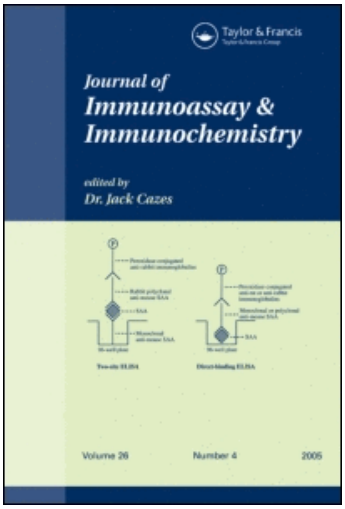

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A RADIORECEPTOR ASSAY FOR DETERMINATION OF NICARDIPINE IN HUMAN SERUM USING $^{3}H(+)PN$ 200-110 AS RADIOLIGAND

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ABSTRACT

A radioreceptor assay for the determination of nicardipine in human serum using the active enantiomer $^{3}H(+)PN$ 200-110 as radioligand is described. The assay is simple to perform, of low cost and requires only a small volume of serum. The standard curve permits measurements in the range 2.5 to 320 nM nicardipine (i.e. 1.2 to 153 ng/ml). The precision and the reproducibility of the method, evaluated from two different concentrations : 120 and 12 nM, show coefficients of variation for within and between assays, of 6 % and 15 % and of 6 % and 9 %, respectively.

Concentrations of nicardipine from 3 ng/ml could be measured with a satisfactory precision. The performances of the method permit the determination of nicardipine concentrations reached after therapeutic administration.

Other dihydropyridine calcium channel antagonists may be measured using this assay if these compounds are used to generate the standard curves. (KEY WORDS : Radioreceptor assay, Nicardipine, $^{3}H(+)PN$ 200-110, $^{3}H(\pm)$Nitrendipine, Dihydropyridine).

INTRODUCTION

Nicardipine, marketed by Sandoz France (Loxen® 20 mg - Loxen® LP 50 mg), for the treatment of systemic hypertension

belongs to the 1-4 dihydropyridine (DHP) group of calcium channel antagonists.

The most commonly used techniques for the determination of nicardipine in plasma or serum are gas chromatography (GC) and high-performance liquid chromatography (HPLC) (1,2,3). These methods, although highly selective, generally require extraction and concentration of the samples and are then time-consuming. Furthermore, the structurally-related specificity of these techniques makes it unable to distinguish between active and inactive metabolites.

Radioreceptor assay (RRA) is a relatively recent analytical method for the determination of drugs in biological samples. The assay is based on the competition between a radioactive ligand and the drug to be dosed for the biological receptor, target of the drug. Since the receptors used in the assay represent the site of action of the drug, the monitored blood concentrations will reflect not only the parent drug but any potentially active metabolite. In addition, for racemate compounds, this method allows the determination of the active enantiomer. Also, the assay will not be restricted to a single drug but to all compounds which act at the same receptor.

To date, 3 radioreceptor assays using $^{3}H(\pm)$-nitrendipine (NTD) as radioligand have been developed for the measurement of a number of calcium antagonists in serum or plasma (4,5,6). Recently, Lee et al. (7) have shown that the optically

active enantiomer $^{3}H(+)PN$ 200-110 binds to rat cardiac homogenates in a specific, reversible and saturable manner, with high affinity and low non-specific binding.

The present paper describes the development of a radioreceptor assay for the determination of nicardipine in human serum, using the pure tritiated isomer $^{3}H(+)PN$ 200-110 as radioligand.

MATERIALS

Reagents

(+)-[methyl-^{3}H]-PN 200-110, specific activity 85 Ci/mmol, was obtained from the Radiochemical Centre, Amersham, England. [5-methyl-^{3}H]-nitrendipine, specific activity 80.9 Ci/mmol was supplied by NEN, Germany.

Unlabelled drugs were kindly provided by Yamanouchi Pharmaceuticals, Japan (nicardipine) and Sandoz Ltd., Basle, Switzerland (isradipine, PY 108-068, nimodipine, nisoldipine and nitrendipine). All other drugs and chemicals were obtained from commercial sources. Unless otherwise indicated, racemic drugs were used throughout the assays.

Receptor preparation

Cardiac membranes were used as a source of dihydropyridine binding sites. Male Sprague-Dawley rats (200-300 g ; Iffa Credo,

France) were sacrificed by decapitation. The heart was quickly removed, freed from fat and placed in a petri dish over ice. The heart was perfused through the aorta with 10 ml of ice cold 50 mM Tris-HCl buffer, (pH = 7.4), and minced finely with scissors. Cardiac tissues were then homogenized in 15 volumes (W/V) of ice-cold Tris-HCl buffer using a Teflon-glass Potter-Elvehjem homogenizer (15 full strokes at 400 rpm). The cardiac homogenate was then centrifuged at 1,000 g for 5 min at +4°C in a Jouan K101 centrifuge, to remove large particles. The pellet was discarded and the supernatant centrifuged at 80,000 g for 15 min at +4°C in a Beckman L8-55 ultracentrifuge. The final pellet was either extemporaneously used or rapidly frozen and stored at -80°C until assay.

Standard curve and control samples

For the calibration curve, a pool of human drug-free serum was prepared and tested. Nicardipine standards were prepared by serial dilution of a 10^{-3}M solution of nicardipine hydrochloride in absolute ethanol, into pooled serum. The standard curve obtained ranged from 2.5 to 320 nM, i.e. 1.2 to 153.3 ng/ml of nicardipine expressed in base.

To assess the performances of the assay, i.e., precision and accuracy, supplemented quality control samples were prepared independently from the standard curves, by addition to the same pooled serum of known amounts of nicardipine standard from

another batch : T_1 = 12 nM (5.75 ng/ml) and T_2 = 120 nM (57.5 ng/ml).

Clinical samples

For the pilot pharmacokinetic application, serum samples from two healthy volunteers who received a single 50 mg oral dose of nicardipine were analyzed by RRA. Blood samples were collected in dry tubes, just before and 0.25, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5, 6, 7, 9, 12 and 24 hours after drug intake. Furthermore, the method was applied to the measurement of nicardipine concentrations in biological samples from 79 healthy volunteers who received a single 20 mg oral dose of nicardipine. Blood samples were collected just before and 1, 2 and 3 hours after drug intake.

For both applications, blood samples were clotted at room temperature and were then centrifuged for 10 min at 2,500 g. The serum was removed and stored at -20°C until analysis.

METHODS

The samples required for an assay were thawed out at room temperature. Triplicate 13 x 75 mm polystyrene hemolysis tubes were set up for measuring non-specific binding (NSB), total

binding (B_0), quality control, standard and clinical samples (Bi). The final pellet of the cardiac homogenate was resuspended and homogenized in an appropriate volume of ice-cold Tris-HCl buffer to achieve a concentration of about 100 µg/ml of proteins. Protein concentration was determined by the method of Lowry et al. (8) with bovine serum albumin as the standard.

To incubation tubes were added, 100 µl of a 10^{-9}M ^{3}H(+)PN solution, 20 µl of serum samples containing known (standards and controls) or unknown (clinical samples) amounts of nicardipine, 80 µl of 50 mM Tris-HCl buffer and 800 µl of receptor preparation. The final concentration of ^{3}H(+)PN in the incubate was 0.1 nM. The assay tubes were incubated in a water bath at 37°C for 60 minutes. The unbound radioactivity was then separated from the membrane bound radioactivity by rapid vacuum filtration of the incubate over Whatman GF/B glass fiber filters, using a Brandel Cell Harvester® (Gaithersburg, MD). Filters were then rapidly washed three times with 3 ml ice-cold buffer, dried and transferred into scintillation vials.

The membrane bound radioactivity trapped by the filters was finally quantified in 4 ml of scintillation fluid (Maxifluor®, Baker) by liquid scintillation counting in a Beckman LS 1800 counter with an efficiency of 50 percent.

The non-specific binding (NSB) was determined following the same procedure in the presence of an excess of unlabelled ligand (10^{-6}M nicardipine) and subtracted from the total binding to yield the specific binding.

Data analysis

All binding values were expressed as specific binding. The standard curve was linearized by a logit-log transformation and the concentration of unknown samples determined from it, using a program developed by Sandoz on a HP 3357 computer.

For specificity studies, the displacement curves were analyzed by computerized iterative nonlinear least squares regression using the "EBDA-LIGAND" program (G.A. Mc Pherson, 1985) adapted for an IBM-PC.

The dissociation constant (K_D) and the number of binding sites (B_{max}) were determined by Scatchard analysis of saturation experiments.

RESULTS

Choice of radioligand

The binding of $^3H(+)$ PN and $^3H(\pm)$ NTD to rat cardiac membranes was studied in order to determine the most suitable radioligand for the RRA. Parallel equilibrium saturation experiments showed that the K_D value of $^3H(+)$ PN was 10 fold lower than that of $^3H(\pm)$ NTD at physiologic temperature and 5 fold at 25°C (table 1).

TABLE 1

Comparison of $^3H(+)PN$ and $^3H(\pm)NTD$ binding parameters in rat cardiac homogenates at different incubation conditions. The results are shown as the mean ± SD of 3 experiments, each performed in duplicate.

	3H(+)PN 200-110 (1)		3H(±)NITRENDIPINE (2)		Kd(2) / Kd(1)
Incubation conditions	Kd (nM)	Bmax (fmol/mg prot)	Kd (nM)	Bmax (fmol/mg prot)	
37°C - 60 min	0.064 ± 0	171 ± 0	0.67 ± 0.30	125 ± 30	10
25°C - 90 min	0.062 ± 0.026	219 ± 1	0.30 ± 0.10	175 ± 5	5

Because of its lower affinity, $^3H(\pm)$ NTD should increase the sensitivity of the radioreceptor assay when the standard curves are generated with this radioligand. In order to confirm this hypothesis, nicardipine standard curves were performed with 3 different concentrations of $^3H(\pm)$ NTD (0.1, 0.2 and 0.4 nM) and their sensitivity compared to a standard curve performed with 0.1 nM $^3H(+)$ PN. At 0.1 nM, the amount of $^3H(\pm)$ NTD bound to the receptors was insufficient and at 0.4 nM the non specific binding was too important to permit the plotting of the standard curves. Thus, among the 3 concentrations of $^3H(\pm)$ NTD tested, only 0.2 nM yielded an appropriate percentage of specific binding. However, parallel nicardipine standard curves performed with 0.1 nM of $^3H(+)$ PN and 0.2 nM of $^3H(\pm)$ NTD showed that the gain of sensitivity obtained with $^3H(\pm)$ NTD was negligible, especially for the low concentrations of unlabelled nicardipine (table 2). On the other hand, the percentage of $^3H(\pm)$ NTD specifically bound was 3 times lower than that of $^3H(+)$ PN (table 2).

These results, added to the fact that the tritiated PN was the pure active enantiomer, prompted us to use routinely the $^3H(+)$ PN as radioligand.

Conservation of the cardiac membranes

Entire hearts as well as pellets were frozen and stored at -80°C. Every week, a heart and a pellet were thawed at room

TABLE 2

Comparison of nicardipine standard curves generated with $^3H(+)$ PN and $^3H(\pm)$ NTD. The assays were performed at 25°C for 90 minutes. The ligand concentrations were 0.1 and 0.2 nM for $^3H(+)$ PN and $^3H(\pm)$ NTD, respectively. The results are shown as the mean of 3 experiments performed in triplicate.

	3H(+)PN 200-110	3H(±)NTD	3H(+)PN / 3H(±)NTD
AT (dpm)	18201 ± 544	32995 ± 9	0.6
NSB (dpm)	515 ± 17	628 ± 23	0.8
BO (dpm)	2665 ± 111	2016 ± 24	1.3
BO-NSB/AT(%)	11.83 ± 1.1	4.21 ± 0.14	2.8
IC 20 (nM)	6.8 ± 0.3	4.3 ± 1.1	1.6
IC 50 (nM)	30 ± 3	19 ± 6	1.6
IC 80 (nM)	132 ± 12	79 ± 32	1.7
Ki (nM)	11.3 ± 1.1	11.1 ± 3.8	1.0

AT : total radioactivity added to the tube
NSB : non specific binding (obtained in the presence of 10^{-6}M nicardipine)
BO : binding obtained in the absence of unlabelled nicardipine

IC_{20}, IC_{50}, IC_{80}, i.e. final nicardipine concentrations producing 20, 50 and 80 % inhibition of the specific binding were calculated from log-logit concentration-response curves.
Ki (inhibition constant) values were calculated according to Cheng-Prussof's equation (14).

temperature and two saturation isotherms were performed. A third saturation isotherm was performed as a control with freshly prepared membranes. The study went on for 4 weeks.

The results showed that the affinity (K_D) and Bmax decreased linearly with time. After 4 weeks at -80°C, both K_D and Bmax decreased by 25 and 40 % in entire hearts and pellet form, respectively.

For practical reasons, the assays were routinely performed using membranes prepared with frozen pellets. The conservation of these membranes did not exceed 2 weeks, the time when the observed loss of affinity (20 %) and number of sites (10 %) had no visible effect on the standard curves and quality control samples.

Choice of the biological fluid

In order to study the influence of the fluid on the ^{3}H(+)PN binding, fixed quantities of ^{3}H(+)PN and cardiac membranes were incubated with increasing volumes of serum or plasma obtained with either citrate or heparin as anticoagulant. The assays were performed as described in the methods.

The binding is variably affected by the type of anticoagulant used for blood sampling : 20 µl of drug-free citrate and heparin plasma reduces the binding by approximately 20 % and 40 %, respectively ; with 50 µl aliquots of these same fluids, the binding was reduced by about 40 % and 75 %,

respectively. The decrease in the binding observed with drug-free serum was less important than with plasma samples, especially up to 20 µl (reduction by only 10-15 %).

The assays were thus routinely performed with serum samples. The volumes were standardized at 20 µl in a total incubation volume of 1 ml which gave adequate sensitivity with minimal effect on the tracer binding.

Standard curves

The results reported hereafter were obtained from 10 consecutive assays carried out over a period of 5 weeks. Each assay was composed of (i) a standard curve (ii) two quality control samples (T1 and T2), each determined 3 times along the assay and (iii) unknown samples. All determinations were performed in triplicate.

A typical standard curve is shown in figure 1 for 20 µl samples of serum supplemented with increasing concentrations of nicardipine. The standard curve was best fitted to a line with mean slope = 0.9920 ± 0.0605 and a mean coefficient of correlation = 0.9918.

Under the assay conditions, the percentage binding of $^{3}H(+)PN$ to cardiac membranes in the absence of unlabelled drug was 8.0 ± 1.5 %. The non-specific component of binding was 1.9 ± 0.4 percent. The proportion of $^{3}H(+)PN$ binding specifically associated with dihydropyridine receptors was thus approximately 76 percent.

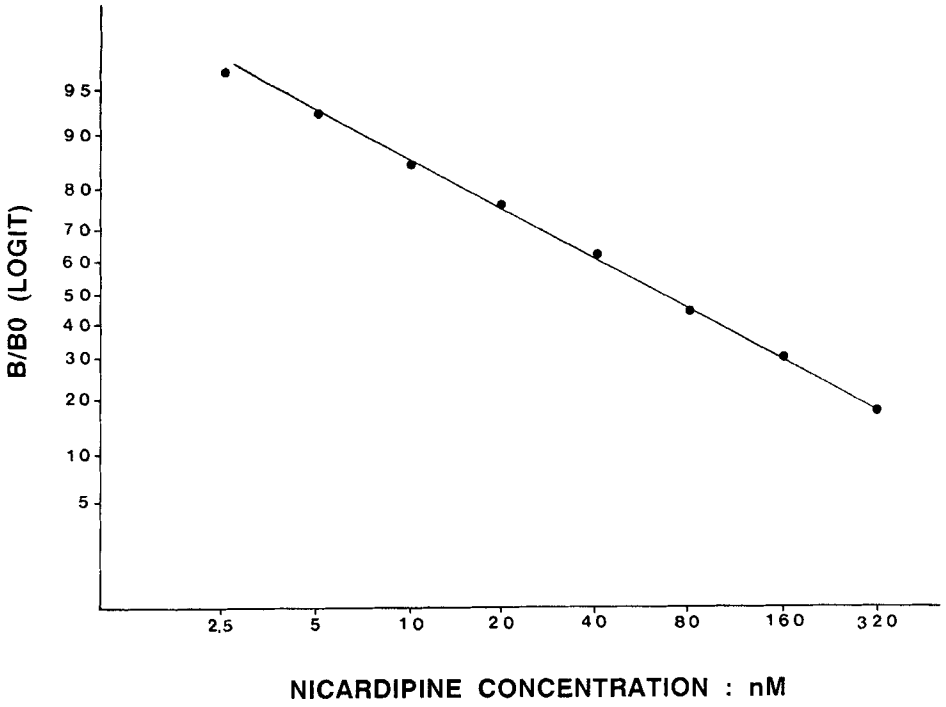

Figure 1 : Radioreceptor assay for nicardipine : a typical standard curve. The data are depicted according to logit-log analysis. Each point is the mean of triplicate determinations.

The between-assay variability (CV %) for the standard curve parameters (slope, intercept, percent of specific binding) was less than 10 %, showing the stability and repeatability of the calibration throughout the period of determinations. Repeated freezing and thawing of the tubes containing known concentrations of nicardipine did not alter the slope or sensitivity of the standard curve.

The final nicardipine concentration producing 50 % inhibition (IC_{50}) of the specific $^{3}H(+)PN$ binding was 1.4 ± 0.24 nM.

Sensitivity

The minimal detectable dose of nicardipine, i.e. the dose which would have an expected response statistically significantly different from the response for zero dose (blank), may be defined according to Rodbard's formula (9). This method, applied to the 10 standard curves led to a theoretical detection limit of 3 ng/ml ($B\phi$ and unknown samples determined in triplicate and $\alpha = 0.05$).

A more real assessment of sensitivity (based on the dose of drug producing 15 percent inhibition of binding on the logit-log standard curve), was applied to the 10 routine assays and led to a mean detection limit of 5 ng/ml.

Precision - accuracy

Within and between assay variability as well as accuracy were assessed by the inclusion of quality control samples (T1 and T2), supplemented with known amounts of nicardipine, in the 10 routine assays. The results are summarized in table 3.

Clinical samples (n=55) were determined on two separate occasions. For each sample, the mean concentration, the standard deviation (s.d.) and the coefficient of variation (CV %) were calculated. The CVs % were then plotted as a function of the

TABLE 3

Precision of nicardipine RRA : Quality control samples
1 nM = 0.479 ng/ml

Theoretical value	Experimental value*	Within assay (CV %)	Between assay (CV %)	Accuracy**
T1 (12 nM)	12.3 (n = 30)	15 %	9 %	+ 2.5 %
T2 (120 nM)	129.7 (n = 30)	6 %	6 %	+ 8.1 %

* Mean of all determinations, each performed in triplicate

**$\frac{\text{Exp. value - Theo. value}}{\text{Theo. value}} \times 100$

mean concentrations (figure 2) and the curve was fitted according to an equation of the form $y = ae^{-bx} + c$ which was shown to be the most suitable equation. The "c" value represents a realistic estimation of the precision with which the unknown samples are routinely determined.

In this assay, nicardipine concentrations from 14 nM (7 ng/ml) were determined with a precision of 20.5 %.

Selectivity

The potencies relative to (±)nicardipine of various calcium channel antagonists and other drugs effective in the treatment

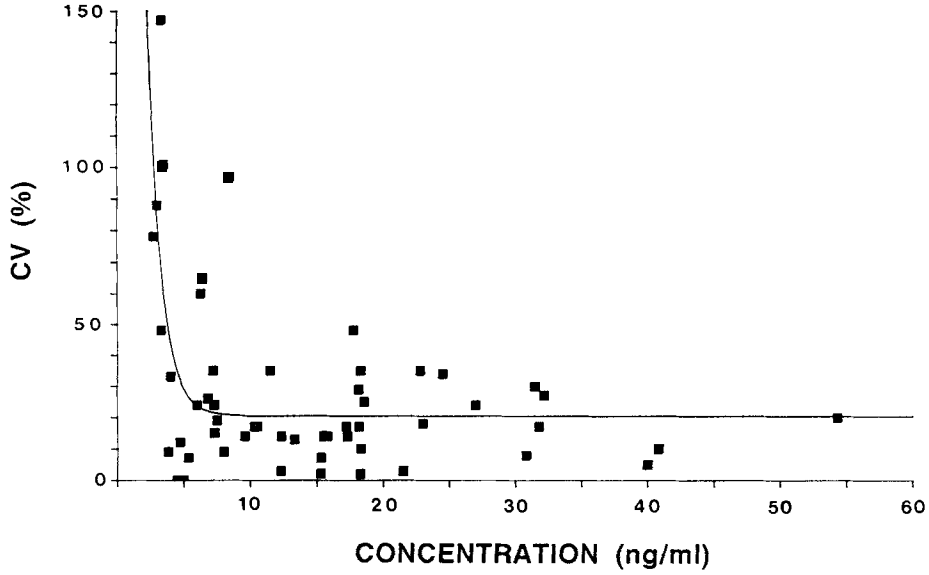

Figure 2 : Precision (CV %) as a function of the mean concentration of unknown samples. The curve was fitted according to the equation : CV % = $ae^{-bconc} + c$ with a = 1240, b = -0.99 and c = 20.5.

of cardiovascular disorders were evaluated from their IC_{50} values estimated from the corresponding fitted displacement curves (table 4).

All 1-4 DHP tested completely inhibited $^3H(+)$ PN binding in the heart with the rank order of : nisoldipine > PN 200-110 > (+)nicardipine > (±)nicardipine > nitrendipine > PY 108-068 > nimodipine > nifedipine > (-)nicardipine.

(-)Nicardipine is approximately 10 fold less potent than (+) nicardipine in inhibiting $^3H(+)$ PN binding (figure 3). These

TABLE 4

Drug specificity of $^{3}H(+)$PN 200-110 binding to rat cardiac membranes.

Drug	IC_{50} (nM)	Ki (nM)	Potency relative to nicardipine
(±)Nicardipine	3.0 ± 0.2	1.1 ± 0.1	100
(+)Nicardipine	1.7 ± 0.3	0.6 ± 0.1	180
(-)Nicardipine	22.9 ± 0.2	8.1 ± 0.2	130
Nisoldipine	1.5 ± 0.2	0.6 ± 0.1	200
PN 200-110	1.6 ± 0.4	0.6 ± 0.1	190
Nitrendipine	5.0 ± 0.1	1.9 ± 0.1	60
PY 108-068	6.2 ± 0.8	2.4 ± 0.3	50
Nimodipine	7.1 ± 2.0	2.8 ± 0.8	40
Nifedipine	17.9 ± 2.1	7.0 ± 0.8	20
Verapamil	877 ± 213	342 ± 83	-
Diltiazem	3435 ± 473	1341 ± 185	-
Bopindolol	-	-	-

IC_{50} and Ki values are shown as the mean ± SD of 3 experiments, each performed in triplicate.
IC_{50} values were estimated from fitted displacement curves and the Ki values calculated according to Cheng and Prusoff's equation (14), using the "EBDA LIGAND" program.
$^{3}H(+)$ PN 200-110 concentration was 0.1 nM and K_D 0.064 nM.

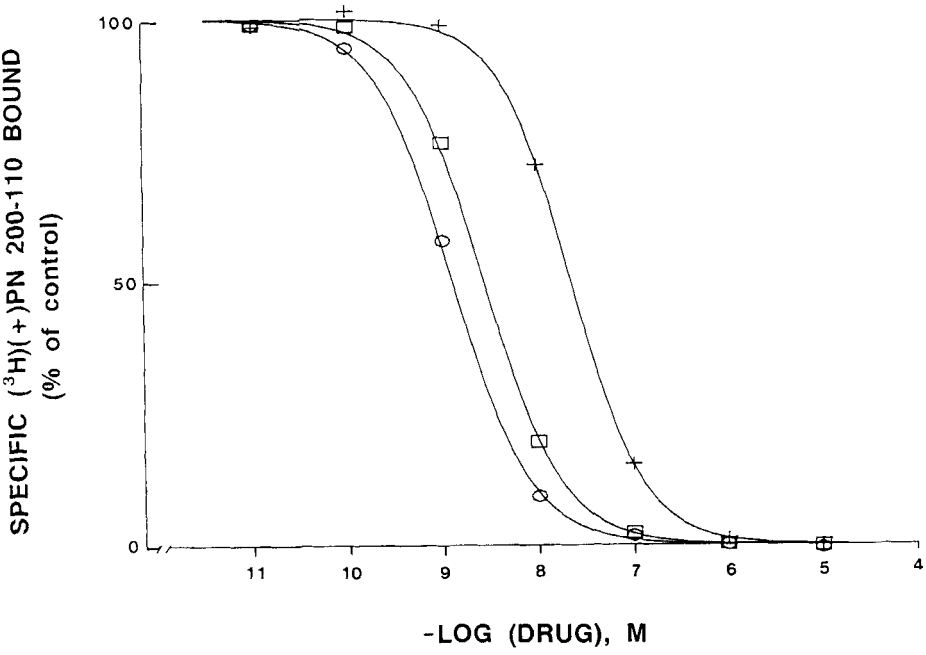

Figure 3 : Inhibition of ^{3}H(+) PN binding by (+)nicardipine (○——○), (±)nicardipine (□——□), and (-)nicardipine (+——+) in rat cardiac homogenates. The ^{3}H(+) PN concentrations were 0.1 nM. Assays were performed at 37°C for 60 minutes with a tissue concentration of 90 µg/ml. Each point is the mean of 3 experiments performed in duplicate. Each line is the best fit using nonlinear least squares analysis (see methods).

results agree well with previous binding studies (7) and physiological experiments (10).

Pharmacokinetic application

The serum concentrations determined by RRA after 50 mg oral administration of nicardipine are illustrated in figure 4.

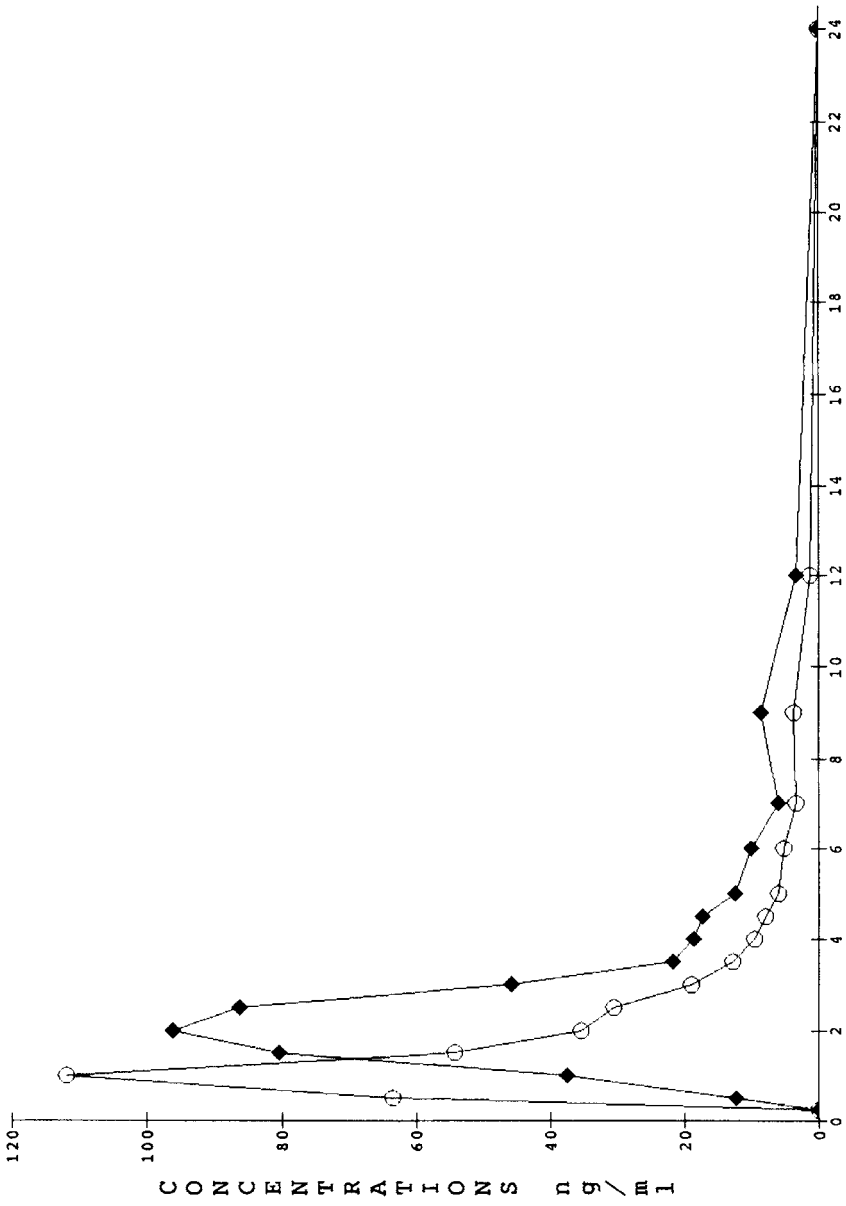

Figure 4 : Serum concentration - time course of nicardipine in two healthy volunteers who received a single 50 mg oral dose.

The peak serum concentration of nicardipine (100-120 ng/ml) occured at 1-2 hours.

After 20 mg oral administration of nicardipine, the peak serum concentration (Cmax) determined by RRA is 32 ± 16 ng/ml (n = 79). The time to reach this concentration (Tmax) is 1.1 ± 0.4 hours (n = 79). These results agree well with those previously reported (11).

DISCUSSION

Radioreceptor assays have proved valuable for measuring concentrations of a variety of drugs in biological samples.

To date, the 3 RRA developed for the measurement of calcium channel antagonists in plasma or serum used as tracer a racemic radioligand, the $^{3}H(\pm)$ NTD (4,5,6). However, in a previous paper Ehlert et al. (12) reported that it is reasonable to assume that only the active (-) enantiomer of the racemic $^{3}H(\pm)$ NTD contributes to the specific binding. Moreover, Hoyer (13) discussed the limitations of the use of racemic radioligands ; briefly, the use of racemic radioligands should be avoided whenever possible and preference should be given to pure enantiomers. The reasons for this are both theoretical and practical ; (i) if both enantiomers bind to the same receptors, there is a competition effect, and the time to reach equilibrium

will be increased ; (ii) since only the "active" enantiomer is supposed to bind, half of the radioactivity is wasted ; (iii) the non specific binding will be increased as both enaniomers contribute to it.

Based upon these findings, and because of the stereoselectivity of the DHP biological receptor, we developed a RRA for the determination of nicardipine in human serum, using the pure tritiated enantiomer $^{3}H(+)PN$ as radioligand.
This compound appears to be an improved ligand over $^{3}H(\pm)$ NTD for the dihydropyridine RRA, especially as no significant difference of sensitivity was observed between the standard curve performed with both radioligands.

The DHP receptor preparations were relatively fragile ; the membrane preparations could neither be lyophilized nor stored over a long period without significant modification of the binding characteristics.

The RRA described in this paper is technically simple to perform, of a relatively low cost and requires only a small sample volume, without any extraction or other purification procedure. $^{3}H(+)PN$ is commercially available. Several clinical samples (>30) can be analyzed in a working day, the total procedure being carried out within 2 days. The assay is selective for calcium antagonist drugs. Amongst the drugs screened, only those which possess pharmacologically relevant calcium antagonist properties influence $^{3}H(+)PN$ binding.

Furthermore, the assay is unaffected by other drugs which may be coadministered with DHP for the treatment of hypertension.

Thus, although this assay has been developed primarily for measuring nicardipine, it should equally be applied to all presently available DHP drugs. The only adaptation of the assay described here would be to establish a standard curve with known concentrations of the compound of interest.

Although the assay does not have the highest level of sensitivity attained with the GC method, the observed performances permit the determination of nicardipine concentrations reached after therapeutic administration.

The major virtue of the assay is that it will detect any metabolite which competes for DHP receptor and thus may be potentially active. Thus, the potential biological specificity of the RRA could be used in conjunction with specific methods (GC, HPLC), to explore the absorption, metabolism and excretion of nicardipine in man.

Further experiments are in progress in order to compare the pharmacokinetic profiles obtained after determination of nicardipine by RRA and GC.

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Footnotes

Abbreviations used :

RRA	: Radioreceptor assay
DHP	: Dihydropyridine
^{3}H(+)PN	: ^{3}H(+)PN 200-110
^{3}H(±)NTD	: ^{3}H(±)nitrendipine
CV	: coefficient of variation
NSB	: non-specific binding

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