Journal of Chromatography, 383 (1986) 405—411
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 3337

Note

Gas chromatographic assay for the quantitation of flordipine in human plasma

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(First received September 18th, 1985; revised manuscript received July 16th, 1986)

Flordipine (I, Fig. 1), diethyl 1,4-dihydro-2,6-dimethyl-1-[2-(4-morpholinyl)-ethyl]-4-[2-(trifluoromethyl)phenyl]-3,5-pyridinedicarboxylate, is a new antihypertensive agent exhibiting calcium antagonist properties and has been shown to be orally effective in various animal studies and during human clinical trials [1—3]. A high-performance liquid chromatographic (HPLC) assay with a lower limit of quantitation of 25 ng/ml [4] was developed for analysis of flordipine in plasma. However, analysis of plasma samples taken from normal human subjects administered flordipine at doses of 50, 100 or 300 mg indicated that flordipine concentrations from most of the samples from the lowest dose group were at or below the quantitation limit of the HPLC assay. It has since been determined, based on results from early clinical trials, that the appropriate clinical dose of flordipine would be $\leqslant$ 50 mg. The objective of the present study, therefore, was to develop a more sensitive assay for the quantitation of flordipine in human plasma using gas chromatography (GC) with a nitrogen-sensitive thermionic specific detector, which would be suitable for routine analysis of samples from clinical studies.

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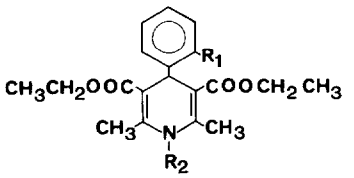
0378-4347/86/$03.50



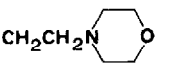
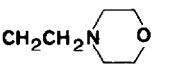
		R_1	R_2
I	flordipine (REV 2906)	CF_3	
II	REV 5956	CH_3	
III	REV 2611	CF_3	H

Fig. 1. Chemical structures of flordipine (I), the internal standard, REV 5956 (II), and REV 2611 (III).

EXPERIMENTAL

Chemicals

Flordipine and the internal standard (II, Fig. 1), REV 5956 [diethyl 1,4-dihydro-4-(2-tolyl)-2,6-dimethyl-1-(2-morpholinoethyl)-3,5-pyridinedicarboxylate], were synthesized by Revlon Health Care Research (Tuckahoe, NY, U.S.A.). Acetone and hexane (both glass-distilled) were supplied by Burdick and Jackson (Muskegon, MI, U.S.A.). Acetonitrile (Fisher Scientific, Pittsburgh, PA, U.S.A.) was HPLC grade. Triethylamine was obtained from J.T. Baker (Phillipsburg, NJ, U.S.A.) and was redistilled before use.

Gas chromatography

A Varian Model 3700 gas chromatograph (Varian Assoc., Palo Alto, CA, U.S.A.), fitted with a 3% OV-17 column (180 cm × 2 mm, 100—120 mesh Supelcoport, Supelco, Bellefonte, PA, U.S.A.) and a Varian thermionic specific detector, was used for the assay. The carrier gas was helium with a flow-rate of 30 ml/min. The operating temperatures were as follows: injector, 300°C; detector 300°C; column, 260°C for 2 min, followed by a programmed rise to 300°C at the rate of 40°C/min. Detector response was measured as peak height using a Hewlett-Packard Model 3390A recording integrator.

Procedure

A series of plasma standards for calibration curves containing 0, 2.5, 5, 10, 20, 40 or 80 ng/ml flordipine and 50 ng/ml internal standard were prepared. Unknown plasma samples (1.0 ml) were added to tubes containing 50 ng of internal standard.

Immediately before extraction of plasma, 100 μl of triethylamine and 10 ml of hexane were added to each tube. The samples were shaken for 20 min on a reciprocating shaker and centrifuged for 10 min at approximately 1500 *g* at 5°C. The tubes were then placed at −20°C to selectively freeze the lower

aqueous layer. A 9-ml volume of the hexane layer was carefully transferred to a clean conical tube and evaporated to dryness under a stream of nitrogen. The side walls of the tube were washed successively with 500 and 100 μl of acetone, followed by evaporation of the solvent with nitrogen after each washing in order to concentrate the contents in the tip of the tube. Samples were reconstituted in 25 μl of acetone, and 5 μl were injected into the gas chromatograph.

The regression equation for the standard curve was determined by linear regression analysis using the relative peak height (peak height of flordipine/peak height of internal standard) and the corresponding theoretical concentration of flordipine. Flordipine concentration in unknown samples was calculated from this regression equation using the corresponding relative peak height.

Extraction efficiencies for flordipine and the internal standard were determined by comparison of peak heights of extracted and unextracted standards.

Gas chromatographic—mass spectrometric (GC—MS) analysis

Plasma samples, taken from two human subjects 0.5 h after oral administration of 50 mg of flordipine, were combined and extracted as above. The sample was analyzed using a Finnigan Model 4000 mass spectrometer fitted with a 3% OV-17 column (previously described). Injector and separator temperatures were 300 and 275°C, respectively. Helium flow-rate through the column was 20 ml/min.

RESULTS AND DISCUSSION

Extraction efficiency

The internal standard (II, Fig. 1) was chosen because of its close structural similarity to flordipine. Comparison of peak heights of flordipine and the internal standard extracted from spiked plasma to those of standards injected directly showed that both compounds were well extracted with hexane. The extraction efficiency for flordipine, measured at each concentration used in the standard curves, averaged 93.3 ± 8.3% and that of the internal standard averaged 99.4 ± 10%. The addition of triethylamine to the samples before extraction has been previously shown to result in more reproducible extraction of flordipine [4].

Selectivity

The temperature programming used in this assay ensured sharp, symmetrical peaks for the analytes which allowed consistent integration of peaks even at flordipine concentrations which were well below the lowest concentration on the standard curve. Typical chromatograms are shown in Fig. 2. The plasma blank contained no endogenous compounds which eluted near either flordipine or the internal standard. The identification of the flordipine peak (peak I, Fig. 2C), observed after administration of the drug to human subjects, was confirmed by GC—MS analysis (molecular ion, *m/z* 510; base peak, *m/z* 100; major fragmentation ions, *m/z* 465 ($M - C_2H_5O$), *m/z* 437 ($M - C_3H_5O_2$), *m/z* 410 ($M - C_5H_{10}NO$) and *m/z* 114 (the N-ethylmorphilino cleavage product).

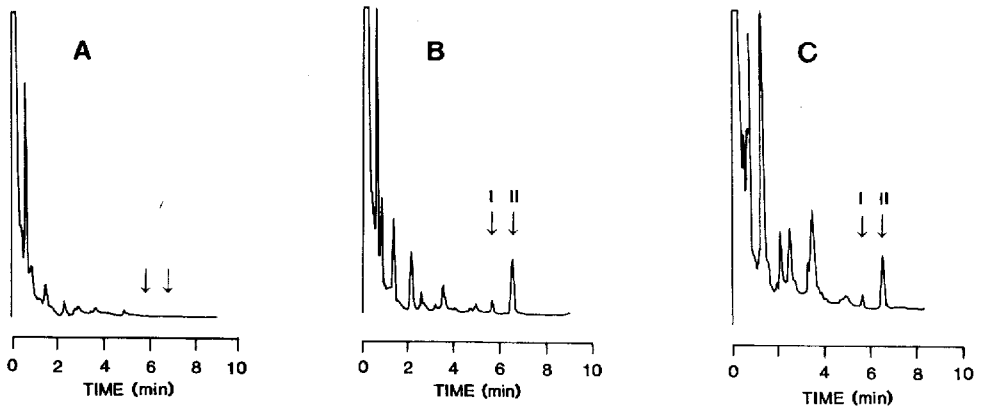


Fig. 2. Chromatograms of hexane extracts of plasma. (A) Plasma blank; (B) plasma spiked with flordipine (10 ng/ml) and internal standard (50 ng/ml); (C) plasma obtained from a human subject 2 h after administration of a 50-mg flordipine tablet. Peaks: I = flordipine; II = internal standard.

Linearity and reproducibility

Assay linearity was demonstrated by the correlation coefficients for five standard curves (0.9982 to 0.9997) and the composite of these curves (0.9970), run over a two-week period.

The inter-day reproducibility (precision) of the chromatographic procedure, as determined by the variation in the relative peak height at each concentration over the range of the standard curve, is shown in Table I. For five daily standard curves the coefficient of variation averaged 7.0 ± 3.4%, ranging from 4.3 to 13.5%, demonstrating that the between-run variation in the chromatographic procedure was small.

The inter-day accuracy of the assay method is presented in Table II. The experimental concentrations were determined over a two-week period by back-calculating each concentration point on five standard curves using the linear

TABLE I

INTER-DAY REPRODUCIBILITY OF THE CHROMATOGRAPHIC SYSTEM

Assays were run over a two-week period; $n = 5$.

Spiked flordipine concentration in plasma (ng/ml)	Relative peak height* (mean ± S.D.)	Coefficient of variation (%)
2.5	0.0675 ± 0.0091	13.5
5	0.1280 ± 0.0088	6.9
10	0.2643 ± 0.0120	4.6
20	0.5229 ± 0.0354	6.8
40	1.0066 ± 0.0431	4.3
80	1.9389 ± 0.1181	6.1
Mean ± S.D.		7.0 ± 3.4

*Ratio of the peak height for flordipine to peak height of the internal standard.

TABLE II

INTER-DAY ACCURACY OF THE QUANTITATION OF FLORDIPINE ADDED TO HUMAN PLASMA

Assays were run over a two week period; $n = 5$.

Theoretical concentration (ng/ml)	Experimentally determined concentration* (mean ± S.D.) (ng/ml)	Accuracy** (mean ± S.D.) (%)
2.5	2.0 ± 0.5	75.2 ± 12.8
5	4.4 ± 0.8	84.4 ± 9.5
10	10.1 ± 0.5	96.0 ± 2.9
20	20.8 ± 1.0	94.3 ± 3.8
40	40.9 ± 1.5	96.6 ± 2.2
80	79.3 ± 0.9	98.9 ± 0.7

* Determined from regression equation of the standard curve and the relative peak heights for the individual data points.

** Accuracy $= \frac{1}{5} \cdot \sum_{1-5} 100 - \left[\frac{\hat{x} - x}{x} \cdot 100\right]$

where $\hat{x}$ = experimental concentration and x = theoretical concentration.

regression equation for each individual standard curve. The mean accuracy varied from 99% at the 80 ng/ml level to 75% at 2.5 ng/ml.

Isomerization of flordipine

During the course of this assay development, a peak, in addition to flordipine, was consistently observed after injection of flordipine. This peak (retention time 3.3 min) consistently represented 4.5% of the peak area of flordipine over the entire working range of the assay. The electron-impact mass spectrum of this compound was very similar to that of flordipine. The spectrum showed the same molecular ion (*m/z* 510) and base peak (*m/z* 100), and similar fragmentation ions (*m/z* 465, 437, 410), as flordipine. The major difference between the spectra was the predominance of an *m/z* 113 ion in the 3.3-min peak; an *m/z* 114 ion, rather than an *m/z* 113 ion, predominated in the flordipine spectrum. The apparent molecular weight of the 3.3-min peak was confirmed with chemical ionization MS using methane as the reagent gas. The M+1 (*m/z* 511) and the M+29 (*m/z* 539) ions were observed, and were shown to be identical to those of floridipine analyzed under the same conditions. Based on the mass spectral data, and results from NMR analysis [5], it was concluded that the compound was most likely an isomer of flordipine, formed via a partial thermal rearrangement of flordipine, possibly in the GC injection port.

GC assays developed for two other dihydropyridines, nifedipine and nicardipine, have involved either direct injection of the compound [6—8], which can result in partial thermal oxidation of the compound to the pyridine form or chemical oxidation of the dihydropyridine ring to the corresponding pyridine before chromatography [9—13] to circumvent the perceived problem of partial thermal oxidation. A similar chemical conversion of flordipine before analysis to prevent the observed partial isomerization would not be

possible, however, since the nitrogen in the dihydropyridine ring of flordipine contains an ethylmorpholino substitution, unlike the other reported dihydropyridines which are unsubstituted in this position. Additionally, chemical removal of the ethylmorpholino substituent, before analysis, would be difficult and undesirable since this would yield the dihydropyridine REV 2611 (III, Fig. 1), which has been shown to be a hexane-extractable metabolite of flordipine [14, 15]. Additionally, the extent of isomerization of flordipine is small and consistent under the conditions of the assay, and this limited isomerization does not affect the precision or accuracy of the assay, as demonstrated by the results reported herein. Direct GC analysis, as described in this report, therefore, was determined to be the best approach for the GC quantitation of flordipine.

Applicability to analysis of clinical samples

The utility of this method in assaying clinical samples was demonstrated by analyzing 0–6 h plasma samples from two subjects administered a 50-mg tablet of flordipine. As seen in Fig. 3, flordipine was detected at concentrations which were within the working range of the assay during the entire 6-h time course for one subject, and up to 2 h post-dose for the other subject.

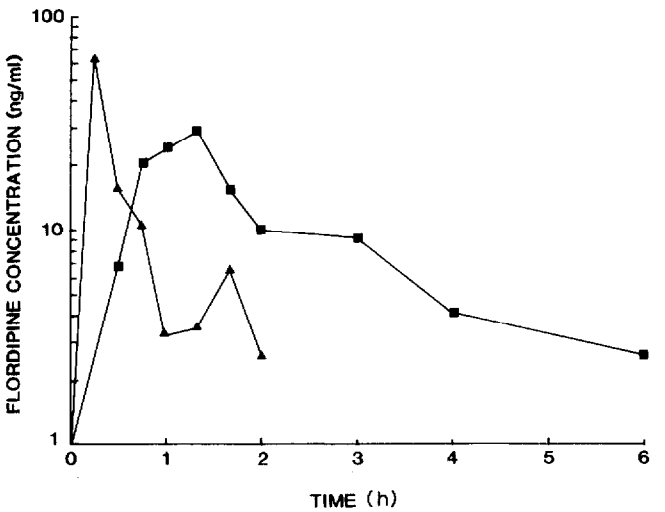


Fig. 3. Concentrations of flordipine determined in plasma from two human subjects administered 50 mg of flordipine.

CONCLUSION

An assay which is suitable for routine analysis of flordipine in clinical samples has been developed. It has been shown to be linear, precise, and reproducible from day to day, and can be accomplished in relatively few steps, thus maximizing daily sample throughput. The applicability of the method to analysis of clinical samples has been demonstrated.

ACKNOWLEDGEMENTS

The authors would like to thank Mr. W. Feeney for providing the mass spectra and Dr. S. Ringel for providing the clinical samples.

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