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Note

Determination of isradipine and the oxidative pyridine metabolite in human plasma by high-performance liquid chromatography

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Isradipine [isopropyl 4-(2,1,3-benzoxadiazol-4-yl)-1,4-dihydro-5-methoxycarbonyl-2,6-dimethyl-3-pyridinecarboxylate, PN 200-110, compound I, Sandoz] is new calcium channel antagonist of the dihydropyridine group [1]. Currently this drug is being investigated for the treatment of hypertension. As with other calcium channel antagonists isradipine, whilst being well absorbed orally, has extensive first-pass metabolism giving a low bioavailability [2]. Because of this, a standard oral dose (2.5–10 mg) yields low plasma concentrations of parent drug. One of the prime metabolites results from the oxidation of the dihydropyridine moiety to give the corresponding pyridine. This is compound II [(203-831, isopropyl 4-(2,1,3-benzoxadiazol-4-yl)-5-methoxycarbonyl-2,6-dimethyl-3-pyridinecarboxylate] and has significant circulating plasma levels [3]. Other metabolites of isradipine result from ester cleavage, with or without concomitant oxidation of the dihydropyridine moiety, giving the corresponding carboxylic acids. These compounds appear to have no significant haemodynamic effects.

Being a relatively new drug, assay procedures for isradipine in plasma or serum have been limited. Studies in humans where plasma concentrations were measured have utilised ^{14}C-labelled isradipine [2], radioimmunoassay [4] and gas chromatography–mass spectrometry [5]. Our aim was to develop a high-performance liquid chromatographic (HPLC) procedure which would be sufficiently sensitive to quantitate isradipine in plasma in the low ng/ml concentration range and simultaneously measure the oxidative pyridine metabolite. The detection sensitivity for the metabolite was not as critical, since plasma concentrations were expected to be greater than for isradipine.

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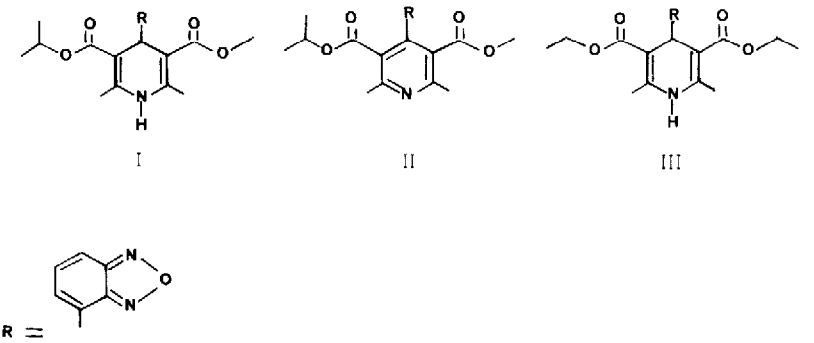

Fig. 1. Structures of isradipine (I), its oxidative pyridine metabolite (II) and the internal standard (III).

EXPRIMENTAL

Materials

Isradipine (I), the oxidative pyridine analogue (II) and the diethyl ester analogue of I (III) (PY 108-068, internal standard) were supplied by Sandoz (Basel, Switzerland) as authentic reference materials. The chemical structures for these compounds are shown in Fig. 1. Dibutylamine phosphate was purchased from Millipore–Waters (Lane Cove, Australia) as D-4 reagent. Methanol and dichloromethane were HPLC grade (Mallinckrodt Australia, Meadowbank, Australia). Water was all-glass double-distilled and pre-filtered through a 0.45-μm Nylon filter.

Apparatus and chromatographic conditions

Chromatographic analyses were performed on a 15 cm × 3.9 mm I.D. Nova-pak 4-μm C_{18} stainless-steel column (Millipore–Waters) with a 2-μm frit pre-column guard filter (Rheodyne, Cotati, CA, U.S.A.). The pump was a Millipore–Waters Model 510 pump and detection was carried out by a Lambda-Max Model 481AZ (Millipore–Waters) ultraviolet–visible detector set at 325 nm with maximum attenuation at 0.001 a.u.f.s. The detector output was recorded on a strip-chart recorder (Servogor 120, Goerz Metrawatt, Vienna, Austria) set at 10 mV full scale. Sample injection was by manual injection (Millipore–Waters Model U6K injector). The mobile phase consisted of 500 ml of methanol and 500 ml water containing 0.0.1 *M* dibutylamine phosphate (Waters D-4 reagent) as mobile phase modifier. The pH of the mobile phase was unadjusted (pH 2.8–3.0). The mobile phase was degassed by vacuum filtration through a 0.45-μm Durapore membrane filter (Millipore), then sparged continually with a slow flow of helium for maximum baseline stability. The flow-rate of the mobile phase through the column was 1.0 ml/min and the column temperature was maintained at 48°C (HPLC Temperature Controller, Nucleus Instruments, Melbourne, Australia).

Sample preparation

Blood samples (10 ml) were collected in heparinized tubes and the plasma was separated by centrifugation. Plasma samples were stored at −20°C prior to anal-

ysis. To each 1-ml plasma sample in screw-cap glass tubes were added 1 ml of water containing 4 ng of III (internal standard, see below) and 100 μl of 2 *M* sodium hydroxide. The samples were extracted with 6 ml of dichloromethane by a rotary mixer for 20 min. The tubes were centrifuged at 1500 *g* for 5 min. (When emulsions had formed these were "broken" by stirring with a glass rod and the tube was re-centrifuged.) The clear organic extract was removed as completely as possible and transferred to a second conical glass tube. The dichloromethane was evaporated under a gentle stream of nitrogen at 40 °C. A further 0.5-ml volume of dichloromethane was added, the tube vortex-mixed and the dichloromethane evaporated as above. The dried extract was then reconstituted in 100 μl of mobile phase by vortex-mixing for 30 s, allowed to stand for 10 min and then vortex-mixed again. For chromatographic analysis 75 μl were injected on to the column.

Calibration

Stock solutions of isradipine (I), its oxidative metabolite (II) and the internal standard (III) were prepared as 1 mg/ml in methanol. The solutions of I and II were diluted in methanol to give a 10 μg/ml working stock. The solution of III was diluted 2500 times in methanol to give a 0.4 μg/ml working stock. A 0.5-ml volume of this solution was further diluted to 50 ml with water to give a working internal standard solution of 4 ng/ml. All solutions were stored at 4 °C and showed no evidence of degradation over two months.

To prepare plasma standards 50 μl of I and 250 μl of II working stock solutions were diluted to 50 ml drug-free plasma to give concentrations of 10 and 50 ng/ml, respectively. This was the highest-concentration plasma standard and was serially diluted with drug-free plasma to give intermediate concentration values to the lowest standard values of 0.5 mg/ml I and 2.5 ng/ml II. Quantitation was done by measuring peak-height ratios of responses of I and II to that of the internal standard (III).

Clinical studies

Patients selected to undergo treatment with isradipine were given a single daily oral dose of 5 mg and serial blood samples to 8 h post-dose were withdrawn. The samples were collected and treated as described previously.

RESULTS AND DISCUSSION

Isradipine did not yield significant native fluorescence when excited at the ultraviolet and visible absorption maxima. Similarly in our hands, isradipine did not exhibit sufficient electrochemical activity for sensitive detection by this means. Therefore ultraviolet detection was used for quantitation.

Isradipine shows intense ultraviolet absorption at pH 3 at 205, 225 and 325 nm with a weaker broad absorption at 390 nm (hence the pale yellow colour of this material). Although the relative absorption intensity at the two shorter-wavelength maxima is greater than at 325 nm the latter was chosen for detection because of the lower chromatographic background from plasma extracts at that

wavelength. Compound II exhibited an absorption maximum at 310 nm, the absorption band being sufficiently broad to give sufficient detection at 325 nm. The internal standard (III) used in the assay was an isomeric compound to isradipine (the diethyl ester analogue) which exhibited the same absorption spectral characteristics as isradipine. To quantitate the extreme low concentrations in plasma, the detector output was set at maximum sensitivity (0.001 a.u.f.s.) with continuous solvent degassing with helium sparge to minimise drift and noise. Extraction from alkalinised plasma with dichloromethane gave the cleanest extract for optimum recovery of isradipine from various procedures attempted, including solid-phase techniques. Extraction recovery at 1 and 10 ng/ml was 75 and 85%, respectively, when compared to aqueous samples. Recovery of compound II was less, being 55% (determined at 5 ng/ml). Optimum chromatographic resolution

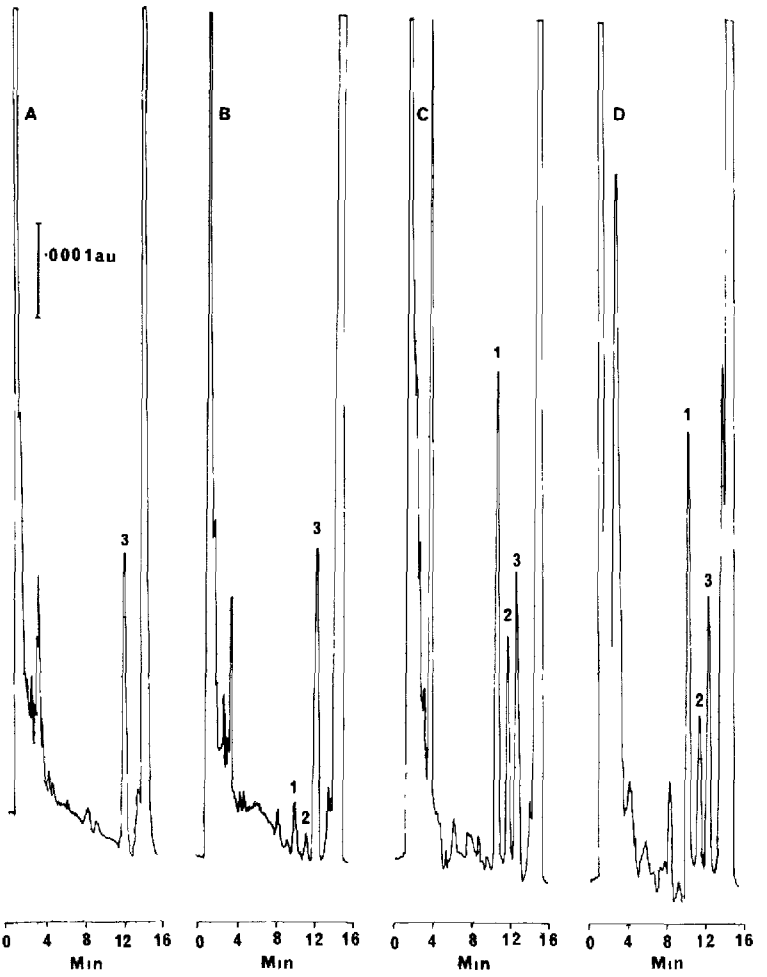

Fig 2. Chromatograms showing (A) drug-free plasma sample containing added internal standard, (B) standard plasma sample containing 0.5 ng/ml I and 2.5 ng/ml II, (C) standard plasma sample containing 5 ng/ml I and 25 ng/ml II and (D) 2.5-h post-dose patient (V.J.) plasma sample. Peaks 1 = metabolite (II); 2 = isradipine (I); 3 = internal standard (III).

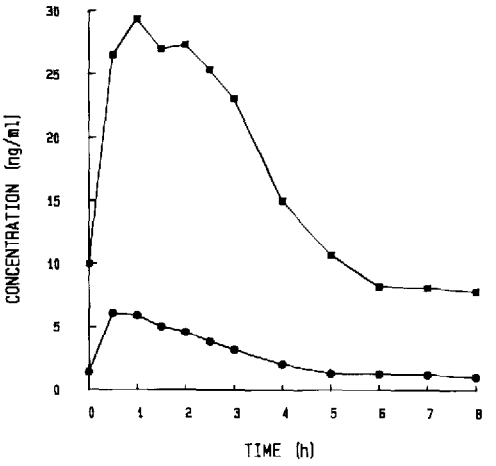

Fig. 3. Plasma concentration versus time profiles of isradipine (●) and metabolite II (■) from a patient (V J.) receiving a 5-mg oral dose. Note· this patient was at steady state and hence there were measurable plasma concentrations at zero time.

was achieved by performing the chromatography at elevated temperature with an elution order of II, I and III. Sample chromatograms from drug-free plasma, standard and patient samples are shown in Fig. 2. In all the plasma sample extracts, amongst the endogenous peaks observed, there was consistently an 'intense' peak eluting after the internal standard but this did not interfere with detection, nor was there interference from other endogenous peaks. Also under the alkaline conditions of the extraction the carboxylic acid metabolites resulting from ester cleavage did not extract into the dichloromethane. It was necessary to assay samples within six months of storage at $-20°$C. Samples deteriorated after this time resulting in additional endogenous peaks making the resolution and quantitation of each analyte difficult.

The assay for isradipine was calibrated from 0.5 to 10 ng/ml and was linear. The minimum concentration for quantitation was the lowest calibrator (0.5 ng/ml). Calibration for the pyridine metabolite (II) was at the higher concentration range of 2.5–50 ng/ml and was linear. The minimum detection was 1 ng/ml but this concentration level was often not observed in the time course of our studies. Serial isradipine (I) and metabolite (II) plasma concentrations up to 8 h after a single 5-mg oral dose at steady state in this same patient are shown in Fig. 3.

The intra-assay reproducibility ($n=6$) for isradipine at concentrations added to plasma of 1.0 and 5.1 ng/ml gave measured concentrations of 1.0 ng/ml (coefficient of variation, C.V., 7.6%) and 5.3 ng/ml (C.V. 2.4%). The inter-assay reproducibility from a quality control sample measured with each assay run gave an isradipine concentration of 2.1 ng/ml with a C.V. of 7.1% ($n=14$). The linear calibration for isradipine determined over five consecutive assays over a period of several weeks gave a slope (mean $\pm$S.D.) of 0.147 ± 0.019 and an intercept (mean $\pm$S.D.) of 0.0064 ± 0.0045, and the regression coefficient for each determination was 0.999 or greater. Similarly, the regression data for the pyridine metabolite (II) for the same five determinations were (mean $\pm$S.D.): slope,

0.0735 ± 0.0092; intercept, 0.0113 ± 0.0061; regression coefficients were 0.999 or greater for each determination.

Isradipine does present an analytical problem for plasma measurement of parent drug due to its extreme first-pass metabolism. Whilst it is not usual to determine such low concentrations with HPLC and ultraviolet detection alone, we were able to satisfactorily achieve this due to the intense ultraviolet chromophore for isradipine and by using the maximum detector sensitivity. We have been using this assay in our clinical studies of 2.5–10 mg doses of isradipine in both acute (single dose) and chronic (steady state) phase for over 24 months with good results for pharmacokinetic measurements of parent drug and metabolite. Such data from these studies will be published elsewhere.

REFERENCES

1 R.P Hof, G. Scholtysik, R. Loutzenhizer, H.J. Vuorela and P. Neumann, J Cardiovasc Pharmacol., 6 (1984) 399.
2 F L.S. Tse and J.M. Jaffe, Eur. J. Clin. Pharmacol., 32 (1987) 361.
3 Sandoz, Basle, personal communication.
4 G.D. Clifton, R.A. Blouin, C. Dilea, H.F. Schran, A.E Hassell, L.M. Gonasun and T.S. Foster, J. Clin Pharmacol., 28 (1988) 36.
5 C. Jean and R. Laplanche, J. Chromatogr., 428 (1988) 61.