical recovery was 94% for nitrendipine and 89% for its pyridine metabolite M1. This GC-ECD method was developed for being used in clinical pharmacokinetic studies.

Keywords: Nitrendipine; Felodipine; Plasma; GC-ECD

1. Introduction

Nitrendipine [ethyl methyl (4*RS*)-2,6-dimethyl-4-(3-nitrophenyl)-1,4-dihydropyridine-3,5-dicarboxylate] (see Fig. 1a) belongs to the group of calcium channel antagonists, which are often used in the therapy of hypertension and angina pectoris [1–3]. The main route of the nitrendipine metabolism is dehydrogenation to the pyridine analogue [4] by cytochrome P450 3A4/5, then cleavage of ester groups by hydrolysis to carboxylic acids, hydroxylation of methyl groups and glucuronidation [5–7]. Pyridine metabolite M1 (see Fig. 1b) exhibits 1000 times less biological activity than nitrendipine [5]. The concentrations of nitrendipine as well as other dihydropyridine calcium channel antagonists in human blood plasma are relatively low as a consequence of extensive first-pass metabolism, poor bioavailability and also relatively high inter- and intra-individual variability [7,8]. Therefore, the determination of nitrendipine requires very sensitive techniques. Several methods have been described for the determination and quantification of nitrendipine in blood plasma. HPLC with ultraviolet detection [9,10] is a generally available technique, but it has relatively low sensitivity and specificity compared with HPLC–MS or LC–MS–MS [11–13]. Gas chromatographic method with electron-capture detection [7,14,16–20,30] or with MS detection [21–23] ensures best the desired analytical parameters.

Some authors employed nitrendipine as an internal standard for determination of other dihydropyridines [13–16,18–21,24–27,30]. Soons et al. [19] used nitrendipine as an internal standard for GC-ECD determination of felodipine in human plasma. On the contrary, we have applied the easily obtainable felodipine as the internal standard for determination of nitrendipine in human plasma replacing 3-(2-hydroxy-2-methyl)ethyl-5-methyl-2,6-dimethyl-4-nitrophenyl)-3,5-pyridine dicarboxylate in the GC-ECD method of Soons and Breimer [17]. For sample preparation we have chosen the robust method of liquid–liquid extraction with toluene as an

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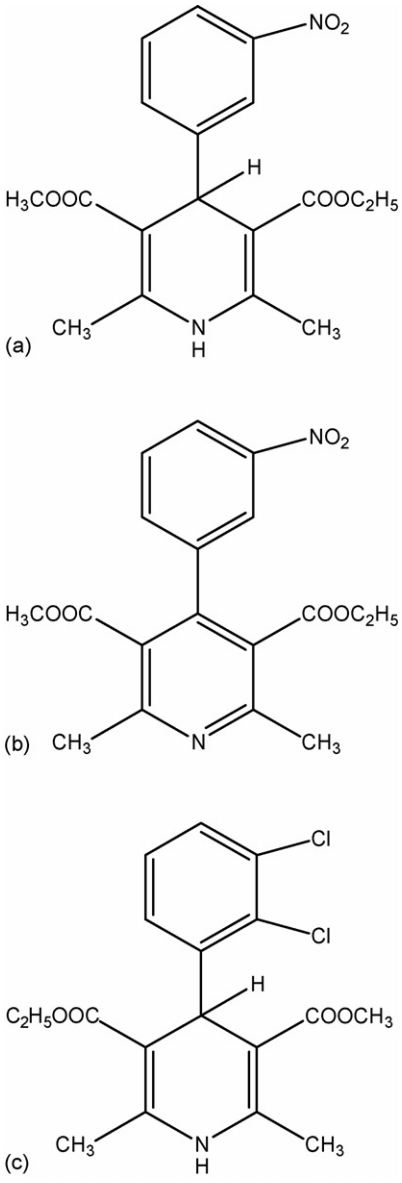

Fig. 1. Structure of: (a) nitrendipine; (b) pyridine metabolite M1 of nitrendipine; (c) felodipine (internal standard).

extraction solvent [14,18,21] followed by direct injection in the split–splitless injection device.

2. Experimental

2.1. Chemicals and reagents

The chemical structures of nitrendipine [ethyl methyl (4*RS*)-2,6-dimethyl-4-(3-nitrophenyl)-1,4-dihydropyridine-3,5-dicarboxylate], its metabolite M1 [ethyl methyl 2,6-dimethyl-4-(3-nitrophenyl)pyridine-3,5-dicarboxylate] and the internal standard felodipine [ethyl methyl (4*RS*)-2,3-dichlorophenyl-2,6-dimethyl-1,4-dihydropyridine-3,5-dicarboxylate] are shown in Fig. 1a–c. All chemicals were of analytical grade or better. Both nitrendipine (order code N0905000) and nitrendipine metabolite M1 (nitrendipine impurity A, order code N0905005) were obtained from European Pharmacopoeia Catalogue. Felodipine (internal standard) was extracted from tablet preparation Plendil® ER, 5 mg (Astra AB, Sweden). Methanol SupraSolv® and toluene SupraSolv® were from Merck (Germany). UHQ water was from PRO.MED.CS Praha a.s. (Prague, Czech Republic).

2.2. Equipment

An Agilent Technologies 6890 Series GC System gas chromatograph with a μ-^{63}Ni-ECD detector, equipped with an Agilent Technologies 7683 Series Injector and 7683 Series Autosampler was used (Agilent Technologies, CA, USA) together with a 30 m × 0.32 mm fused silica column coated with polydimethylsiloxane (DB-1) with a film thickness of 0.25 μm (J&W Scientific, USA). Helium at the flow rate of 2.2 ml/min was used as the carrier gas. The inlet temperature was set at 260 °C. Splitless injection (1 μl) was used with a deactivated injection liner, which was replaced after every 80 injections. The electron-capture detector was set at 260 °C with a nitrogen makeup flow of 25 ml/min. The initial column temperature was 100 °C. After 3 min at 100 °C, the temperature was gradually raised to 230 °C at the rate of 50 °C/min, and this temperature was maintained for 15 min. A Hewlett-Packard ChemStation (Agilent Technologies) was used to control the GC apparatus and to acquire and process the data. Calibration curves obtained with a known concentration range of nitrendipine or its M1 metabolite and internal standard (felodipine) were used for quantification of unknown samples.

2.3. Extraction procedure

Fifty microlitres of the internal standard solution (felodipine, concentration: 1 μg/ml) was added to 1.0 ml of the human blood plasma, and the solution whirl-mixed for 10 s. For the extraction, toluene (1.0 ml) was added, and the tube was shaken in the horizontal shaker (90 cycles/min) for 30 min and centrifuged (3000 × *g*) for 5 min. 0.2 ml of the organic layer was transferred into the glass insert tube, which was placed in the brown autosampler vial. One microlitre of the extract was injected into the gas chromatograph.

2.4. Preparation of standards, calibration and clinical samples

The entire treatment and analytical procedures had to be performed in the dark room using yellow sodium light to prevent degradation of dihydropyridine analytes [28]. For sample preparation only glassware was used to prevent the loss of nitrendipine by absorption [30]. The human blood samples were withdrawn from a forearm vein, collected into lithium–heparin tubes, and immediately centrifuged (3000 × *g*, 10 min); blood plasma was transferred into the brown glass tubes for further treatment or storage (at −70 °C).

2.4.1. Preparation of the stock and calibration solutions

One tablet of Plendil® ER containing 5 mg of felodipine was disintegrated and diluted with 100 ml of methanol. Methanol

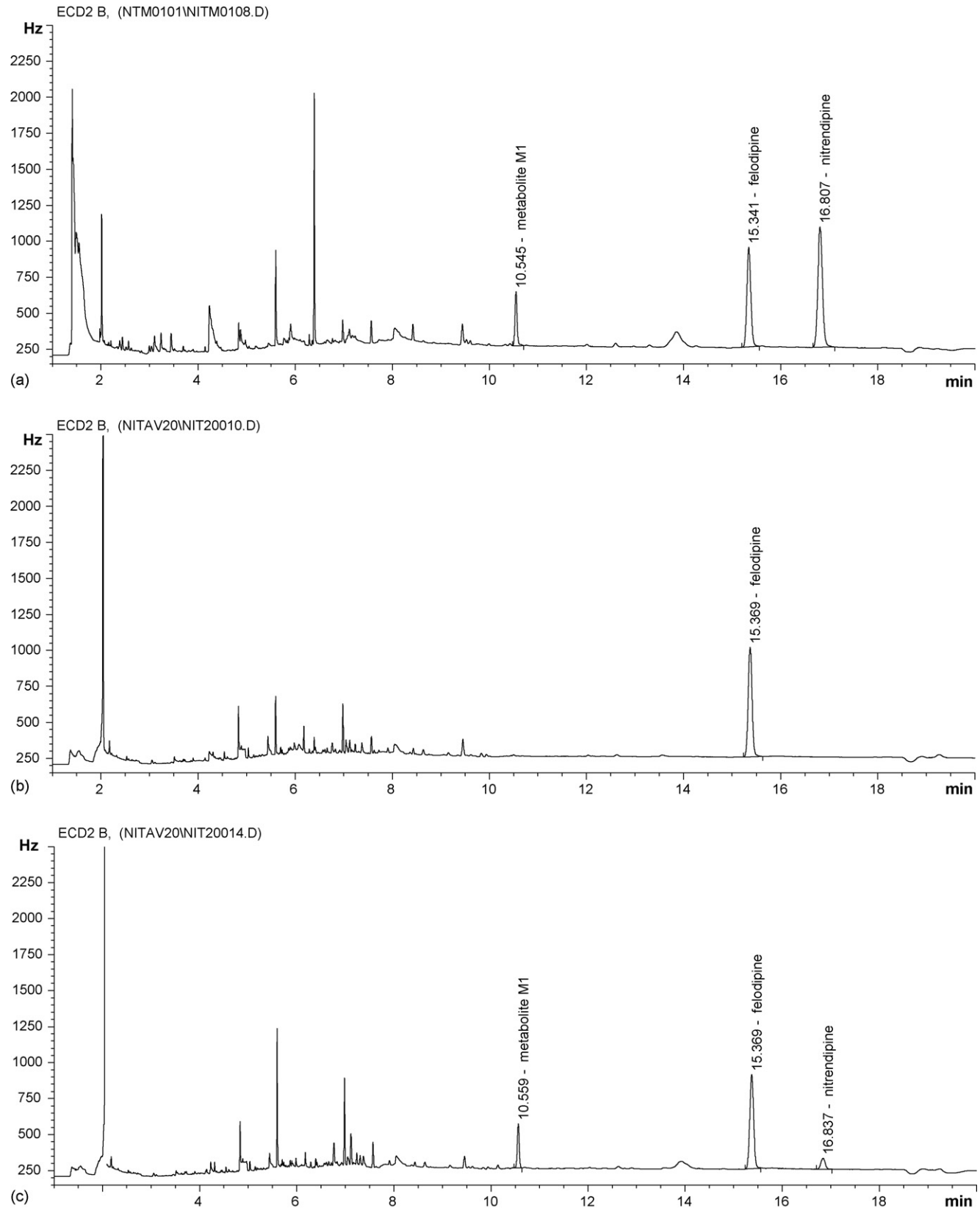

Fig. 2. Chromatogram of: (a) 1.0 ml human drug-free plasma extract spiked with 50 ng of nitrendipine (Rt = 16.8 min), 15 ng of its pyridine metabolite M1 (Rt = 10.55 min) and 50 ng of felodipine (internal standard, Rt = 15.35 min); (b) 1.0 ml human drug-free plasma extract spiked only with 50 ng of felodipine (internal standard, Rt = 15.35 min); (c) 1.0 ml of the real sample from the clinical study. Human plasma obtained 40 min after an oral administration of 20 mg of nitrendipine spiked with 50 ng of felodipine (internal standard, Rt = 15.35 min).

solution was centrifuged (4000 × *g*) for 10 min and supernatant was filtered using Milipore® 0.45 μm filters. The internal standard solution was obtained by the dilution of the stock solution with water to the final concentration of 1 μg/ml.

The nitrendipine and its metabolite M1 stock solutions were prepared by dissolving the compounds in methanol. The calibration solutions were obtained by the dilution of the stock solutions with water.

2.4.2. Calibration and validation

For calibration and validation, aliquots of nitrendipine and its metabolite M1 solutions were added to 1.0 ml of drug-free human plasma to obtain reference samples in the concentration range 0.25–70 ng/ml for nitrendipine, and 0.3–61 ng/ml for its M1 metabolite. Intra- and inter-day precision was determined with the spiked plasma standards. The statistical evaluation of the inter-day assay method performance includes the data from an intra-day validation and additional measurements performed independently in three consecutive days.

3. Results and discussion

3.1. Selectivity and linearity

Typical chromatograms of the drug-free plasma sample spiked with the analytes and the plasma sample from the clinical study are shown in Fig. 2. The analytes (nitrendipine and its metabolite M1) are well separated from the other components of the plasma extract. The linearity of the detector response for the 1.0 ml plasma samples was confirmed in the concentration range 0.25–70 ng/ml for nitrendipine ($r = 0.99$, $n = 36$) and 0.3–61 ng/ml for its metabolite M1 ($r = 0.99$, $n = 36$).

3.2. Precision, accuracy, LLOQ and limit of detection

The limit of quantification was evaluated, using decreasing concentrations of nitrendipine and its pyridine metabolite until nitrendipine concentration 0.25 ng/ml. This value had been deduced from the presupposed requirement of the clinical study. Limit of the quantification had to be 1/20 of the expected maximum concentration in fast metabolizers, usually around 5 ng/ml. The lower limits of quantification of 0.25 ng/ml for nitrendipine and 0.3 ng/ml for its pyridine metabolite M1 were fully sufficient for the evaluation of plasma samples from the clinical study. The lower limit of detection, defined as three times the signal-to-noise ratio was 0.1 ng/ml for both analytes.

Even if the value of LLOQ for nitrendipine seems to be worse than results obtained by authors Soons and Breimer [17] 18 years ago (in the cited paper, the limit of quantification was 50 pg/ml for nitrendipine), our method gives fully satisfactory results using much simpler procedure. The above authors have concentrated the extract twentyfold before the injection on the column. We have used simple liquid–liquid extraction (1 ml of the plasma sample and 1 ml of toluene) and direct injection of the toluene extract without further treatment. The lower limits of quantification, we can achieve, are in full accordance with practical purposes of clinical trials. The method was found to be precise with R.S.D. values lower than 8% except from the concentrations near LLOQ, where R.S.D. value was lower than 11%. The accuracy of the method was satisfactory (<15%) for both analytes (see Tables 1 and 2).

Table 1
Intra-day accuracy and precision for nitrendipine and its pyridine metabolite M1

Compound	Concentration added (ng/ml)	Concentration found[a] (ng/ml)	Precision (R.S.D., %)	Accuracy (%)
Nitrendipine	70	72.56 ± 1.66	2.3	104
	50	48.1 ± 1.41	2.9	96
	20	19.75 ± 0.60	3.0	99
	5	4.92 ± 0.08	1.6	98
	1	1.00 ± 0.03	3.2	100
	0.5	0,50 ± 0.03	5.5	99
	0.25	0.25 ± 0.02	6.6	99
Pyridine metabolite M1	61.2	62.40 ± 2.8	4.5	102
	30.6	29.06 ± 1.13	3.9	95
	15.3	13.75 ± 0.41	3.0	90
	1.22	1.38 ± 0.07	5.2	113
	0.61	0.55 ± 0.06	10.1	90
	0.30	0.29 ± 0.03	10.0	97

[a] Mean ± standard deviation; $n = 6$.

3.3. Extraction recovery

Thanks to lipophilic properties of nitrendipine and its M1 metabolite both analytes can be easily extracted from plasma samples by toluene. On the other hand, the shaking must be done very carefully—with a slow speed, because there is a danger of gel formation with the blood plasma. In agreement with Mück and Bode [14] we observed similar increase in total recovery, when the pure toluene solutions were used. The authors explained this phenomenon by thermal oxidative degradation of dihydropyridines in pure toluene solutions. Probably the extracted plasma components are able to protect nitrendipine as well as other dihydropyridines against catalytic activities of contacting hot surfaces in a GC apparatus [14,29]. To cope with this difficulty we spiked toluene extract of the drug-free plasma with nitrendipine and its M1 metabolite for evaluation of total extraction recovery. The determined extraction recov-

Table 2
Inter-day accuracy and precision for nitrendipine and its pyridine metabolite M1

Compound	Concentration added (ng/ml)	Concentration found[a] (ng/ml)	Precision (R.S.D., %)	Accuracy (%)
Nitrendipine	50	50.15 ± 3.02	6.0	100
	20	19.21 ± 0.63	3.3	96
	5	5.03 ± 0.31	6.1	101
	1	1.01 ± 0.7	7.2	101
Pyridine metabolite M1	61.2	61.87 ± 2.65	4.3	101
	30.6	28.35 ± 1.28	4.5	93
	15.3	13.32 ± 0.48	3.6	87
	1.22	1.33 ± 0.07	8.0	109

[a] Mean ± standard deviation; $n = 18$.

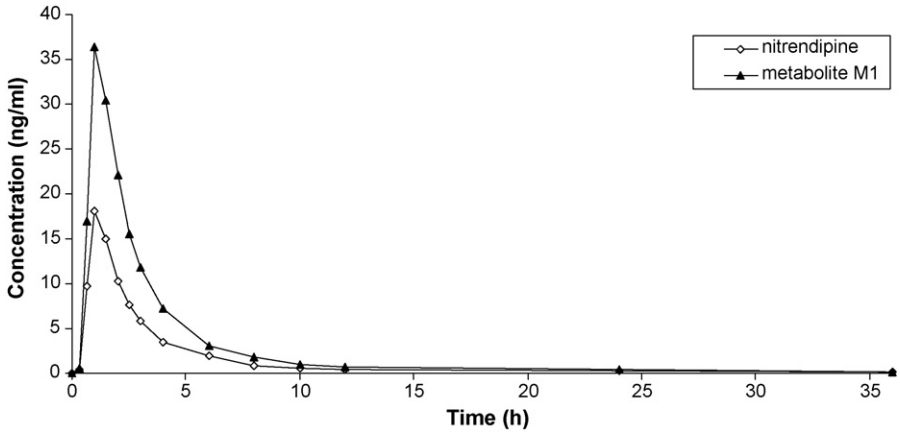

Fig. 3. Representative nitrendipine and its pyridine metabolite M1 plasma concentration–time profile obtained in one healthy female volunteer after an oral administration of 20 mg of nitrendipine.

eries for nitrendipine and its M1 metabolite were 94 and 89%, respectively.

3.4. Stability

Stability studies were carried out with drug-free plasma samples spiked with nitrendipine and its pyridine metabolite M1.

3.4.1. Autosampler stability

The stability of nitrendipine and its pyridine metabolite M1 in the autosampler was checked for 24 and 48 h at carusel temperature for 5, 20 and 50 ng/ml spiked samples of nitrendipine and 15.3, 30.6 and 61.2 ng/ml for its pyridine metabolite M1. No significant differences were found between $t = 0$, 24 and 48 h.

3.4.2. Freeze and thaw stability

Spiked samples with 5, 20 and 50 ng/ml of nitrendipine and 15.3, 30.6 and 61.2 ng/ml for its pyridine metabolite M1 were stored at −70 °C and were subjected to two freeze–thaw cycles. No significant differences were found between the first and the second freeze–thaw cycle.

3.4.3. Long-term stability

The quality control, plasma standards and samples from the clinical study were stable at −70 °C for at least 3 months. All samples were stored in amber glass autosampler vials and placed in the black plastic box (to prevent any light exposition).

4. Conclusions

A sensitive and relatively simple method based on liquid–liquid extraction [14,18,21] and GC-ECD detection was employed for the determination of nitrendipine and its pyridine metabolite M1 in human blood plasma from the clinical study. To prevent photodegradation of nitrendipine and internal standard felodipine all procedures and analyses had to be carried out in the dark room under sodium light. We have confirmed also the thermal oxidative degradation of dihydropyridines in pure toluene solutions, observed by Mück and Bode [14]. The LLOQ values were 0.25 ng/ml for nitrendipine respectively 0.3 ng/ml for its metabolite M1, which are similar to other GC-ECD methods [17,19,31] and also satisfactory for measuring of samples from the clinical study.

This GC-ECD technique is relatively cheap comparing with GC–MS. The applicability of the GC-ECD method is demonstrated in Fig. 3—nitrendipine and its pyridine M1 plasma concentration–time profile obtained from the pharmacokinetic clinical study of nitrendipine.

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