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# A High-Performance Liquid Chromatographic Method for The Simultaneous Determination of Nicardipine and Its Pyridine Metabolite II in Plasma

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**Abstract** □ A rapid and specific method in which reverse-phase high-performance liquid chromatography (HPLC) with UV detection was used for the simultaneous determination of nicardipine and its pyridine metabolite II in human plasma is described. Nicardipine, its pyridine metabolite II, and the internal standard were extracted from plasma and partially purified by acid–base partitioning. Final purification and quantitation were achieved by HPLC by using a reverse-phase column and a UV detector (254 nm). The extraction efficiencies for nicardipine and its pyridine metabolite II from 1 mL of plasma were 77.4 and 81.1%, respectively. The sensitivity of the assay was 5 ng/mL for both nicardipine and its pyridine metabolite II, and the linear concentration range of the assay was 5–150 ng/mL for both compounds. The low coefficients of variation (≤5%) for samples spiked with nicardipine and its pyridine metabolite II in this concentration range demonstrate good reliability and reproducibility of the assay. The HPLC procedure has been validated by comparison with a GC–electron-capture detection (ECD) procedure, which gives the combined concentration of nicardipine–its pyridine metabolite II (total) and with an HPLC/GC–ECD procedure, which gives the concentration of its pyridine metabolite II. All three methods, which were developed in our laboratory, were used to analyze nicardipine and its pyridine metabolite II in specimens of plasma from subjects treated with nicardipine hydrochloride. Good correlations were found for concentrations of nicardipine, its pyridine metabolite II, and nicardipine plus the metabolite determined by these three procedures. The HPLC procedure is suitable for use in pharmacokinetic studies following administration of nicardipine hydrochloride to humans.

**Keyphrases** □ Nicardipine—simultaneous determination with its pyridine metabolite II, reverse-phase HPLC □ HPLC—reverse-phase simultaneous determination of nicardipine and its pyridine metabolite II in human plasma

Nicardipine hydrochloride, 2-(*N*-benzyl-*N*-methylamino)ethyl methyl 1,4-dihydro-2,6-dimethyl-4-(*m*-nitrophenyl)-3,5-pyridinedicarboxylate monohydrochloride (I), is a new calcium entry-blocking agent with potent oral vasodilating activity. The therapeutic efficacy of this compound for the treatment of angina, hypertension, and cerebrovascular disease is under investigation.

Nicardipine hydrochloride, which is effective at relatively low doses, undergoes extensive first-pass metabolism to produce several metabolites (1). Therefore, a sensitive and specific method is required for its determination in human plasma. Various methods have been reported for the determination of nicardipine in plasma. These include gas chromatography (GC) with either electron-capture detection (GC–ECD) (2) or with mass spectrometric detection (GC–MS) (3). However, these methods are relatively nonspecific because they require oxidation of nicardipine prior to GC analysis. Since the product of this oxidation is the pyridine metabolite II of nicardipine, these methods measure the combined concentration of nicardipine plus its pyridine metabolite II (total). Pharmacokinetic analyses of data generated by these methods can be misleading in view of the fact that the vasodilative activity of the pyridine metabolite II is only $^1/_{300}$ that of the parent drug (4). The individual concentrations of nicardipine and its metabolite in human plasma have been investigated recently using a TLC–GC–MS method (5), in which nicardipine was separated from its pyridine metabolite II by TLC prior to analysis by GC–MS. These investigators have found that in healthy volunteers and hypertensive patients, the concentration of nicardipine relative to that of the combined concentrations of nicardipine plus its pyridine metabolite II ranged from 74% to 99% (5). Recently, we developed a method using high-performance liquid chromatography (HPLC) in conjunction with GC–ECD which allowed the individual concentrations of nicardipine and its pyridine metabolite II to be determined[1]. In this HPLC/GC–ECD method, which was developed prior to the availability in our laboratory of a high-sensitivity HPLC detector, HPLC was used to separate nicardipine from the pyridine metabolite II prior to analysis of the latter by GC–ECD. The concentration of nicardipine in a sample of plasma was obtained by subtracting the concentration of the pyridine metabolite II as determined by the HPLC/GC–ECD assay from the combined concentration of nicardipine plus the pyridine metabolite II as determined by the GC–ECD method

[1] Unpublished results (a brief description of this method is given in the text).



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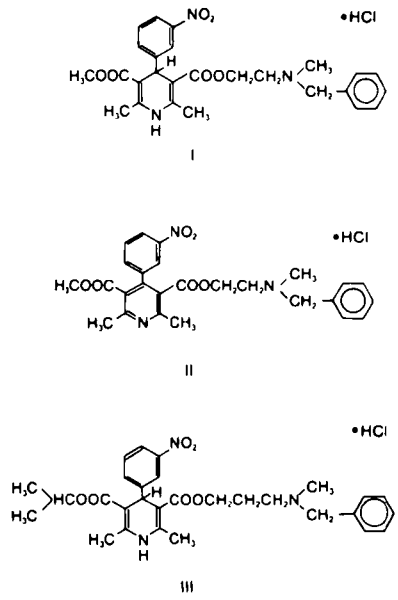

(2). Based on our HPLC/GC-ECD assay, the pyridine metabolite II accounted for 28.58 ± 8.73% ($n$ = 36) of the combined concentration of nicardipine plus the pyridine metabolite II in plasma from subjects treated orally with nicardipine hydrochloride. In view of the presence of varying proportions of nicardipine and its pyridine metabolite II in plasma, a specific method for the determination of the concentrations of nicardipine should be used to provide data for pharmacokinetic analysis. Although the TLC-GC-MS and HPLC/GC-ECD methods have this specificity, they are relatively laborious and do not provide a practical means for the specific determination of nicardipine and its pyridine metabolite II in plasma on a routine basis.

The method described here is a simple HPLC method which allows the simultaneous determination of the individual concentrations of nicardipine and its pyridine metabolite II in human plasma. The assay, which has a sensitivity of 5 ng/mL for nicardipine and the pyridine metabolite II is suitable for the analysis of these compounds in plasma collected from subjects treated orally with nicardipine hydrochloride at doses as low as 20 mg.

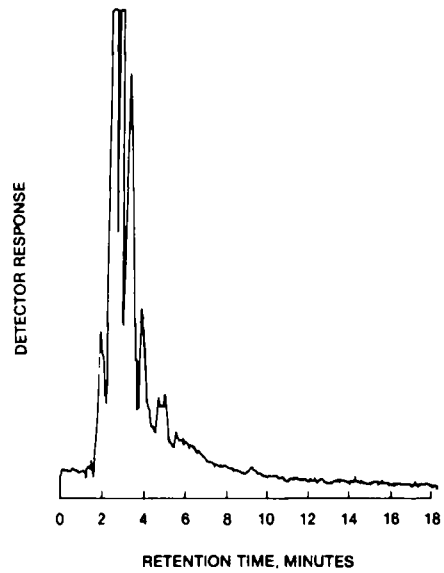


**Figure 1**—*HPLC chromatogram of a 1-mL blank human plasma sample. Extracted residue was dissolved in 70 μL of mobile phase, and 55 μL was injected onto the liquid chromatograph.*

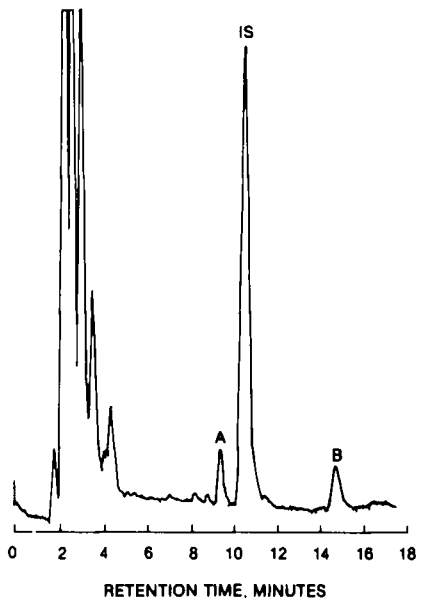


**Figure 2**—*HPLC chromatogram of a 1-mL blank human plasma sample spiked with nicardipine hydrochloride (A), 5 ng; internal standard (IS), 75 ng; pyridine metabolite II hydrochloride (B), 5 ng. Extracted residue was dissolved in 70 μL of mobile phase, and 55 μL was injected onto the liquid chromatograph.*

## EXPERIMENTAL SECTION

**Apparatus**—A high-performance liquid chromatograph[2] equipped with a fixed UV (254 nm) detector[3] and an automatic injector[4] was used. Separations were performed on a reverse-phase column[5] (250 × 4.6 mm i.d.). A precolumn[6] (70 × 2.1 mm i.d.) was used. Chromatograms were traced on a

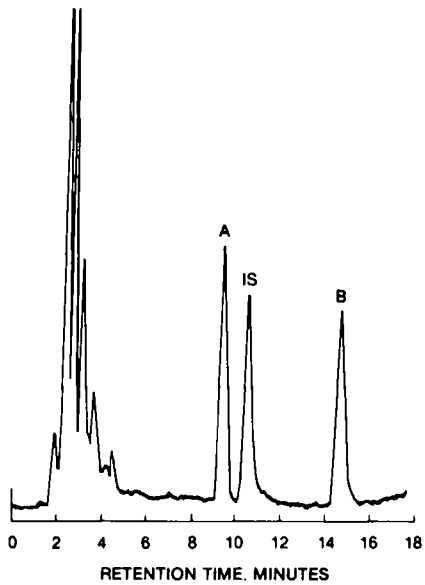


**Figure 3**—*HPLC chromatogram of a 1-mL blank human plasma spiked with nicardipine hydrochloride (A), 50 ng; internal standard (IS), 75 ng; pyridine metabolite II hydrochloride (B), 50 ng. Extracted residue was dissolved in 70 μL of mobile phase, and 25 μL was injected onto the liquid chromatograph.*

[2] Model 204; Waters Associates, Milford, Mass.
[3] Model 160; Beckman Instruments, Palo Alto, Calif.
[4] Model 710B, Waters Intelligent Sample Processor (WISP); Waters Associates.
[5] Partisil-5, ODS-3, 5 μm; Whatman Inc., Clifton, N.J.
[6] HC pellosil, 30–38 μm; Whatman Inc.

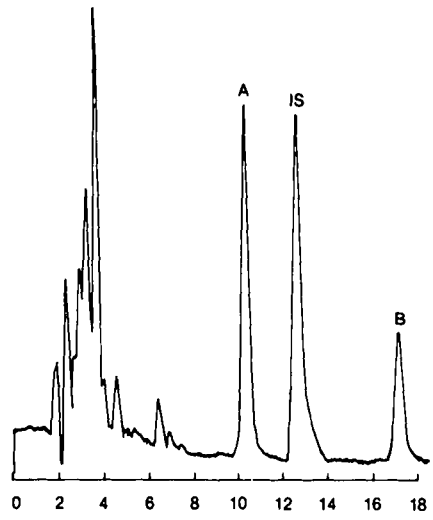


**Figure 4**—*HPLC chromatogram of a 1-mL sample of plasma collected 40 min after an oral dose of 20 mg of nicardipine hydrochloride. Extracted residue was dissolved in 70 μL of mobile phase, and 40 μL was injected onto the liquid chromatograph.*

strip-chart recorder[7]. The system was operated at ambient temperature, and the detector sensitivity was set at 0.002 AUFS.

**Chemicals and Reagents**—Nicardipine hydrochloride (I)[8], 2-(*N*-benzyl-*N*-methylamino)ethyl methyl 2,6-dimethyl-4-(*m*-nitrophenyl)-3,5-pyridinedicarboxylate monohydrochloride (II)[9], 2-(*N*-benzyl-*N*-methylamino)-ethyl isopropyl 1,4-dihydro-2,6-dimethyl-4-(*m*-nitrophenyl)-3,5-pyridinedicarboxylate monohydrochloride (III)[8], methanol[10] (HPLC grade), acetonitrile[10] (HPLC grade), absolute ethanol[11], and ether[12] (HPLC grade) were obtained commercially. All other chemicals and reagents were reagent grade.

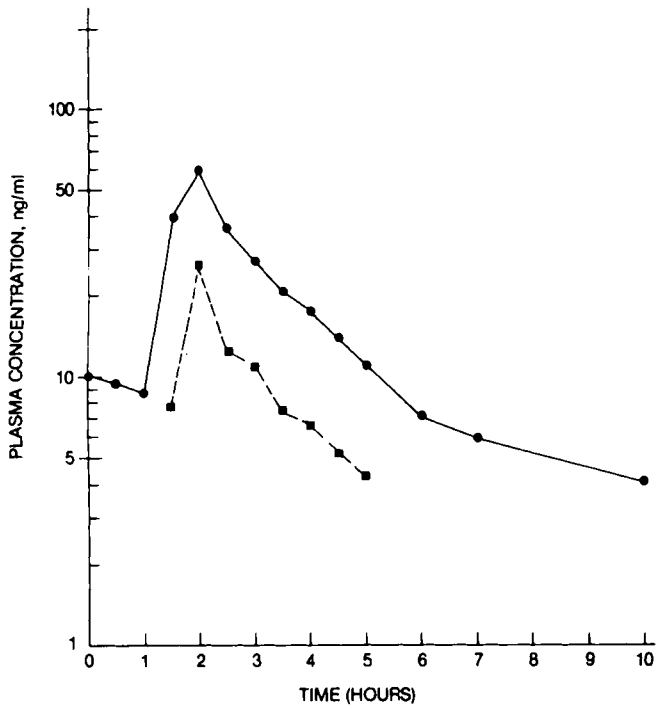


**Figure 5**—*Semilogarithmic plot of the plasma concentration–time profile for nicardipine (●) and its pyridine metabolite II (■) in one subject following an oral dose of 20 mg of nicardipine hydrochloride on day 4 of a multiple-dose study.*

[7] Model 7130A dual-channel strip chart recorder; Hewlett-Packard, Santa Clara, Calif.
[8] Yamanouchi Pharmaceutical Co. Ltd., Tokyo, Japan.
[9] Syntex Research Center, Edinburgh, Scotland.
[10] J. T. Baker Chemical Co.
[11] U.S. Industrial Chemical Co.
[12] Burdick and Jackson Laboratories.

**Table I—Nicardipine Recovery from Spiked Plasma Samples**[a]

| Nicardipine Hydrochloride Recovered, ng/mL | Nicardipine Hydrochloride Added, ng/mL | | | | | |
|---|---|---|---|---|---|---|
| | 5 | 10 | 20 | 50 | 100 | 150 |
| Mean | 4.9 | 9.2 | 19.2 | 51.1 | 103.4 | 148.1 |
| *SD* | 0.2 | 0.5 | 0.9 | 2.2 | 1.1 | 2.9 |
| *CV*, % | 4.1 | 5.0 | 4.5 | 4.3 | 1.1 | 1.9 |

[a] Total of four assays.

**Table II—Pyridine Metabolite II Recovery from Spiked Plasma Samples**[a]

| Pyridine Metabolite II Hydrochloride Recovered, ng/mL | Pyridine Metabolite II Hydrochloride Added, ng/mL | | | | | |
|---|---|---|---|---|---|---|
| | 5 | 10 | 20 | 50 | 100 | 150 |
| Mean | 5.1 | 9.6 | 20.3 | 51.1 | 98.8 | 151.3 |
| *SD* | 0.3 | 0.0 | 0.3 | 1.9 | 0.5 | 4.2 |
| *CV*, % | 4.9 | 0.0 | 1.4 | 3.7 | 0.5 | 2.8 |

[a] Total of four assays.

The aqueous 2.0 M NaOH solution was washed once with an equal volume of acetonitrile and once with an equal volume of ether before use. Acid buffer was prepared by mixing equal volumes of 0.1 M sodium acetate solution and 0.1 M hydrochloric acid.

**Chromatographic Conditions**—HPLC was performed by using a mobile phase of acetonitrile–0.02 M $KH_2PO_4$ (pH 4.8; 53:47 v/v) with a flow rate of 1.5 mL/min (2000 psi).

**Standard Solutions**—Stock solutions of the internal standard III, nicardipine hydrochloride (I), and the monohydrochloride of its pyridine metabolite II at a concentration of 1 mg/mL were prepared in ethanol. The working solution of internal standard III contained 75 ng/500 μL of 2% ethanolic acid buffer. Spiking solutions, containing 5, 10, 20, 50, 100, and 150 ng of the hydrochloride salts of nicardipine (I) or its pyridine metabolite II per 500 μL of 4% ethanolic acid buffer, were prepared by serial dilution of the stock solutions.

**Sample Preparation for the Determination of Nicardipine and Its Pyridine Metabolite II**—Human plasma (0.1–1.0 mL) was transferred into a 15-mL culture tube (16 × 125 mm) fitted with a polytef-lined screw cap. The volume was adjusted to 1 mL by the addition of blank human plasma[13]. Ethanolic acid buffer (4%, 1 mL), 500 μL of internal standard solution (III, 75 ng), and 500 μL of 2.0 M NaOH were added to the sample. The mixture was agitated on a vortex mixer for 15 s. The mixture was extracted with 4 mL of ether by shaking on a mechanical shaker for 5 min and centrifuging for 5 min at 5000 rpm. The organic layer was transferred to another 15-mL tube. To the organic phase was added 2 mL of 0.1 M HCl, and the mixture was shaken for 5 min. The mixture was centrifuged, and the organic layer was removed and discarded by using a vacuum aspirator. The aqueous fraction was made alkaline by the addition of 500 μL of 2.0 M NaOH. The mixture was extracted with 4 mL of ether as described previously. After centrifugation, the organic layer was transferred to another 15-mL culture tube, and the solution was evaporated to dryness under a stream of nitrogen in a water bath (45°C). The residue was immediately dissolved in 70 μL of HPLC mobile phase, and the solution was transferred to an autosampler vial[14]. Of this final solution, 20–55 μL was injected onto the liquid chromatograph for analysis.

**Quantitation**—Concentrations of nicardipine and the pyridine metabolite II in unknown samples were calculated with reference to their corresponding calibration curves, which were generated by using a 1-mL sample of blank plasma spiked with nicardipine hydrochloride and the monohydrochloride of the pyridine metabolite II at levels of 5, 10, 20, 50, 100, and 150 ng/mL. The samples of spiked plasma were processed as described above.

Calibration curves were obtained by plotting the ratio of the peak height for the analyte to that of the internal standard against the amount of analyte added.

**HPLC/GC-ECD Assay for the Pyridine Metabolite II**—This assay employed as the internal standard the pyridine form[1] of the internal standard III used for the direct HPLC assay described here. Preparation of the samples for HPLC was as described above. HPLC was performed as described above,

[13] Venous blood from four untreated volunteers was drawn into heparinized containers and centrifuged to generate a plasma pool.
[14] 1-Dram vial, 0.4-mL glass insert, compression spring, self-sealing septum, and self-sealing septum assembly; Waters Associates.

Table III—Comparisons of the Concentrations (ng/mL) of Nicardipine, Its Pyridine Metabolite II, and Total [a] in Plasma Determined by Different Methods

| Sample | Nicardipine | | II | | Total | |
|---|---|---|---|---|---|---|
| | GC-ECD + HPLC/GC-ECD ($y$) | HPLC ($x$) | HPLC/ GC-ECD ($y$) | HPLC ($x$) | GC-ECD ($y$) | HPLC ($x$) |
| 1 | 292.3 | 287.7 | 71.7 | 75.8 | 364.0 | 363.5 |
| 2 | 124.2 | 129.5 | 40.3 | 48.9 | 164.5 | 178.4 |
| 3 | 98.0 | 96.2 | 33.6 | 27.2 | 131.6 | 123.4 |
| 4 | 91.0 | 80.5 | 28.5 | 27.4 | 119.5 | 107.9 |
| 5 | 64.6 | 59.7 | 19.0 | 25.9 | 83.6 | 85.6 |
| 6 | 33.4 | 23.6 | 13.0 | 14.1 | 46.4 | 37.7 |
| 7 | 21.3 | 20.1 | 9.3 | 10.2 | 30.6 | 30.3 |
| 8 | 18.9 | 21.2 | 9.9 | 8.7 | 29.0 | 29.9 |
| 9 | 18.2 | 17.3 | 6.6 | 7.9 | 24.8 | 25.2 |
| 10 | 9.8 | 8.5 | 5.4 | 5.6 | 15.2 | 14.1 |
| Correlation equation | $y = 1.0022x + 2.5781$ | | $y = 0.9168x + 0.6541$ | | $y = 0.9883x + 2.4848$ | |
| Correlation coefficient | $r = 0.9984$ | | $r = 0.9825$ | | $r = 0.9978$ | |

[a] Total = Nicardipine plus its pyridine metabolite II.

except that a mobile phase of acetonitrile–0.01 M $KH_2PO_4$ (pH 4.8; 1:1) was employed. Under these conditions, the pyridine metabolite II and the internal standard coeluted and were well separated from nicardipine. The HPLC effluent containing the pyridine metabolite II and the internal standard was collected, made alkaline by the addition of 0.5 mL of 2.0 M NaOH, and extracted with ether (4 mL). The ether extract was evaporated to dryness, and the residue was reconstituted in 50 μL of toluene. A portion of the extract was subjected to GC-ECD analysis (2). The concentration of the pyridine metabolite II in plasma was determined by reference to suitable calibration curves which were constructed by using a procedure analogous to that described above.

## RESULTS AND DISCUSSION

The UV absorption maximum of nicardipine was at 237 nm. However, in this assay, the absorption at 254 nm was used for quantitation. Although the use of 254 nm reduces the theoretical sensitivity to some extent, it has the advantage of providing a cleaner chromatogram for blank plasma. A typical chromatogram for 1 mL of blank human plasma processed by this method is shown in Fig. 1. There were no components which eluted with nicardipine, the pyridine metabolite II, or the internal standard. To achieve a clean chromatogram of this type, we found that it was necessary to perform the acid–base partitioning step prior to the HPLC analysis. In addition, we found that the 2.0 M NaOH used in this procedure must be washed once with acetonitrile and once with ether prior to use. If unwashed 2.0 M NaOH is used, potentially interfering components appear in the chromatogram. By using the extraction procedure described above, the extraction efficiencies for nicardipine and its pyridine metabolite II from 1 mL of plasma were 81.1 and 79.6%, respectively.

Representative chromatograms for 1 mL of blank human plasma spiked with nicardipine hydrochloride and the monohydrochloride of the pyridine metabolite II, each at levels of 5 or 50 ng and processed by this procedure, are shown in Figs. 2 and 3, respectively. A typical chromatogram for 1 mL of plasma taken from a subject who received nicardipine hydrochloride is shown in Fig. 4.

A good separation of nicardipine, the pyridine metabolite II, and the internal standard from each other and from other components was obtained. During the HPLC separation, an acidic mobile phase (pH 4.8) with a relatively high buffer concentration (0.02 M $KH_2PO_4$) was used. The acidic mobile phase causes nicardipine, the pyridine metabolite II, and the internal standard to exist as the corresponding protonated species during chromatography, thereby leading to improved chromatographic peak shapes and reduced retention times. The high buffer concentration was found to be necessary to ensure a good separation between the pyridine metabolite II and the internal standard. At a lower buffer concentration (0.01 M $KH_2PO_4$), the separation of these three compounds and even their order of elution was found to vary as the column aged. Even with the higher buffer concentration, as the column aged, it was necessary to add a small quantity of methanol to the solvent system [*e.g.*, acetonitrile–0.02 M $KH_2PO_4$–methanol (43:49:8 v/v/v)], to maintain the elution order and the quality of the separation.

Calibration curves for nicardipine hydrochloride and the monohydrochloride of the pyridine metabolite II showed good linearity. Data for the recovery of nicardipine and the pyridine metabolite II from samples of plasma fortified with these compounds in the range of 5–150 ng/mL are shown in Tables I and II, respectively. The low coefficients of variation (≤5% at all levels) provide evidence of the reproducibility of the method.

The HPLC method was validated against a GC-ECD method which gave the combined concentration of nicardipine plus the pyridine metabolite II and against an HPLC/GC-ECD method, which gave the concentration of the pyridine metabolite II. All three methods were applied to samples of plasma obtained from subjects treated orally with nicardipine hydrochloride (Table III). A comparison of the levels of nicardipine, the pyridine metabolite II, and nicardipine–the pyridine metabolite II determined by the three methods is shown in Table III. The overall agreement between the results obtained by all three methods is good.

A plasma concentration–time profile for nicardipine and the pyridine metabolite II obtained for one subject on day 4 of a multiple-dose study (20 mg, three times per day) is presented in Fig. 5 as evidence of the usefulness of this method for pharmacokinetic analyses.

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