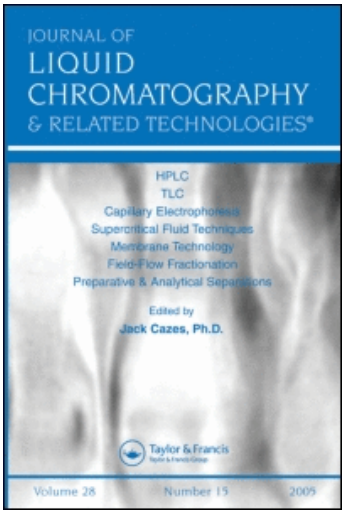

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ROUTINE DETERMINATION OF A NEW 1-4 DIHYDROPYRIDINE, OXODIPINE, IN HUMAN PLASMA BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY WITH ELECTROCHEMICAL DETECTION

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ABSTRACT

A rapid, specific and reproducible high-performance liquid chromatographic routine assay with electrochemical detection was developed for the determination of Oxodipine in human plasma.

After extraction at alkaline pH by cyclohexane, Oxodipine and its internal standard were chromatographied on a reversed-phase column.

Calibration curves were linear over a concentration range of 1-50 ng/ml with relative errors within-day or between-day not exceeding 8 % at any level.

The limit of detection was 30 pg injected based on a signal-to- noise ratio of 7. However, the reliable limit of quantification was 1 ng/ml using 1 ml of human plasma.

A dual-electrode coulometric detector was operated in a screening mode of oxidation, providing a greater specificity and reducing background noise.

This method allowed the complete follow-up of clinical pharmacokinetic studies and drug monitoring in patients.

INTRODUCTION

Oxodipine (1-4-dihydro-2,6-dimethyl-4-(2',3'-methylenedioxyphenyl)-3,5-pyridine carboxylic acid, methyl, ethyl ester) (figure 1) is a new dihydropyridine calcium channel blocker evaluated for potential clinical use in hypertension.

Very stable to light and high temperatures, Oxodipine shows a much longer duration of pharmacological action than most of the 1-4 dihydropyridines (1).

In man, Oxodipine is extensively absorbed and slowly eliminated (half-life of elimination : 10 to 16 hours).

Previous HPLC methods using UV detection did not provide sufficient sensitivity to follow plasma levels until 24 hours post-dose after oral administration of 20 mg in man.

In accordance with the chemical structure of 1-4 dihydropyridines (2) a sensitive and selective HPLC method using electrochemical detection was developed to allow accurate determination of Oxodipine plasma levels in man.

EXPERIMENTALS

Materials

Oxodipine and its internal standard were supplied by IQB (Madrid, Spain). HPLC grade acetonitrile, cyclohexane, and water

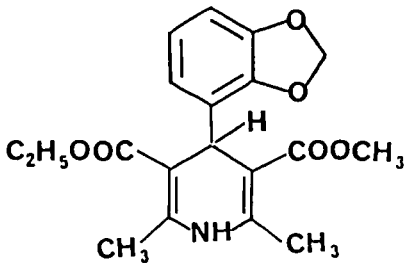

FIGURE 1 : Oxodipine

were obtained from FSA (Loughborough, U.K.). Methanol, of analytical grade, was obtained from Prolabo (Paris, France).

Tetrahydrofuran for U.V. and I.R. spectrophotometry was supplied by Carlo-Erba (Milan, Italy). Sodium hydroxide, di-sodium hydrogen phosphate, and potassium dihydrogen phosphate, of analytical grade, were supplied by Merck (Darmstadt, F.R.G.).

Standard Solution

Stock solutions of Oxodipine were prepared in methanol at a concentration of 1000 ng/ml. Working standard solutions (1 and 100 ng/ml) were obtained by serial dilutions in methanol. A working solution of the internal standard (1000 ng/ml) was prepared in the same manner. Both stock and working solutions were stable at 4°C.

Instrumentation and Chromatographic Conditions

The chromatographic system consisted of an isocratic 114M pump (Beckman, Berkeley, USA), a pulsation damper (Touzart & Matignon, Vitry-sur-Seine, France), and a SIL-6A automatic sample injector (Shimadzu, Kyoto, Japan).

Detection was carried out with a 5100 A Coulochem electrochemical detector equipped with a 5020 guard cell and a 5011 dual analytical cell (E.S.A., Bedford, U.S.A.).

The potential of the guard cell was at + 1 V, while the detectors 1 and 2 of the analytical cell were set at + 0.39 V and 0.70 V respectively. Separation was performed on a Novapak C18, 4 µm, 150 mm X 3.9 mm I.D. column (Waters, Milford, U.S.A.) protected by a C18, 7 µm, 15 mm X 3.2 mm I.D. precolumn (Brownlee, Santa Clara, U.S.A.). The mobile phase was composed of a mixture (60/40, V/V) of phosphate buffer (13 mM pH = 7) and acetonitrile containing 0.5 % tetrahydrofuran, filtered and degassed before use. The flow rate was 1.2 ml/min (1.9 Kpsi) at ambient temperature (ca

21°C). The mobile phase was recycled and renewed every 2 to 3 weeks. Chromatograms were recorded by a CR4A integrator (Shimadzu, Kyoto, Japan).

Sample Preparation

In round bottom glass tubes of 10 ml capacity fitted with a glass top, were introduced 1 ml of human plasma, 0.1 ml of a 0.1 M sodium hydroxide solution and 15 µl of the internal standard solution (1 ng/ul). The mixture was vortexed for 5 seconds and extracted twice with 4 ml of cyclohexane (15 minutes linear agitation, REALIS). After centrifugation (5 min ; 2500 rpm) the organic phase was collected into conical glass tubes and the cyclohexane was evaporated for 45 min using a "Speed-Vac" system equipped with a liquid nitrogen trap. The dry residue was dissolved by vortexing in 100 µl of the HPLC eluent and transferred into conical glass automatic sample injector vials. An aliquot (5-25 µl) was injected into the HPLC system.

Calibration

Calibration curves were constructed daily by addition of aliquots (5-50 µl) of the respective standard solutions of Oxodipine to blank plasma to give final concentrations of 1, 2, 5, 10, 15, 25, and 50 ng/ml. Pools of spiked plasma were also prepared, transferred into 1 ml vials and stored at -20°C until analysis ; at least two spiked samples of different concentrations were analysed daily. The calibration standards and the spiked samples were extracted as described above. Calibration curves were obtained by linear regression analysis (unweighted) of the Oxodipine/internal standard peak-height ratios against Oxodipine concentrations.

RESULTS AND DISCUSSION

Electrochemistry

Electrochemical detection techniques are coupled with HPLC for a lot of electroactive compounds assays (3) and in particular for Nifedipine, a drug from the same chemical class as Oxodipine (4).

The electrochemical detector used for this method consisted of two coulometrically efficient porous graphit electrodes working in series.

The screen mode of operation was selected in order to improve the detector selectivity.

The operating potentials were optimized by generating current-voltage curves for the oxidation of Oxodipine into its pyridine analogue under the HPLC conditions described above. The first electrode potential was set at + 0.39 V resulting in a reduced background noise from the impurities of the mobile phase and the sample extracts. An applied potential of 0.7 V was chosen for the second electrode.

Just before the injector, in a recirculation process of the mobile phase, a high potential (+ 1 V) applied by a guard cell, allowed the oxidation of all the compounds and impurities present in the mobile phase.

However, the background noise of basic current increases over a period of time. In order to maintain optimical detector performance, analytical cells were cleaned, according to the manufacturer's procedure, monthly or after analysis of approximately 500 samples.

Selectivity and Specificity

Under the chromatographic conditions described, retention times for Oxodipine and the internal standard were 6.6 and 10.2 minutes respectively.

In pure solution, the detection limit, based on a signal-to-noise of 7 is 30 pg. However, the limit of quantification of the assay from 1 ml of plasma was 1 ng/ml. Generally concentrations of 0.5 ng/ml could be detected.

Examples of chromatograms, blank human plasma, spiked plasma, and plasma sample from a subject are shown in figure 2.

No interfering peaks were detected in the control human samples or in the clinical samples from patients who had received concomitant drugs, such as Paracetamol, Metoclopramide, Cimetidine, Ranitidine, Oxazepam, Temazepam, Carbamazepine, Diazepam, Salbutamol, Allopurinol, Glibenclamide, or Tolbutamide.

Main metabolites of dihydropyridines are described for most of them as pyridine homologous (5, 6, 7). Since these compounds do not give a response in the conditions used for electrochemical detection, this method would be specific for Oxodipine in presence of the expected main metabolites.

Linearity

Calibration curves (n = 46) were linear over the range of 1 to 50 ng/ml. The mean equation (± SD) being (n = 46) :

$$y = (0.07554 \pm 0.00782)x - (0.02229 \pm 0.06444)$$

with a correlation coefficient of 0.9995 ± 0.00049.

Inter-day assay variation (for 2 or 3 analysts using different HPLC systems) was estimated by comparing the linear regression slopes of the calibration curves.

Over a period of 2 months, the mean slope was 0.07554 with a coefficient of variation of 10.4 % (n = 46).

Reproducibility and Accuracy

The reproducibility and accuracy of the method, expressed as the mean relative error of all concentrations from the theoretical value, ranged from 0.4 % to 8 % for 50 and 1 ng/ml respectively (table 1).

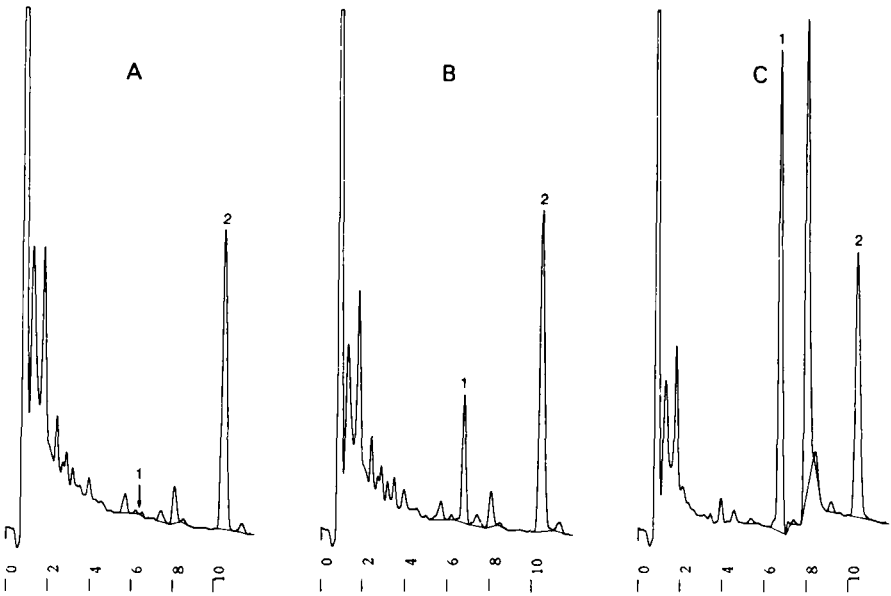

FIGURE 2 : Chromatograms of extracts from (A) blank human plasma, (B) spiked plasma (oxodipine 5 ng/ml), and (C) plasma sample from a subject (20 ng/ml).
PEAKS : 1 = Oxodipine ; 2 = Internal Standard

TABLE 1

Reproducibility and Accuracy of the Method for Calibration Curves in Human Plasma (n = 46)

Theoretical concentration (ng/ml)	Concentration found (mean ± S.D.) (ng/ml)	Coefficient of variation (%)	Relative error of the mean (%)
1.0	1.08 ± 0.247	22.9	+ 8.0
2.0	2.17 ± 0.284	13.1	+ 8.5
5.0	5.15 ± 0.336	6.5	+ 2.6
10.0	9.70 ± 0.375	3.9	- 3.0
15.0	14.70 ± 0.482	3.3	- 2.0
25.0	24.90 ± 0.766	3.1	- 0.4
50.0	50.20 ± 0.480	1.0	+ 0.4

TABLE 2

Precision and Accuracy of the Method for Spiked Plasma

Concentrations of Oxodipine added	Number of samples	Concentrations found (mean ± S.D.) (ng/ml)	Coefficient of variation (%)	Relative error of the mean (%)
Within-day				
1	6	1.13 ± 0.027	5.5	13.0
2	6	1.88 ± 0.112	6.0	6.0
5	6	5.18 ± 0.087	1.7	3.6
Day-to-day				
1	30	1.23 ± 0.374	30.4	23.0
2	12	2.04 ± 0.291	14.3	2.0
10	44	9.50 ± 0.814	8.6	5.0
25	12	22.80 ± 1.412	6.2	10.0

From spiked plasma samples at different concentrations with respect to the calibration curve, within-day coefficients of variation ranged from 1.7 to 6 % and day-to-day variation of spiked samples analysed by several analysts using different HPLC systems were 6.2 to 30.4 % (table 2).

Replicate determinations of in vivo samples (n = 46) from subjects having received Oxodipine showed good results. No significant difference was observed between the concentrations found.

These results demonstrate that the method is very reproducible and accurate in routine analysis.

Recovery

Althought absolute recoveries of Oxodipine and its internal standard were low, extraction with cyclohexane gave cleaner plasma extracts than other solvents (toluene, diethyl-ether, heptane) which gave greater recoveries (table 3).

TABLE 3

Absolute Recoveries of Oxodipine and its Internal Standard from Spiked Plasma Samples

	Concentration (ng/ml)	Number of samples	Recovery (%)	Coefficient of variation (%)
Oxodipine	2	5	52.5	10.6
	10	5	42.6	2.9
	25	5	43.6	6.6
Internal Standard	15	4	59.0	2.6

TABLE 4

Stability of Oxodipine in Frozen Plasma

Initial Concentrations (ng/ml)	Concentrations found* (ng/ml) 1 day	15 days	30 days	60 days	120 days
2	2.1	2.1	2.2	2.0	2.1
10	9.3	8.4	9.3	9.3	9.5
23	23.1	21.0	23.2	22.5	23.3

* mean of 3 spiked samples

Stability

The stability of Oxodipine in spiked plasma at -20°C was determined over a period of 4 months (120 days). The results in table 4 indicate that no significant degradation had occured over the period studied.

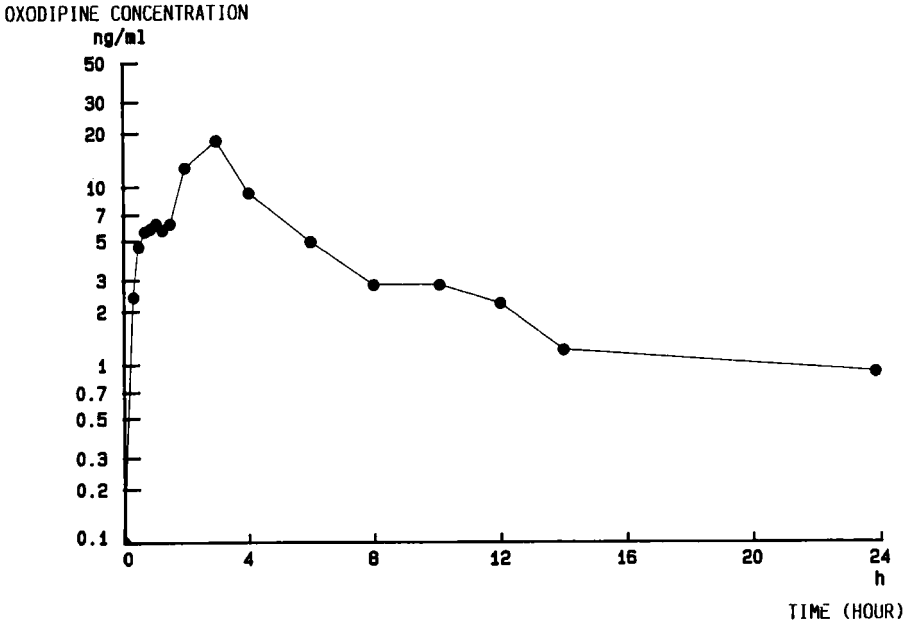

FIGURE 3 : Plasma concentration versus time curve following a single oral dose administration of 20 mg of Oxodipine to a healthy volunteer (semi-logarithmic plot)

Application of the Method

This HPLC method is now intensively used in our Laboratories and more than 2000 samples collected from healthy volunteers and patients receiving oral doses of Oxodipine have now been analysed.

Figure 3 shows an example of the plasma concentration versus time profile of a subject receiving 20 mg of Oxodipine by oral route. Maximum concentration of 18.1 ng/ml are reached at 3 h ; terminal half-life is about 14.4 h.

CONCLUSION

The method described for the determination of Oxodipine is sufficiently easy, sensitive and rapid to be applied to routine analysis. It allows the complete follow up of clinical pharmacokinetic studies and drug monitoring in hypertensive patients.

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