

High-Performance Liquid Chromatographic Determination of Pyridostigmine Bromide, Nicotine, and Their Metabolites in Rat Plasma and Urine

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Abstract

This study reports on the development of a rapid and simple method for the determination of the antinerve agent drug pyridostigmine bromide (3-dimethylaminocarbonyloxy-*N*-methyl pyridinium bromide) (PB), its metabolite *N*-methyl-3-hydroxypyridinium bromide, nicotine (*S*-1-methyl-5-(3-pyridyl)-2-pyrrolidine), and its metabolites nornicotine (2-(3-pyridyl)pyrrolidine) and cotinine (*S*-1-methyl-5-(3-pyridyl)-2-pyrrolidone) in rat plasma and urine. The compounds are extracted and eluted by methanol and acetonitrile using C₁₈ Sep-Pak cartridges and separated using high-performance liquid chromatography by a gradient of methanol, acetonitrile, and water (pH 3.2) at a flow rate of 0.8 mL/min in a period of 14 min. UV detection was at 260 nm for nicotine and its metabolites and at 280 nm for PB and its metabolite. The limits of detection ranged between 20 and 70 ng/mL, and the limits of quantitation were 50–100 ng/mL. The average percent recovery of five spiked plasma samples were 85.7 ± 7.3%, 80.4 ± 5.8%, 78.9 ± 5.4%, 76.7 ± 6.4%, and 79.7 ± 5.7% and for urine were 85.9 ± 5.9%, 75.5 ± 6.9%, 82.6 ± 7.9%, 73.6 ± 5.9%, and 77.7 ± 6.3% for nicotine, nornicotine, cotinine, PB, and *N*-methyl-3-hydroxypyridinium bromide, respectively. The calibration curves for standard solutions of the compounds of peak areas and concentration are linear for a range between 100 and 1000 ng/mL. This method is applied in order to analyze the previously mentioned chemicals and metabolites following their oral administration in rats.

Introduction

Pyridostigmine bromide (PB) is used in the treatment of myasthenia gravis patients (1,2). PB was used during the Gulf War to protect military personnel from a possible attack by nerve agents (3,4). People are exposed to nicotine, which is a natural alkaloid, during smoking or inhalation of environmental tobacco smoke (5,6). PB has been reported to absorb into plasma and excrete in urine following an oral or intravenous dose in rats (7–11),

humans (12,13), and dogs (14). Cigarette smoking increases the risk of lung and heart diseases (15) as well as induced oxidative stress and DNA damage (16,17). Nicotine primarily metabolizes to cotinine in the human liver (17). Nicotine and its metabolites have been determined in the plasma and urine of smokers or people who were exposed to smoke in public places (18,19). The following analytical methods have been used for the determination of *N*-methyl-3-hydroxypyridinium bromide, PB, nicotine, nornicotine, and cotinine when applied alone in biological matrices: high-performance liquid chromatography (HPLC) (7,10,19–22), HPLC–mass spectrometry (MS) (23,24,25), gas chromatography (GC) (13,26,27), and GC–MS (28,29). Veterans are commonly treated with PB and exposed to nicotine. For this study, we hypothesize that a combined exposure to both compounds could result in toxic interactions. We plan to study the pharmacokinetic profile of nicotine and PB, alone and in combination. In order to achieve this task, a method was needed for the simultaneous determination of the parent compounds and their metabolites in plasma and urine. This study presents a simple method for the determination of PB, nicotine, and their metabolites in rat plasma and urine using solid-phase extraction (SPE) and reversed-phase (RP) HPLC.

Experimental

Chemicals and materials

PB (3-dimethylaminocarbonyloxy-*N*-methyl pyridinium bromide), nicotine, nornicotine, and cotinine (Figure 1) were purchased from Sigma Chemical (St. Louis, MO). *N*-methyl-3-hydroxypyridinium bromide was prepared in our laboratory following the method of Somani et al. (12). Water (HPLC-grade) and acetonitrile were obtained from Mallinckrodt Baker (Paris, KY). C₁₈ Sep-Pak^R cartridges were obtained from Waters Corporation (Milford, MA).

Animals

Rats (Sprague Dawley) were purchased from Zivic Miller

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(Zelienople, PA). The animals were kept in plastic metabolic cages. Five rats were treated with the combination of a single oral dose of 13 mg/kg PB and 10 mg/kg nicotine. Five untreated control rats were treated with an oral dose of water. The animals were held in metabolic cages that allowed for the collection of urine samples. Urine samples were collected from treated and control rats after 12 h of dosing. The animals were anesthetized with halothane and sacrificed by heart exsanguinations at 12 h. Blood was collected via the heart puncture with a heparinized syringe and centrifuged at 2400 rpm for 15 min at 5°C in order to separate plasma. Urine and plasma samples were stored at -20°C prior to analysis.

Instrumentation

The liquid chromatographic system (Waters 2690 separation module) consisted of Waters 600E multisolvent delivery system pumps, a Waters Ultra WISP 715 autoinjector, and a Waters 2487 Dual λ absorbance detector. A guard column (2 cm \times 4.0 mm, 5 μ m) (Supelco Park, Bellefonte, PA) and an RP μ Bondapak C₁₈ column (Waters Corporation) (10 μ m, 3.9 \times 300 mm) were used.

Sample preparation

A 0.5-mL plasma and 1-mL urine sample from untreated rats were spiked with concentrations ranging between 100 and 1000

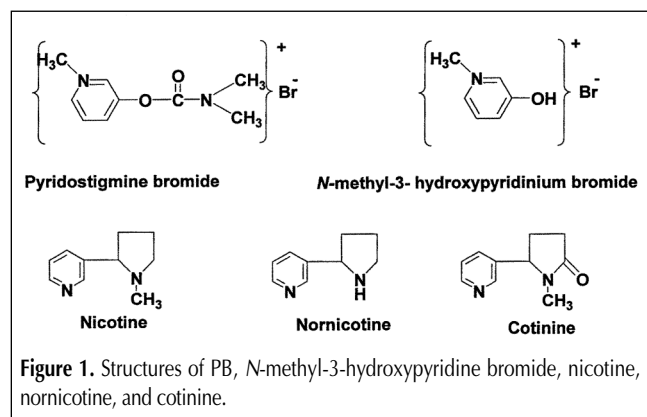


Figure 1. Structures of PB, *N*-methyl-3-hydroxypyridine bromide, nicotine, normnicotine, and cotinine.

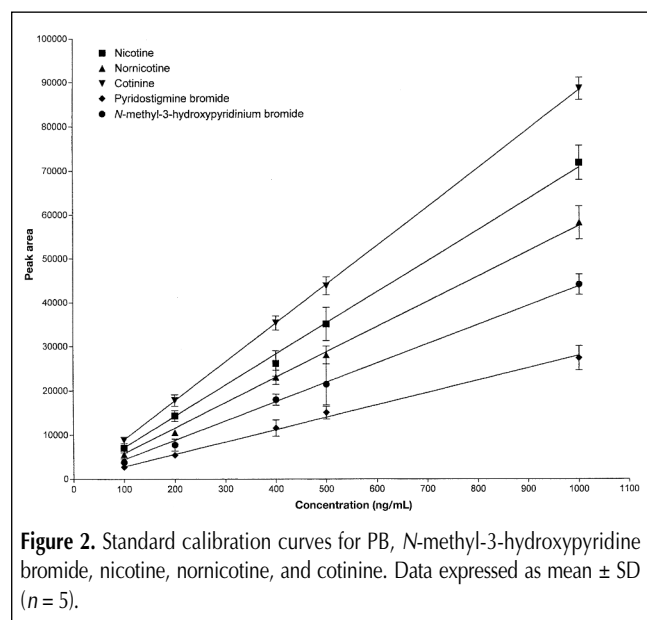


Figure 2. Standard calibration curves for PB, *N*-methyl-3-hydroxypyridine bromide, nicotine, normnicotine, and cotinine. Data expressed as mean \pm SD ($n = 5$).

ng/mL each of PB, *N*-methyl-3-hydroxypyridinium bromide, nicotine, normnicotine, and cotinine. Spiked and treated samples were acidified with 1N acetic acid (pH 5.0). Disposable C₁₈ Sep-Pak Vac 3 cm³ (500 mg) cartridges (Waters Corporation) were conditioned with 3 mL of acetonitrile then equilibrated using 3 mL water prior to use. Prior to analysis by HPLC, the spiked urine and plasma samples were vortexed for 30 s and centrifuged for 5 min at 1000 g. The supernatant was then loaded into the disposable cartridges, washed with 3 mL of water, and then eluted twice by 1 mL of methanol, twice using 2 mL of acetonitrile, and reduced to 500 μ L using a gentle stream of nitrogen at room temperature.

Accuracy and precision

Intraday precision and accuracy of the method were determined in plasma and urine samples. The plasma and urine samples ($n = 5$) were spiked with concentrations of 100, 200, 400, 500,

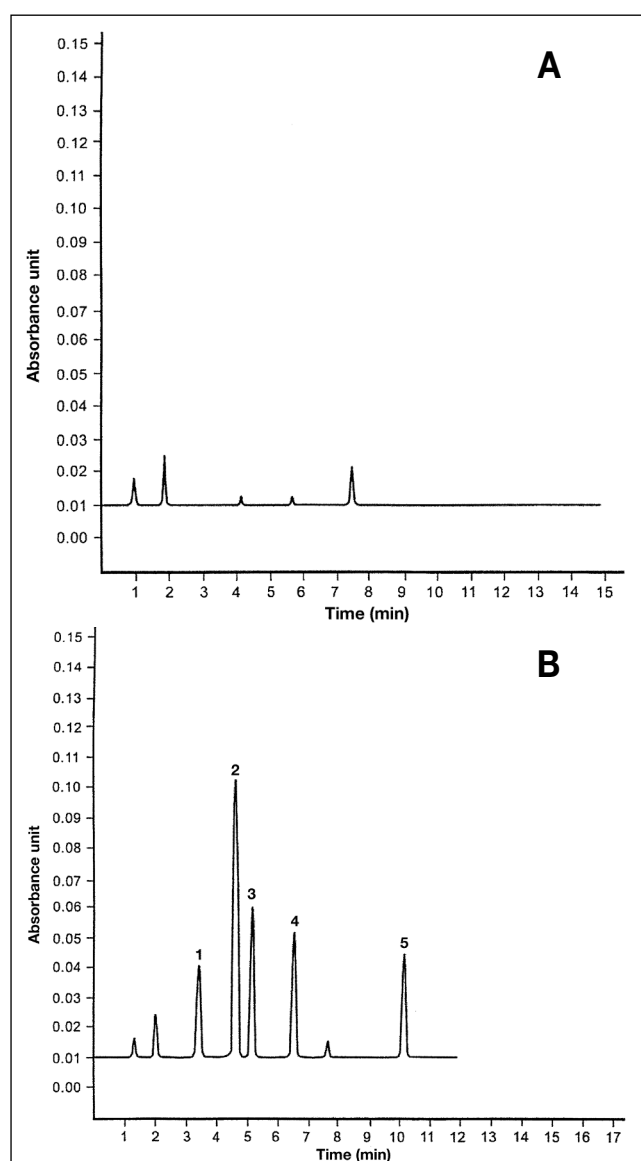


Figure 3. Chromatograms of (A) a blank plasma sample and (B) a spiked plasma sample with 500 ng/mL of (1) normnicotine, (2) nicotine, (3) *N*-methyl-3-hydroxypyridine bromide, (4) cotinine, and (5) PB under established HPLC conditions. Compounds 1, 2, and 4 were detected at 260 nm, and compounds 3 and 5 were detected at 280 nm.

and 1000 ng/mL. The samples were analyzed on the same day. The relative error percent accuracy was determined as the mean of the detected concentration divided by the added concentration and then multiplied by 100. For the determination of precision, the coefficient of variation (CV) was calculated.

Chromatographic conditions

A 10- μ L solution of plasma or urine residues was injected into the HPLC. The mobile phase was 10% methanol in water (adjusted to pH 3.2 using 1N acetic acid) for the 5-min mark. Then, it was changed to a gradient of acetonitrile in water (adjusted to pH 3.2 using 1N acetic acid) at 6 min. The gradient started at 20% acetonitrile, increased to 35% acetonitrile at 10 min, and then the system returned to 10% methanol in water (adjusted to pH 3.2 using 1N acetic acid) at 12 min in which it was

kept under this condition for 2 min to re-equilibrate. The flow rate was 0.8 mL/min, and the eluents were monitored by the UV detection of a 280-nm wavelength for PB and *N*-methyl-3-hydroxypyridinium bromide and a 260-nm wavelength for nicotine, nornicotine, and cotinine. The chromatographic analysis was performed at ambient temperature.

Calibration procedures

Five different calibration standards of a PB, *N*-methyl-3-hydroxypyridinium bromide, nicotine, nornicotine, and cotinine mixture were prepared in acetonitrile. Their concentrations ranged from 100 to 1000 ng/mL. Linear calibration curves were obtained by plotting the average peak areas of five replicants of the individual chemicals as a function of the concentration using the GraphPad Prism program for Windows (GraphPad Software, San Diego, CA). The standard curves were used to determine the recovery of the chemicals from plasma and urine samples.

Limits of detection and limits of quantitation

The limits of detection (LODs) were determined at the lowest concentration to be detected taking into consideration a 1:3 baseline noise–calibration point ratio. A reproducible lowest possible concentration was considered as the limit of quantitation (LOQ), and it was repeated five times for confirmation.

Results

Standard calibration curves

The standard calibration curves of the peak area versus the concentration of PB, *N*-methyl-3-hydroxypyridinium bromide, nicotine, nornicotine, and cotinine are shown in Figure 2. The linearity of the calibration curves of the parent compounds and their metabolites was achieved at concentrations ranging from 100 to 1000 ng/mL.

Chromatogram

Chromatographic profiles of blank and spiked plasma and urine samples after SPE using C_{18} Sep Pak^R cartridges under HPLC conditions described previously are shown in Figures 3A, 3B, 4A, and 4B. The retention times were 3.3 min, 4.5 min, 4.9 min, 6.9 min, and 10.3 min for nornicotine, nicotine, *N*-methyl-3-hydroxypyridinium bromide, cotinine, and PB, respectively. The total run time was 14 min. A clean chromatogram shows no interference from endogenous substances in plasma and urine samples. This suggests an efficient sample-preparation and clean-up method.

Extraction efficiency

The average extraction recoveries for PB, *N*-methyl-3-hydroxypyridinium bromide, nicotine, nornicotine, and cotinine were determined at concentrations ranging between 100 and 1000 ng/mL (Tables I and II). Spiked plasma and urine samples were extracted and analyzed for each concentration in five replicates. The average percent recoveries from plasma were $85.7 \pm 7.3\%$, $80.4 \pm 5.8\%$, $78.9 \pm 5.4\%$, $76.7 \pm 6.4\%$, and $79.7 \pm 5.7\%$ and from urine were $85.9 \pm 5.9\%$, $75.5 \pm 6.9\%$, $82.6 \pm 7.9\%$, $73.6 \pm 5.9\%$,

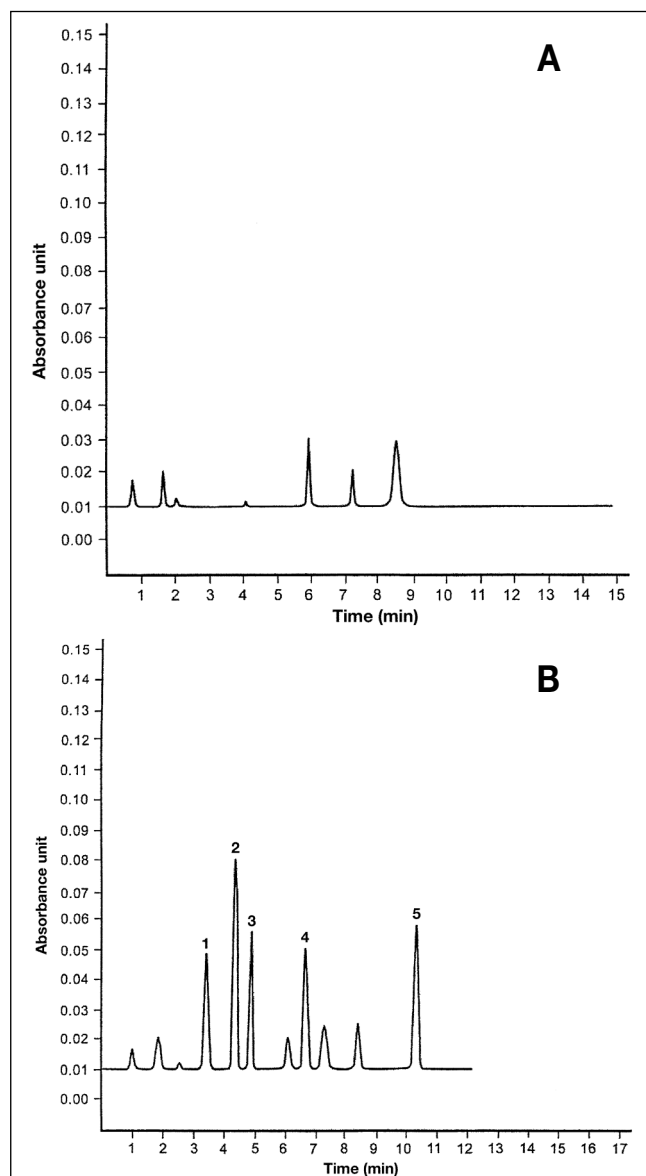


Figure 4. Chromatograms of (A) a blank urine sample and (B) a spiked urine sample with 500 ng/mL of (1) nornicotine, (2) nicotine, (3) *N*-methyl-3-hydroxypyridine bromide, (4) cotinine, and (5) PB under established HPLC conditions. Compounds 1, 2, and 4 were detected at 260 nm, and compounds 3 and 5 were detected at 280 nm.

and $77.7 \pm 6.3\%$ for nicotine, nornicotine, cotinine, PB, and *N*-methyl-3-hydroxypyridinium bromide, respectively.

Accuracy and precision

Results from the intraday accuracy and precision were calculated as described previously. The average percent relative error of accuracy for all of the added concentrations to the plasma samples were $3.2 \pm 0.8\%$, $2.6 \pm 1.0\%$, $2.9 \pm 1.2\%$, $3.8 \pm 1.4\%$, and $4.1 \pm 1.2\%$ and for the urine samples were $2.4 \pm 0.6\%$, $2.8 \pm 0.9\%$, $3.2 \pm 1.1\%$, $3.2 \pm 1.0\%$, and $3.7 \pm 1.4\%$ for nicotine, nornicotine, cotinine, PB, and *N*-methyl-3-hydroxypyridinium bromide, respectively. Intraday precision was determined as the %CV for plasma and urine samples ranging between $1.3 \pm 0.8\%$ and $2.6 \pm 1.1\%$.

LODs

Blank plasma and urine samples from untreated rats were used as references for plasma and urine collections. The LODs were calculated from a peak signal-to-noise ratio of 3:1. The resulting LODs were 20, 50, 30, 50, and 70 ng/mL for nicotine, nornicotine, cotinine, *N*-methyl-3-hydroxypyridinium bromide, and PB, respectively.

LOQs

The LOQs from plasma and urine were determined to be 50, 100, 50, 100, and 100 ng/mL for nicotine, nornicotine, cotinine, *N*-methyl-3-hydroxypyridinium bromide, and PB, respectively.

Application of the method to biological samples

For the purpose of validation, the method was applied for the analysis of the previously mentioned chemicals and their metabolites following an oral dose in rats. Following the administration of PB and nicotine, the animals developed toxic symptoms such as tremors, seizures, weakness, and difficulty in standing. The rats were sacrificed 12 h after the dosing. Nicotine, cotinine, *N*-methyl-3-hydroxypyridinium bromide, and PB were detected in plasma. Their levels were 408 ± 171 ng/mL, 236 ± 118 ng/mL, 379 ± 164 ng/mL, and 248 ± 92 ng/mL for nicotine, cotinine, *N*-methyl-3-hydroxypyridinium bromide, and PB, respectively. Cotinine and *N*-methyl-3-hydroxypyridinium bromide were identified in the rat urine. Their concentrations were 946 ± 278 ng/mL and 542 ± 182 ng/mL, respectively. Nornicotine was not detected in the urine or plasma samples.

Discussion

This study reports the development of an HPLC method for the simultaneous analysis of PB, nicotine, and their metabolites in rat plasma and urine.

Recoveries of the chemicals and metabolites were suitable for the application of the method for the analysis of treated plasma and urine samples for parent compounds or their metabolites. A low recovery for PB might have resulted from the use of a solvent system that was not quite suitable for extracting PB; however, it was needed for extracting and analyzing the other two chemicals and metabolites under similar conditions. Also, the hydrolysis of PB during the extraction was possible. In a previous study, Aquilonius and Hartvig (2) reported that the hydrolysis of PB could take place in buffer solutions, plasma, and blood. Hennis et al. (14) reported a recovery of 50% of *N*-methyl-3-hydroxypyridinium from dog plasma and urine, and Chan et al. (30) reported a recovery of 82% of PB. Also in a previous report, Oddoze et al. (22) reported that a recovery of nicotine from human and rat urine was between 47% and 86% and for cotinine between 92% and 100%. In our method, recoveries differed with individual compounds. The recovery of the analyzed chemicals was between 74% and 86%. This range lies within the reported values in the literature, taking into consideration simultaneous analysis of the parent chemicals and their metabolites.

The linearity of standard calibration curves for the chemicals in the studied method was consistent with previous reports. Chan et al. (30) reported a linear range for PB in human plasma over concentrations between 50 and 1000 ng/mL.

The chromatogram obtained following SPE and HPLC analysis showed no interference from plasma and urine components, indicating an efficient clean-up method used.

The LODs reported in our method allowed for

Table I. %Recovery* of Nicotine, PB, and Their Metabolites from Rat Plasma

<i>N</i> -methyl-3-hydroxypyridinium Concentration (ng/mL)	Nicotine	Nornicotine	Cotinine	PB	bromide
100	85.8 ± 7.4	73.2 ± 6.3	81.0 ± 4.5	73.2 ± 5.6	75.5 ± 4.1
200	83.9 ± 8.6	80.1 ± 6.5	78.2 ± 6.3	75.7 ± 8.2	79.3 ± 5.6
400	85.9 ± 4.7	83.2 ± 6.0	80.6 ± 6.4	77.2 ± 6.2	80.4 ± 9.6

Table II. %Recovery* of Nicotine, PB, and Their Metabolites from Rat Urine

<i>N</i> -methyl-3-hydroxypyridinium Concentration (ng/mL)	Nicotine	Nornicotine	Cotinine	PB	bromide
100	82.8 ± 5.4	71.1 ± 9.0	81.6 ± 8.6	73.1 ± 3.8	71.5 ± 6.1
200	83.6 ± 9.2	74.2 ± 6.5	80.1 ± 7.3	74.0 ± 8.0	75.7 ± 8.2
400	85.1 ± 6.2	73.9 ± 6.2	82.1 ± 6.3	70.6 ± 7.2	78.2 ± 7.8

the analysis of samples from treated animals following doses resembling real exposure. The ability to detect the compounds and metabolites in plasma after 12 h of dosing is evidence of the method suitability. The LODs and LOQs depended upon the nature of the matrix, rate of application, and method of analysis (14,30). Hennis et al. (14) reported 50 ng/mL as an LOD for *N*-methyl-3-hydroxypyridinium in dog plasma using ion-exchange liquid chromatography, and Miller and Verma (9) reported 2.5 ng/mL for PB in tissues using the radioimmunoassay method. When using an HPLC technique, the LOD for PB in plasma was 10 ng/mL (10). The LOD for cotinine in smokers' serum was 50 ng/L using HPLC-MS (23). According to James et al. (20), the LOD for nicotine and cotinine in human urine and serum was 0.16 ng/mL, and the LOQ was 1.25 ng/mL using GC-MS. Using HPLC-UV detection, Nakajima et al. (19) found that the LOD for nicotine and cotinine in human plasma was 0.2 ng/mL and 1 ng/mL, respectively. In another study, the LOD for cotinine was 10 ng/mL in smokers' plasma using HPLC-UV (20). The reported LODs in the literature are consistent with our results for the simultaneous analysis of the combined chemicals and their metabolites, which ranged between 20 and 70 ng/mL.

A simple HPLC method was developed in this study for the separation and quantitation of PB, nicotine, and their metabolites in rat plasma and urine samples. The method could be used in pharmacokinetic studies to assess the distribution of the parent compounds and metabolites in body tissues and fluids. The main advantage of the method is the ability to analyze simultaneously the chemicals and their metabolites under similar conditions, thus saving time and expenses for sample preparation.

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