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Validation of a high-performance liquid chromatographic–radioimmunoassay method for the determination of lacidipine in plasma

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ABSTRACT

A sensitive and reproducible method for the determination of lacidipine, a new potent antihypertensive dihydropyridine, is reported. The method involves solid-phase extraction, reversed-phase high-performance liquid chromatography and radioimmunoassay of the collected fraction. The assay provides a limit of detection of 20 pg/ml of plasma, allowing the determination of trough (24 h) plasma concentrations. The method is suitable for pharmacokinetic studies in man.

INTRODUCTION

Lacidipine, (*E*)-4-{2-[3-(1,1-dimethylethoxy)-3-oxo-1-propenyl]phenyl}-1,4-dihydro-2,6-dimethyl-3,5-pyridine dicarboxylic acid diethyl ester (Fig. 1), is a dihydropyridine calcium antagonist possessing potent and long-lasting antihypertensive properties [1–5]. The suggested therapeutic

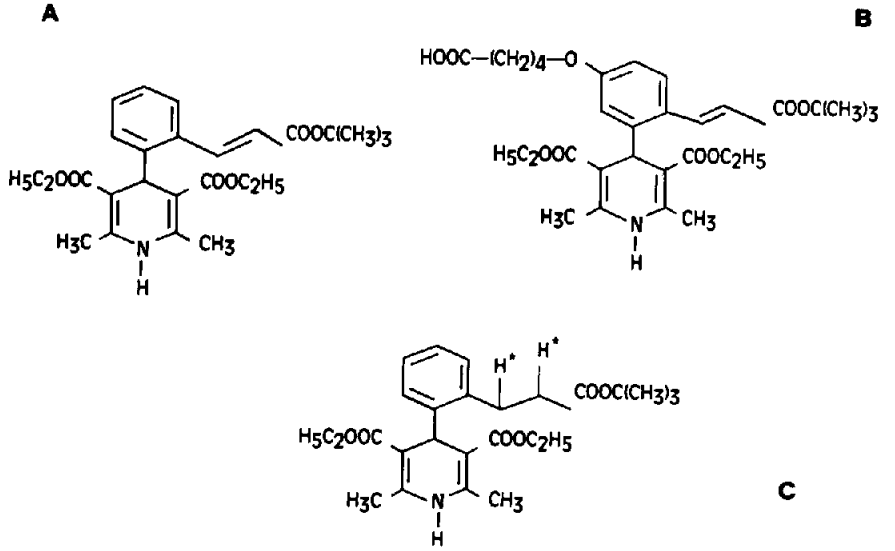

Fig. 1. Chemical structures of lacidipine (A), hapten (B) and tracer (C). Asterisks indicate the position of tritium atoms.

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dosage is one 4-mg tablet daily; therapeutic plasma levels are very low, making the determination of the full pharmacokinetic profile extremely difficult. The routine assay, high-performance liquid chromatography (HPLC) with ultraviolet (UV) detection, has a limit of quantitation of 0.5 ng/ml from a 3-ml plasma sample [6]. A variety of other methods have been tried: only with HPLC–mass spectrometry (MS) is the limit of quantification improved (100 pg/ml) [6]. HPLC–MS, however, is not suitable for assaying a large number of samples.

Radioimmunoassay (RIA) can reach very high sensitivity but is often subject to interference from endogenous substances. An antiserum to lacidipine, raised in the rabbit, shows good antibody titer and no cross-reactivity with the known metabolites. Nevertheless, non-specific interferences make direct RIA of plasma or of plasma extracts impossible. Combined HPLC–RIA is a very effective method for achieving both high sensitivity and high specificity, combining the best of both systems [7]. It has been applied both to endogenous compounds [8,9] and to drugs [10]. This paper describes the application of HPLC–RIA to the determination of plasma concentrations in the low picogram range of lacidipine.

EXPERIMENTAL

Apparatus

The HPLC apparatus consisted of a Waters Model 510 pump, a TCM oven, a refrigerated WISP 712, a variable-wavelength UV detector (Jasco UVIDEC, Jasco, Tokyo, Japan) and a fraction collector (Gilson Model 202, Gilson, Villiers-le-Bel, France).

An analytical column, Hypersil ODS, 3 μm particle size, 60 mm × 4.6 mm I.D. (Hewlett-Packard, Padua, Italy), protected by a dry-packed 30 mm × 4.6 mm I.D. guard column filled with RP-8, 30 40 μm particle size (Merck-Bracco, Milan, Italy) was used throughout the study.

Evaporation of solvents was performed by heating at 40°C in a Savant (Hicksville, NY, USA) RT100A apparatus. Radioactivity was measured with a Packard (Milan, Italy) 1900 CA scintillation counter.

Materials

Lacidipine (Fig. 1A), its metabolites (Fig. 2), (*E*)-4-{2-[3-(1,1-dimethylethoxy)-3-oxo-1-propenyl-5-[4-carboxybutyloxo]phenyl}-1,4-dihydro-2,6-dimethyl-3,5-pyridine carboxylic acid diethyl ester (hapten, Fig. 1B) and unlabeled diethyl-2,6-dimethyl-4-[2-(2-terbutoxycarbonylethyl)phenyl]-1,4-dihydropyridine-3,5-dicarboxylate (tracer, Fig 1C) were synthesized and supplied by the Chemical Development Lab., Glaxo (Verona, Italy). The radiolabeled tracer, prepared by the catalytic hydrogenation of lacidipine, was supplied 98% pure by the Amersham Radiochemical Centre (Amersham, UK) at an initial specific activity of 3.61 GBq/mg. The tracer, supplied in ethanolic solution at the concentration of 10.25 μg/ml and stored at −25°C, was stable for at least six months.

Bovine thyroglobulin (BT), bovine serum albumin (BSA), neomycin sulphate, Charcoal Norit A, tri-*n*-butylamine, isobutylchloroformate and dimethylformamide were supplied by Sigma (London, UK), Dextran T70 by Pharmacia (Uppsala, Sweden), kallikrein KIR-Lichter by Lepetit (Milan, Italy) and sodium merthiolate by Merck. The scintillation cocktail Picofluor 40 was supplied by Packard and Freund's complete adjuvant was supplied by Difco (Detroit, MI, USA). Bondelut C_{18} cartridges were supplied by Analytichem International (Harbor City, CA, USA). All the other reagents were of analytical or HPLC grade. Purified water was obtained through a Millipore (Milan, Italy) Milli-Q system.

Preparation of solutions

RIA buffer. Disodium hydrogenphosphate–disodium ethylenediaminotetraacetate (Na_2 EDTA) (0.05 *M*, pH 7.4) containing 0.1% BSA and 0.1% sodium azide. The solution was stable for two weeks at 4°C.

Tracer working solution. The stock solution of tritiated tracer was diluted 1:50 in ethanol, and this diluted solution (A) was stable for up to six months when stored at −25°C. Purity was regularly checked by thin-layer chromatography, spotting 5 μl on silica gel F_{254} plates (Merck) and developing in dichloromethane–ethanol (50:1, v/v) in parallel with the "cold" standard. A fur-

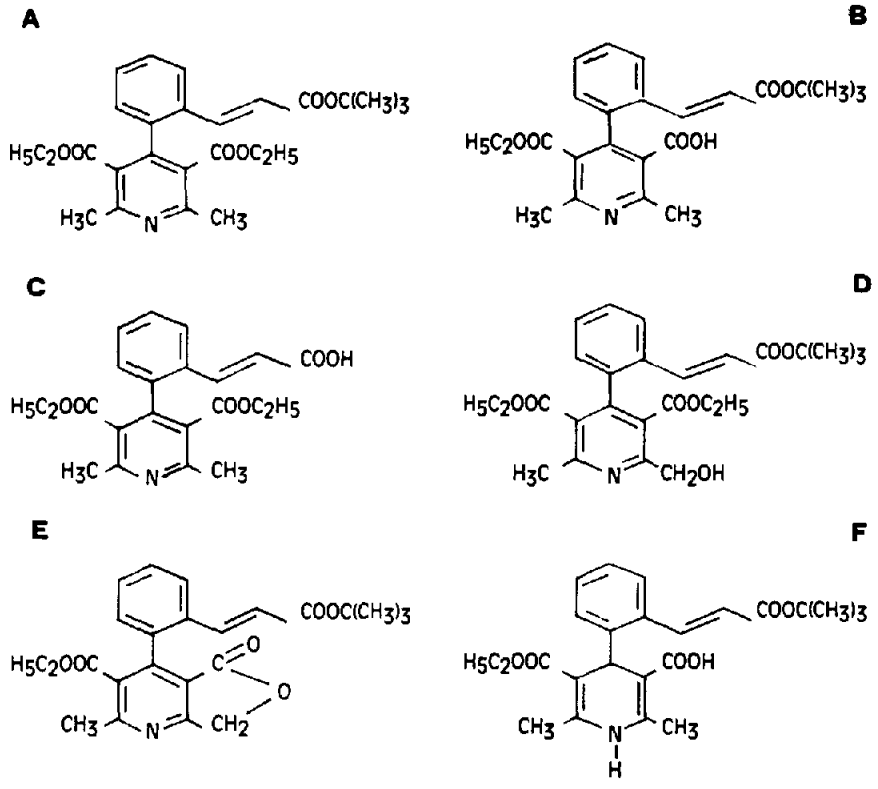

Fig. 2. Chemical structures of the principal metabolites of lacidipine.

ther 1:50 dilution (B) was freshly prepared on each experimental day; solution B was stable for up to 8 h when stored at 4°C. The final concentration of the tracer was 4.1 ng/ml, equivalent to 15 kBq/ml.

Antiserum working solution. The original antiserum was stored in small aliquots at −25°C. Samples of 0.5 ml were diluted 1:35 with RIA buffer containing 17 500 I.U. of kallikrein: this solution was stored at 4°C for up to twelve months. On each experimental day this solution was appropriately diluted (approximately 1:200) in RIA buffer to bind 60–70% of the tracer in the experimental conditions.

Charcoal–dextran suspension. A suspension of charcoal–dextran (10:1, w/w) was prepared in RIA buffer (5 mg/ml) each day and kept stirred at 4°C until used.

Standard solutions. Lacidipine and metabolites for cross-reactivity studies were dissolved in ethanol and stored at appropriate concentrations at −25°C.

Heparinised horse plasma samples of 3 ml were spiked every day with up to 100 μl of ethanolic solutions of lacidipine to final concentrations of 0, 20, 40, 80, 160, 320, 640, 1280 and 2560 pg/ml.

Solid-phase extraction washing solutions. Acid washing solution, acetonitrile–water–orthophosphoric acid, 88% (10:89:1, v/v/v); basic washing solution, acetonitrile–water–ammonia, 33% (10:88:2, v/v/v).

Synthesis of the antigen

Lacidipine is completely unreactive in its native form. A congener was therefore synthesized and coupled to BT by the mixed anhydride reaction [11].

Isobutylchloroformate (13 μl) in dry dimethylformamide (0.5 ml) was added to a solution of 60 mg of hapten in dry dimethylformamide (5.0 ml) under ice cooling, and stirred for 30 min.

BT (210 mg) dissolved in 7.0 ml of 30% dimethylformamide in water at 4°C was added drop by drop. The mixture was stirred at 4°C, and the pH was maintained at 9.0–9.5 by addition of 0.1 *M* sodium hydroxide. After allowing

to stand at 4°C overnight, the resulting conjugate was dialyzed extensively (for two days) and lyophilized. Ultraviolet absorption measurements at 280 nm on the conjugate indicated coupling of approximately 12–15 mol of hapten per mol of BT.

Immunization procedure

The immunization procedure has been fully described previously [12]. Briefly, male and female albino New Zealand rabbits were inoculated intradermally at 20–25 sites with conjugate emulsified in 0.9% saline and Freund's complete adjuvant (1:1, v/v). Animals were boosted at one-month intervals; blood was drawn from the central ear vein at ten-day intervals after booster injections. Blood was allowed to clot and serum, stabilized with 0.01% sodium merthiolate and 0.05% neomycin sulphate, was divided into aliquots and stored at −25°C.

Assay procedure

Blood collection and plasma conservation. The method has been applied to human, dog and rat plasma; horse plasma was always used for spiked samples, calibration curves and quality controls. Blood was collected in heparinised tubes and plasma separated after centrifuging at 4°C at 1000 *g* for 15 min. Plasma was stored at −25°C until assayed.

Extraction. As the compound is slightly photosensitive, all the operations were performed avoiding direct exposure to light.

Plasma samples (3 ml) were thawed and deproteinised by adding the same volume of acetonitrile. After centrifugation the supernatant was applied onto a 500-mg Bondelut C_{18} extraction cartridge preactivated with 3 ml of methanol and 2 ml of acetonitrile–water (1:1, v/v). The cartridge was then washed with 2 ml of acetonitrile–water, 1.5 ml of basic washing solution, 1.5 ml of acid washing solution and again 2 ml of acetonitrile–water. Lacidipine was eluted with 2 × 1.5 ml of acetonitrile. The samples were dried and redissolved in 100 μl of HPLC mobile phase and stored at 4°C until analysed by HPLC.

Chromatographic method. Column temperature: 40°C. Mobile phase: acetonitrile–methanol–water (6:66:28, v/v/v). Flow-rate: 1 ml/min. Detection wavelength: 300 nm. Injection volume: 82 μl.

A fraction of 1.2 ml was collected in 8-ml glass tubes at the authentic retention time previously determined for lacidipine standard (approximately 4.5 min). The total analysis time was 9 min. Lacidipine is not stable in the mobile phase when exposed to air or oxygen, so a Gilson pipette tip was placed onto the top of the collection tubes to minimise the exposure to air. In these conditions there was no loss of lacidipine for at least 2 h. The samples were dried and reconstituted with 75 μl of ethanol; the reconstitution volume was higher if the plasma concentration of lacidipine, measured approximately with UV absorption, exceeded 1 ng/ml.

Radioimmunoassay procedure. The appropriate volume of RIA buffer was placed in the tubes, kept in an ice bath, and then 25 μl of the redissolved HPLC fraction, 25 μl of the tracer solution (approximately 23 000 dpm) and 100 μl of the antiserum solution were added. The final volume was 500 μl. The samples were vortex-mixed and incubated for 19 h at 4°C.

The separation of bound lacidipine from free lacidipine was achieved by addition to each tube of 250 μl of charcoal–dextran suspension. The tubes were incubated for 15 min at 4°C, then centrifuged at 1000 *g* for 15 min at 4°C. The supernatant was transferred into scintillation minivials and counted after adding 4.5 ml of Picofluor 40.

Each determination was made in duplicate and each set included a calibration curve from which the concentrations of the unknown samples were calculated.

The antibody-bound fraction was expressed as the percentage B/B_0 (the amount bound relative to the amount bound at 0 concentration) *versus* log concentration. The dose–response curve was a four-parameter logistic function.

Human oral dosing studies

Two healthy volunteers, fasted overnight, received a single oral administration of lacidipine as a 4-mg tablet. Plasma levels were determined with the usual HPLC–UV method and with HPLC–RIA at 0, 0.5, 1.0, 1.5, 2.0, 3.0, 4.0, 6.0, 8.0, 12.0 and 24.0 h post dosing.

RESULTS

Production and assessment of antisera

Ten weeks after the primary immunisation, circulating antibodies to lacidipine were already detectable in sera obtained from all rabbits ($n = 6$) at a titer of at least 1:1000 (initial dilution). The titer of the antiserum was expressed as the reciprocal of the dilution necessary to achieve 50% binding of the tracer alone.

The antibody response showed a great individual variability; after seven months of treatment, the antiserum of one of the immunised rabbits (code CM 766) attained a 1:7000 titer and was chosen for the present assay. The relative specificity was tested by determining the amount of the tracer displaced by 200 ng of each of the known metabolites reported in Fig. 2: none elicited a significant interference, not even the pyridine analogue of lacidipine. In the absence of antiserum, the non-specific binding of the ligand was on average 7.25%.

Validation

Recovery. The overall recovery of the method was calculated by comparing the concentrations found in plasma samples spiked with known amounts of lacidipine and the concentrations found in ethanolic samples containing the same amount of lacidipine in 75 μl (*i.e.* the reconstitution volume). Recovery was 67.5 ± 9.2% (mean ± S.D.) throughout the calibration range.

Calibration curves. The values of B/B_0 were constant within 10% in the calibration range (Fig. 3).

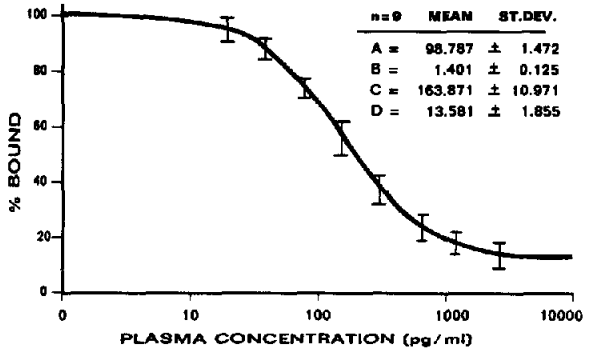

Fig. 3. Standard radioimmunoassay curve for lacidipine. (A) Response for zero concentration; (B) slope; (C) mid-range; (D) response extrapolated at infinite concentration.

Within-batch validation. Within-batch variability was evaluated by analysing eight-fold replicate samples prepared in horse plasma at each of the calibration curve concentrations against a duplicate calibration line.

As reported in Table I, the precision (coefficient of variation, C.V.) was always better than 15% except at the lowest concentration where the coefficient of variation was 30%. Bias was more than 15% only at the two lowest concentrations (20 and 40 pg/ml).

Between-batch validation. Plasma samples at three different concentrations were assayed on six different days. The coefficient of variation, as reported in Table II, never exceeded 16% and the bias 6%.

Limit of detection. The lowest calibration point was 20 pg/ml. This concentration, from 3 ml of plasma, gave a response consistently different from 0, as shown in Table I. Therefore it was assumed to be the limit of detection of the assay.

Dilution test. A sample of human plasma of unknown concentration was diluted 1:1, 1:2, 1:4, 1:8, 1:16 and 1:32 with "blank" human plasma. Table III shows that the measured concentra-

TABLE I

HPLC–RIA ASSAY OF LACIDIPINE IN PLASMA: WITHIN-BATCH VALIDATION

C.V. = standard deviation × 100/mean; bias = (measured − theoretical) × 100/theoretical. Values are means of eight samples. ND = not determined.

Theoretical concentratrion (pg/ml)	Concentration measured (mean ± S.D.) (pg/ml)	C.V. (%)	Bias (%)
0	ND		
20	30 ± 7.53	25.37	48.33
39	50 ± 4.17	8.31	28.63
79	80 ± 3.43	4.28	1.48
158	158 ± 10.91	6.90	0.11
315	331 ± 27.78	8.39	5.08
630	536 ± 79.77	14.89	−14.95
1261	1060 ± 101.43	9.57	−15.91
2521[a]	2586 ± 55.24	2.14	2.57
5042[b]	4728 ± 169.32	3.58	−6.23

[a] These samples were reconstituted with 750 μl.

[b] These samples were reconstituted with 1500 μl.

TABLE II

HPLC–RIA ASSAY OF LACIDIPINE IN PLASMA: BETWEEN-BATCH VALIDATION

	Measured concentration (pg/ml)		
	QC 66 pg/ml	QC 663 pg/ml	QC 2651 pg/ml
	67	688	2943
	79	680	2815
	69	624	2635
	70	663	2639
	61	794	2719
	59	678	2811
	97	645	2622
	70	630	2788
	61	729	3109
	61	632	3170
	61	744	2782
	75	580	2754
Mean	69	674	2816
S.D.	10.79	59.22	176.90
C.V. (%)	15.61	8.79	6.28
Bias (%)	4.80	1.65	6.21

TABLE III

HPLC–RIA OF LACIDIPINE IN PLASMA: DILUTION TEST

Dilution of plasma sample	Measured concentration (mean ± S.D.) (pg/ml)
Undiluted	450 ± 30.7
1:2	203 ± 7.3
1:4	91 ± 9.5
1:8	46 + 6.1
1:16	24 ± 5.6
1:32	12 ± 4.5

tions, corrected for the dilution factors, were similar excluding any effect of the matrix.

Throughput. A total of 300 unknown samples can be assayed by two persons in a month. The throughput could be improved automating some steps of the assay.

Human plasma levels comparison with HPLC–UV

Plasma concentrations measured with the usual HPLC–UV assay and with HPLC–RIA are reported in Table IV. The percentage relative deviation [(HPLC- RIA results − HPLC–UV results) × 100/HPLC–UV results] was +7.42%: the results of the two methods were superimposable above the limit of detection of the HPLC–

TABLE IV

PLASMA LEVELS IN TWO VOLUNTEERS AFTER ORAL ADMINISTRATION OF 4 mg OF LACIDIPINE: COMPARISON OF TWO DIFFERENT ANALYTICAL METHODS

ND (HPLC–UV): < 500 pg/ml.

Time after administration (h)	Concentration (pg/ml)			
	Volunteer 1 (4 mg)		Volunteer 2 (4 mg)	
	HPLC–UV	HPLC–RIA	HPLC–UV	HPLC RIA
Pre-dose	ND	ND	ND	ND
0.5	1025	820	1261	1657
1	1918	1887	3081	3392
1.5	1422	1378	2626	2668
2	1257	1111	1401	1776
3	694	715	980	1427
4	ND	468	700	745
6	ND	332	ND	430
8	ND	220	ND	281
12	ND	105	ND	119
24	ND	40	ND	54

UV method (0.5 ng/ml). HPLC–RIA allowed the determination of plasma concentration of lacidipine at all the collection times, *i.e.* up to 24 h.

DISCUSSION

Lacidipine is a drug administered once daily at low doses (2–4 mg): peak plasma levels are in the low ng/ml range, whereas trough levels are in the tens of picograms range. While in experimental animals the pharmacokinetics of lacidipine has been fully investigated [13], the usual HPLC method is not sensitive enough to allow a proper pharmacokinetic characterization of this drug in man. This sensitivity can only be achieved by methods such as LC MS or RIA.

The antiserum raised in the rabbit did not cross-react with metabolites, but interferences of unknown origin were present in plasma and/or produced during the extraction procedure: the same problem has been frequently reported [7].

The solution was to combine the high specificity of HPLC and the sensitivity of RIA in a coupled method. The resulting assay was specific, sensitive, accurate and reproducible. Despite its complexity, a reasonable number of samples can be processed (150 per man per month). The plasma levels during the 24-h interval between administrations can be determined. Therefore this method will be used to define the pharmacokinetic characteristics of lacidipine in man.

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