Determination of a Novel Calcium Channel Antagonist, Mepirodipine, in Plasma by Radioreceptor Assay Using (+)-[^{3}H]PN 200-110

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Received September 25, 1991; accepted March 10, 1992
KEY WORDS: calcium channel antagonist; mepirodipine; plasma concentration; radioreceptor assay; gas chromatography.

INTRODUCTION

Mepirodipine hydrochloride, (+)-(3′*S*,4*S*)-3-(1′-benzyl-3′-pyrrolidinyl)-methyl 2,6-dimethyl-4-(*m*-nitrophenyl)-1,4-dihydropyridine-3,5-dicarboxylate hydrochloride, a potent and long-lasting dihydropyridine calcium channel antagonist, has been tested for the treatment of hypertension (1–4). The plasma concentration of mepirodipine is low, owing to its low effective dose (5) and extensive metabolism (6), and thus, a sensitive procedure for the determination of mepirodipine in biological fluids is required. Recently, Teramura *et al.* (7) developed a sensitive and specific assay of mepirodipine in human plasma by specific capillary column gas chromatography–negative-ion chemical ionization mass spectrometry (GC-NICI-MS).

A number of assay procedures for dihydropyridine calcium channel antagonists in biological fluids have been reported. They include high-performance liquid chromatography (HPLC) and the combination of HPLC with gas chromatography (GC) (8,9), GC with electron-capture detection (ECD) (10), and GC with electron-impact mass spectrometry (EI-MS) (11,12). These methods have several shortcomings such as poor sensitivity, large sample size, and time-consuming determinations. In contrast, the radioreceptor assay (RRA) is useful for the determination of drug concentration in biological fluids by the competitive inhibition of specific binding of radioligand by administered drugs (13–15). The assay procedure is more simple and less time-consuming than GC or HPLC methods. Several investigators have reported RRA procedures for dihydropyridine calcium channel antagonists in plasma, e.g., nifedipine, nicardipine, nitrendipine, and benidipine (16–19), with [^{3}H]nitrendipine as the radioligand. (+)-[^{3}H]PN 200-110 has been developed to label the dihydropyridine receptors in the cerebral cortex, heart, and blood vessels with an approximately 10 times higher affinity than [^{3}H]nitrendipine (4,20). We report here a simple and highly sensitive RRA procedure using (+)-[^{3}H]PN 200-110 for the determination of mepirodipine in the plasma.

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MATERIALS AND METHODS

Materials

(+)-[^{3}H]PN 200-110 (87.0 Ci/mmol; radiochemical purity, 99%) was purchased from New England Nuclear (Boston, MA). Mepirodipine hydrochloride and nicardipine hydrochloride were kindly donated by Yamanouchi Pharmaceutical Company (Tokyo), and nifedipine hydrochloride by Bayer Pharmaceutical Company (Ohsaka, Japan). These drugs were dissolved in methanol, and mepirodipine hydrochloride was dissolved in 10% polyethyleneglycol 400 and 10% ethanol for oral administration in rats. The stock solution (1 m*M*) of mepirodipine hydrochloride was prepared once per 2 to 3 weeks, kept at −20°C, and diluted before use. The solution was properly diluted with methanol, and it was added to the RRA system for the construction of standard curves. All other chemicals were obtained from commercial sources.

For the determination of blood levels, male Wistar rats (250–300 g) or male Sprague–Dawley rats (400–450 g) were administered mepirodipine hydrochloride orally (5.68 μmol/kg). After 5 min to 6 hr, the blood was taken from the tail vein of Sprague–Dawley rats for pharmacokinetic analysis. In the second series of experiments for the comparison of plasma concentrations of mepirodipine hydrochloride by RRA and GC-NICI-MS methods, the blood was taken from the aorta of Wistar rats at 0.5 to 12 hr after administration. The plasma was isolated by centrifugation.

Extraction

Mepirodipine was extracted from plasma by a modification of the method described for benidipine hydrochloride by Ishii *et al.* (18). Two volumes of methanol was added to 1 vol of the plasma sample. After stirring, the mixture was centrifuged at 15,000*g* for 15 min. The supernatant was transfered to the glass centrifuge tube by decantation, and 0.01 *N* NaOH (1 ml) and ethyl ether (2 ml) were added and vortexed for 1 min. After brief centrifugation, the ethyl ether was transfered to a glass assay tube and evaporated under nitrogen stream. The residue was dissolved in 50 μl methanol and used as radioreceptor sample. For the standard curve, 50 μl of drug solution was added to the drug free plasma. The deproteinization and extraction procedures were performed as described above. The determination of nicardipine and nifedipine was performed as described for mepirodipine.

The determination of mepirodipine hydrochloride in the plasma by GC-NICI-MS was carried out as described previously (7).

Receptor Preparation

Rat cerebral cortical membranes, which are rich in dihydropyridine binding sites (20), were prepared and utilized as a source of dihydropyridine receptors. The cerebral cortex from male Wistar rats weighing 250–300 g was homogenized in 10 vol of ice-cold 50 m*M* Tris–HCl buffer (pH 7.4). The homogenate was centrifuged at 40,000*g* for 15 min at 4°C and the pellet was suspended in the original volume of Tris–

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HCl buffer. It was recentrifuged at 40,000*g* for 15 min at 4°C, and the resulting pellet was finally resuspended in the original volume of buffer. Aliquots were kept at −70°C until RRA for no more than 10 days. After thawing, the homogenate suspension was diluted with Tris–HCl buffer and used in the (+)-[^{3}H]PN 200-110 binding assay.

Binding Assay

The binding assay of (+)-[^{3}H]PN 200-110 was performed according to the method of Lee *et al.* (20) and Yamada *et al.* (4). The assay was performed in a total volume of 1 ml, adding rat cortical membranes (200 μg protein/assay, 800 μl) to test tubes containing (+)-[^{3}H]PN 200-110 (final concentration, 0.2 n*M;* 50 μl) and "plasma extract" (dissolved in 50μl methanol) in 50 m*M* Tris–HCl buffer (pH 7.4). After 60 min of incubation at 25°C, the reaction was terminated by rapid filtration (Cell Harvester, Brandel Co. Gaithersburg, MD) through Whatman GF/B glass fibers, and filters were rinsed three times with 3 ml of ice-cold buffer. Tissue-bound radioactivity was extracted from the filters overnight in scintillation fluid (2 liters of toluene, 1 liter of Triton X-100, 15 g of 2,5-diphenyloxazole, 0.3 g of 1,4-bis[2-(5-phenyloxazolyl)]benzene) and the radioactivity was determined in a liquid scintillation counter. Specific (+)-[^{3}H]PN 200-110 binding was determined experimentally from the difference between counts in the absence and counts in the presence of 3 μ*M* nifedipine (100 μl). Assays were carried out under a sodium lamp. The specific (+)-[^{3}H]PN 200-110 binding to rat cerebral cortex under this analytical condition was approximately 93.3 ± 11.9% (n = 6) of the total binding. Also, the effect of rat plasma on specific (+)-[^{3}H]PN 200-110 binding was examined by the direct addition to the RRA assay tube without an extraction procedure.

Data Analysis

The concentration of mepirodipine in the plasma was estimated using a computer program for radioimmunoassay (RIA 1, Kodensha).

The plasma concentration of mepirodipine after oral administration was analyzed according to the two-compartment open model with first-order absorption process. Data were fitted by the nonlinear least-squares regression program, MULTI (21). The area under the plasma concentration–time curve ($AUC_{0-\infty}$) after oral administration was calculated by the trapezoidal rule for the observed values and extrapolation to infinity. Total plasma oral clearance (Cl) was calculated using the equation Cl = dose/$AUC_{0-\infty}$.

RESULTS AND DISCUSSION

Previous investigators have assayed the plasma concentrations of nifedipine and nitrendipine by the direct addition of human plasma to the RRA tube (16,17), but the application of these assays is limited to small volumes of (highly concentrated) samples, because of the inhibition of ligand binding by plasma. In the present study, the direct addition of rat plasma (10–500 μl) to the assay tube was shown to reduce the cortical (+)-[^{3}H]PN 200-110 binding by 10–80% in proportion to the amount of plasma. The methanol deproteinization of plasma (500 μl) and the ethyl ether extraction under alkaline conditions markedly reduced the inhibitory effect of plasma itself on specific (+)-[^{3}H]PN 200-110 binding, yielding 96.4 ± 2.9% (n = 6) of the specific binding in the absence of plasma extract.

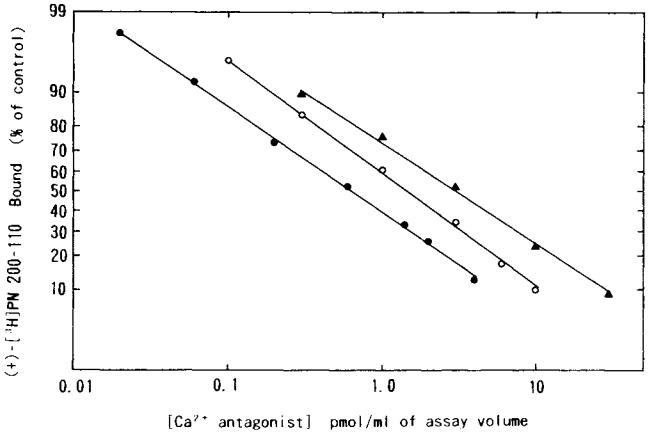

Fig. 1. Standard curves (logit-log scale) in the radioreceptor assay of mepirodipine hydrochloride (●), nicardipine hydrochloride (○), and nifedipine hydrochloride (▲). Different concentrations of mepirodipine hydrochloride, nicardipine hydrochloride, and nifedipine hydrochloride were added to rat blank plasma, and the radioreceptor assay of these drugs was performed as described under Materials and Methods. The points represent a typical standard curve for each drug. Each standard curve fits to the linear equation. Similar standard curves were obtained in seven (mepirodipine) and three (nicardipine, nifedipine) experiments.

The reliability of RRA for mepirodipine was evaluated by testing its linearity over a range of concentrations from 0.06 pmol (31.7 pg) to 4.0 pmol (2.1 ng) of mepirodipine hydrochloride/assay in the presence of rat blank plasma (300 μl). As shown in Fig. 1, the linear correlation between the probit of the percentage inhibition of specific (+)-[^{3}H]PN 200-110 binding and the logarithm of the rat plasma mepirodipine concentration was observed. Similar linear curves on a logit-log scale were obtained in the presence of human or dog plasma (data not shown). The lower limit of detection by this method, defined as the amount of unlabeled mepirodipine hydrochloride displacing approximately 25% of the control (+)-[^{3}H]PN 200-110 binding, was about 0.2 pmol/assay. When highly concentrated samples are used, the amount of plasma could be decreased. The standard curves for nicardipine hydrochloride (0.1–10.0 pmol/assay) and nifedipine hydrochloride (0.3–30.0 pmol/assay) were also lin-

Table I. Precision of the Radioreceptor Assay for Mepirodipine Hydrochloride

	Intraassay		Interassay	
Added (pmol/ml)	Found (pmol/ml)[a]	CV (%)	Found (pmol/ml)[a]	CV (%)
0.20	0.19 ± 0.02	9.9	0.20 ± 0.02	10.0
1.40	1.36 ± 0.12	8.8	1.37 ± 0.06	4.4

[a] Mean ± SD of nine (intraassay) or four (interassay) determinations.

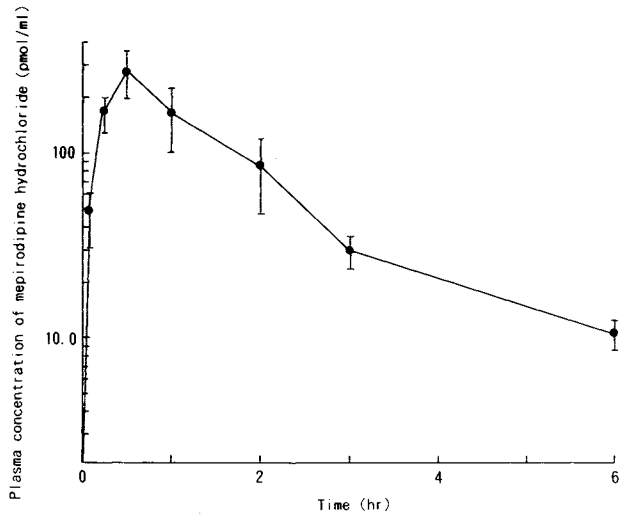

Fig. 2. Plasma concentration–time curve measured by radioreceptor assay after oral administration of mepirodipine hydrochloride to rats. The blood was taken from the tail vein at 5 min to 6 hr after oral administration of mepirodipine hydrochloride (5.68 μmol/kg) to Sprague–Dawley rats. Each point represents the mean ± SD of four rats.

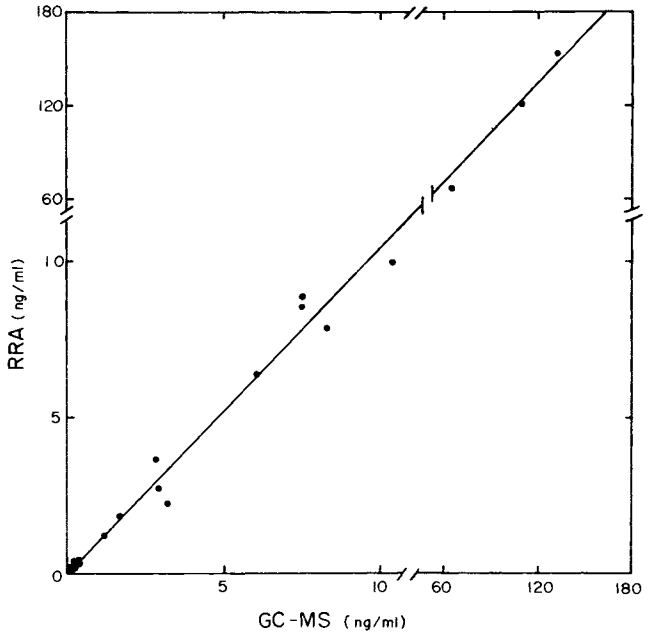

Fig. 3. Correlation between plasma concentrations of mepirodipine hydrochloride determined by radioreceptor assay (RRA) and gas chromatography–negative-ion chemical ionization mass spectrometry (GC-MS) procedures in rats. The plasma samples taken from the aorta at 0.5 to 12 hr after oral administration of mepirodipine hydrochloride (5.68 μmol/kg) to Wistar rats were analyzed for mepirodipine by both procedures. The points represent single data pairs from 18 rats. The values fit the linear equation $y = 1.16x - 0.08$ ($r = 0.998$).

ear in the presence of rat plasma (Fig. 1). The detection limits of nicardipine hydrochloride and nifedipine hydrochloride were approximately 0.3 and 1.0 pmol/assay, respectively.

To examine the reproducibility and reliability of the RRA procedure, two different concentrations of mepirodipine hydrochloride were added to the rat plasma. The coefficient of variation (CV) for the concentrations of 0.20 and 1.40 pmol/ml were 9.9 and 8.8%, respectively, in the intraassay and 10.0 and 4.4%, respectively, in the interassay (Table I).

Figure 2 shows the plasma concentration–time curves after the oral administration of mepirodipine hydrochloride at a dose of 5.68 μmol/kg. Following the administration, mepirodipine was rapidly absorbed, the plasma concentration attained the maximum level (269 ± 88 pmol/ml; $n = 4$) at 0.5 hr, and thereafter it gradually disappeared, showing 10.4 ± 1.0 pmol/ml after 6 hr. The pharmacokinetic parameters derived from the nonlinear least-squares regression program (MULTI) are shown in Table II.

The plasma samples (0.5–12 hr) from rats administered mepirodipine hydrochloride orally (5.68 μmol/kg) were analyzed by RRA, and the values were compared with those measured by GC-NICI-MS method of the same samples. As shown in Fig. 3, there was a good agreement in the values for mepirodipine hydrochloride between the two assay methods. This finding coincides with the previous observation that active metabolites of mepirodipine either are not produced or do not accumulate to significant levels (6). The relative sensitivity of the RRA and GC-NICI-MS methods for measurement of mepirodipine appears to be similar.

It was reported previously that the detection limits of nicardipine and nifedipine by RRA were approximately 10 pmol/ml in human plasma (16,19). The higher sensitivity and accuracy of measurements of drugs by RRA could be obtained by using selective radioligands with a high affinity. [^{3}H]Nitrendipine has been utilized as a ligand previously to measure the plasma level of nifedipine, nicardipine, and nitrendipine by RRA (16–19). We and others have demonstrated that (+)-[^{3}H]PN 200-110 binds to the dihydropyridine receptors, with an approximately 10 times higher affinity and lower proportion of nonspecific binding than [^{3}H]nitrendipine (4,20). In the present study, the sensitivity of RRA procedure for calcium channel antagonists has been shown to be significantly enhanced by the extraction and

Table II. Pharmacokinetic Parameters After Oral Administration of Mepirodipine Hydrochloride to Rats

K_a (hr^{-1})[a]	α (hr^{-1})[a]	β (hr^{-1})[a]	C_{max} (pmol/ml)[a,b]	Cl (L/hr/kg)[a]	$AUC_{0-\infty}$ (pmol · hr/ml)[a]
5.29 ± 3.08	4.98 ± 3.80	0.43 ± 0.26	269 ± 88	17.2 ± 2.4	381 ± 80

[a] Mean ± SD of four rats.
[b] Concentration of mepirodipine hydrochloride at 0.5 hr (T_{max}).

concentration steps in the plasma and also by the use of (+)-[^{3}H]PN 200-110 as a radioligand. In fact, the sensitivity of the present RRA for nicardipine and nifedipine is at least 10 times greater than that of previous RRA procedures (16,19).

Consequently, the RRA developed in the present study is a simple and sensitive procedure for the determination of calcium channel antagonists in biological fluids. Particularly, this method is less time-consuming than the GC or HPLC method. The plasma concentrations of drugs determined by this method may correlate well with their pharmacological effects *in vivo*. We have recently demonstrated a significant correlation among plasma concentration by RRA, occupancy of calcium channel antagonist receptors, and hypotensive effect following oral administration of mepirodipine in spontaneously hypertensive rats (SHR) (22). Thus, the plasma concentration of calcium channel antagonists measured by RRA in human may reflect well their clinical effects.

ACKNOWLEDGMENT

The authors would like to thank Dr. O. Inagaki (Yamanouchi Pharmaceutical Company) for kindly measuring mepirodipine by gas chromatography–negative-ion chemical ionization mass spectrometry.

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