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Note

High-performance liquid chromatographic assay of flunarizine in plasma and its application to biopharmaceutic investigations

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Flunarizine [(*E*)-1-[bis(4-fluorophenyl)methyl]-4-(3-phenyl-2-propenyl)-piperazine] is the fluoro analogue of cinnarizine. It is widely used in the treatment of cerebral and peripheral vascular insufficiencies, and acts as a Ca^{2+} channel blocker and antagonist to histamine.

The usual daily doses of 5.9—11.8 mg of flunarizine dihydrochloride result in plasma levels in the low nanogram range. In the literature methods using gas chromatography—mass fragmentography [1, 2] or gas chromatography with nitrogen-sensitive flame ionization detection [3] are published. High-performance liquid chromatography (HPLC) has only been used for metabolite research and isolations from animals [4]. For pharmacokinetic studies, with numerous samples generated, the published methods are cumbersome and time consuming. The use of reversed-phase HPLC promises sensitive, accurate, fast and automated sample analysis.

The procedure described here is simple and sensitive, requires no derivatization and concomitantly administered drugs such as tricyclic antidepressives or beta-blocking agents do not interfere with the assay. The method comprises extraction of alkalinized, diluted plasma with methylene choride, with almost quantitative recovery. The application of this procedure to a single-dose pharmacokinetic study with ten healthy volunteers clearly demonstrates its utility.

EXPERIMENTAL

Materials

Flunarizine dihydrochloride and meclizine dihydrochloride were supplied by

0378-4347/86/$03.50

Sigma (Munich, F.R.G.). Methylene chloride was purified by filtration through activated basic alumina; all chromatographic solvents were HPLC grade and all other chemicals were analytical reagent grade.

Apparatus

A Gyncotek Model M 600 liquid chromatographic pump (Gynkotek, Munich, F.R.G.) and a Waters 710 B WISP autosampler (Waters Millipore, Eschborn, F.R.G.) were employed. A LiChrosphere 100 CH B, 125 × 4.0 mm I.D. column (particle size 4 μm; Merck, Darmstadt, F.R.G.) was connected to a Kratos SF 757 variable-wavelength detector (Kratos, Karlsruhe, F.R.G.) operated at 255 nm and 0.005 a.u.f.s. The mobile phase was 500 g of methanol, 300 g of water, 2.5 g of sodium acetate and 2.0 g of acetic acid, pumped at a constant flow-rate of 1.2 ml min^{-1}. The mobile phase was degassed with helium prior to use. All chromatography was carried out at ambient temperature.

Internal standard

A stock solution of 10 μg ml^{-1} meclizine (free base) was made weekly in water and stored at 4°C.

Spiked calibration samples and quality controls

For preparation of calibration samples, plasma from ten healthy volunteers was pooled and spiked with 1.00 mg of flunarizine per ml of water to give a concentration of 50.0 ng of flunarizine per ml of plasma. Samples at concentrations of 25.0, 10.0, 5.0 and 2.0 ng ml^{-1} were prepared by appropriate dilution with additional blank plasma. The samples were divided into 1-ml portions, frozen and stored with the subject samples of the study. Quality controls at concentrations of 5 and 25 ng ml^{-1} were prepared daily and blinded to the analyst.

Extraction of plasma samples

To a 10-ml screw-capped glass test tube, 1.00 ml of plasma, 100 μl of internal standard solution, 1 ml of 1 *M* sodium hydroxide and 6.00 ml of methylene chloride were added. The sample was treated for 30 min in the overhead-shaker and centrifuged at 2000 *g* for 10 min. The aqueous phase was aspirated, and the organic phase was transferred to another conical glass tube and evaporated at 40°C under nitrogen in the water-bath. A 150-μl volume of mobile phase was added and the sample was mixed for 1 min. An aliquot of 120 μl was injected into the liquid chromatograph. The samples were extracted in sequences. One sequence consisted of 42 study samples, a control blank, calibration samples and quality controls.

Recovery from plasma

For the determination of recovery, three replicate samples at levels of 2.0, 10.0 and 50.0 ng ml^{-1} for flunarizine were run through the procedure with exactly controlled volumes, as described for extraction of samples. The peak areas obtained for the extracted samples were compared with those of fresh standards of the analyte in mobile phase, regarding the volumes handled during extraction.

Quantitation

The integrator determined the peak areas of analyte and internal standard. After the run, it calculated the peak area ratio of analyte to internal standard. This result was fed into a computer (Commodore CBM 8032, Commodore, Frankfurt, F.R.G.). The results of the calibration samples were used for calculation of the calibration curve with linear regression, after $1/x$ concentration weighing. This was done by LAB-CAL software. The calibration curve was characterized by regression coefficient, slope and intercept. Using the calibration curve, the concentrations of the samples and the quantitation coefficients (QC) were calculated. The calibration is valid from the lower limit of quantitation of 2 ng ml^{-1} to the upper limit of quantitation of 50.0 ng ml^{-1}. Blanks are not included in the calibration.

Method validation

The procedure ws evaluated in terms of sensitivity, linearity of response, accuracy, precision, recovery and selectivity. For this purpose, five calibration curves in five different sequences were measured. The sensitivity of the method was evaluated by analysing plasma samples at the presumed lower limit of quantification (LLQ). The linearity of response was checked by means of the regression coefficients. The accuracy of the method was assessed by statistical evaluation of the mean value of five spikes of the same nominal concentration. The selectivity was tested by injection of structural related compounds, as well as common drugs, and evaluating their retention times.

Pharmacokinetic study

In the pharmacokinetic study, ten healthy volunteers (one male, nine female, aged between 18 and 52) were dosed in randomized two-fold crossover with two different formulations of flunarizine dihydrochloride. Dosage A was two tablets, each containing 5.9 mg of flunarizine dihydrochloride. Dosage B was two capsules with the same drug amount. The dosed amount equalled 10 mg of flunarizine free base. The volunteers fasted overnight and received the drug together with 200 ml of water in the morning. Four hours after drug administration, a breakfast followed. Blood samples (10 ml) were obtained by venipuncture and collected in heparinized tubes (Vacutainers, Becton and Dickinson, Kassel, F.R.G.) at 0, 0.25, 0.5, 1.0, 1.5, 2.0, 3.0, 4.0, 6.0, 8.0, 10.0 12.0, 24.0 and 48.0 h following each dose. The blood samples were centrifuged immediately and the plasma was removed and stored at −20° C until analysis.

RESULTS AND DISCUSSION

Analytical results

Flunarizine and internal standard gave sharp, symmetrical, well-resolved peaks under the conditions described in Experimental, with retention times of 3.8 and 5.3 min, respectively. Fig. 1a shows the chromatogram of a predose plasma extraction. Fig. 1b shows the chromatogram of a real sample with an analyte concentration near the LLQ, where the signal-to-noise ratio is approximately 20. Fig. 1c shows the chromatogram of a sample taken at a maximal plasma concentration (C_{max}), calculated as 49 ng ml^{-1}. Fig. 1d shows

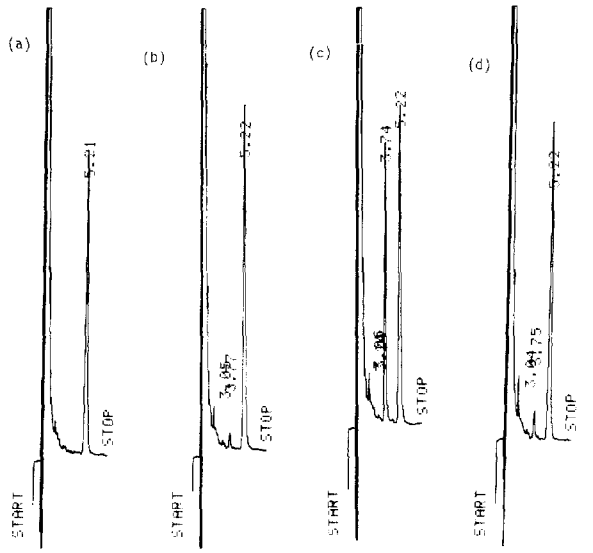

Fig. 1. Chromatograms of extracts from 1.00 ml of plasma. (a) Predose sample; (b) plasma sample 24 h post-dose with 2.3 ng/ml flunarizine; (c) plasma sample 4 h post-dose with 27 ng ml^{-1}; (d) quality control sample of 5.0 ng/ml^{-1}. The peak at 3.77 min is flunarizine, that at 5.22 min is internal standard.

TABLE I

STATISTICAL EVALUATION OF THE ANALYTICAL PARAMETERS DURING VALIDATION

QC = quality control.

Flunarizine added (ng ml^{-1})	Flunarizine calculated (ng ml^{-1})	S.D. (n = 5)	Accuracy (%)	Precision (%)
2.0	2.2	0.19	+ 9.9	8.6
5.0	5.3	0.41	+ 6.1	7.7
10.0	10.0	0.47	0.0	4.7
25.0	25.0	0.81	0.0	3.3
50.0	50.0	1.42	+ 0.2	2.8
5.0 (QC)	5.1	0.45	+ 2.0	8.8
25.0 (QC)	24.4	1.00	− 2.4	4.1
Mean r^2:	0.999 ± 0.0004			
Mean slope:	0.102 ± 0.006			
Mean intercept:	0.0062 ± 0.0025			
Equation:	$y = 0.0062 + 0.102x$			

a quality control sample at a concentration of 5.0 ng ml^{-1}. Table I shows the results of the validation for accuracy and precision of the calibration and quality control samples, as well as slopes, intercepts and regression coefficients of the calculated calibration curves. The calibration curves of the validation were linear from 2.0 to 50.0 ng ml^{-1}, with a mean slope value of 0.102, a mean intercept of 0.0062 and a mean r^2 of 0.999. Table II shows the respective results for the calibration and quality control samples of the clinical study. The

TABLE II

STATISTICAL EVALUATION OF THE ANALYTICAL PARAMETERS DURING STUDY

QC = quality control.

Flunarizine added (ng ml^{-1})	Flunarizine calculated (ng ml^{-1})	S.D. (n = 7)	Accuracy (%)	Precision (%)
2.0	2.1	0.14	+ 4.6	6.6
5.0	4.8	0.40	− 3.5	8.3
10.0	9.9	0.68	− 0.9	6.9
25.0	24.7	0.78	− 1.1	3.1
50.0	50.5	1.24	+ 1.1	2.5
5.0 (QC)	5.0	0.70	− 0.60	14.1
25.0 (QC)	26.1	2.16	+ 4.3	8.3

Mean r^2:	0.999 ± 0.0005
Mean slope:	0.099 ± 0.004
Mean intercept:	− 0.020 ± 0.018
Equation:	$y = -0.020 + 0.099x$

TABLE III

RECOVERY OF FLUNARIZINE FROM PLASMA

Flunarizine added (ng ml^{-1})	Flunarizine recovered (ng ml^{-1})	Mean recovery (%)	S.D. (n = 5)
2.0	1.89	94.5	1.6
10.0	9.64	96.4	1.3
50.0	47.35	94.7	1.7

calibration curves of the study were linear from 2.0 to 50.0 ng ml^{-1}, with a mean slope value of 0.099, a mean intercept of −0.02 and a mean r^2 of 0.999. Table III gives the results of the recovery study; at all concentrations investigated, the recovery from plasma was higher than 90%.

The selectivity was tested by carrying therapeutic concentrations of salicylic acid, propranolol, theophylline, ibuprofen, diazepam and fluphenazine through the method. None of the cited drugs interfered with either the internal standard or the analyte.

Pharmacokinetic results

Fig. 2 shows the mean plasma concentrations versus time profile for all ten subjects and two dosages. The collected pharmacokinetic parameters are listed in Table IV.

In the absorption phase, both formulations have the same plasma level vs. time profile. Formulation A (tablets) leads to a mean value of C_{max} of 49.0 ng ml^{-1} after a mean maximal time (T_{max}) of 3.4 h. Formulation B (capsules) causes a C_{max} of 45.6 ng ml^{-1} after T_{max} of 3.6 h. After T_{max}, the mean plasma concentration curve for formulation A decreases slightly more than the curve for formulation B. This can be manifested by the fact that the terminal

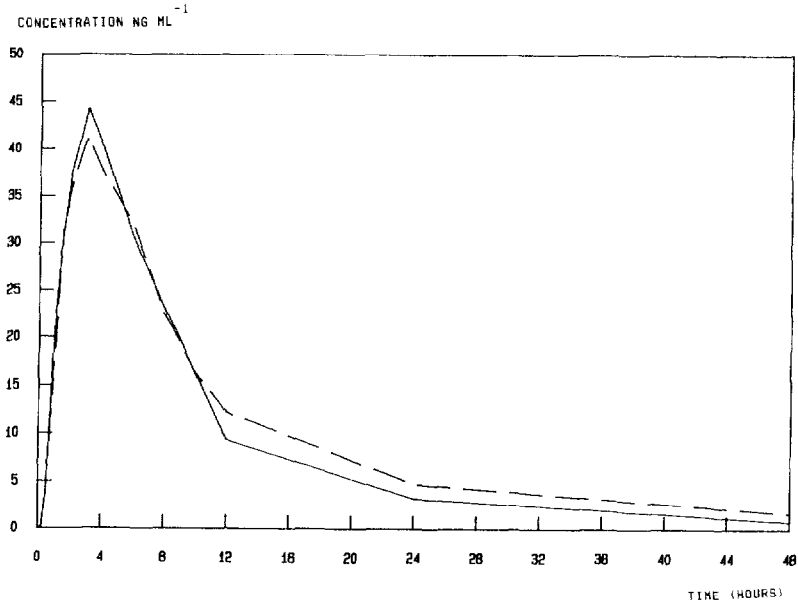

Fig. 2. Plasma concentration versus time profile. Mean over ten subjects for two formulations: ——, Treatment A (2 × 5.9 mg of flunarizine dihydrochloride in tablet form); — — —, treatment B (2 × 5.9 mg of flunarizine dihydrochloride in capsule form).

TABLE IV

PHARMACOKINETIC PARAMETERS OF TWO FLUNARIZINE FORMULATIONS

Formulation A: 2 × 5.9 mg of flunarizine dihydrochloride in tablet form. Formulation B: 2 × 5.9 mg of flunarizine dihydrochloride in capsule form.

Parameter	Treatment A		Treatment B	
	Mean	S.D.	Mean	S.D.
AUC^1 (h ng ml^{-1})	415.3	189.6	458.6	173
AUC^3 (h ng ml^{-1})	449.3	209.4	517.8	213.5
C_{max} (ng ml^{-1})	48.98	16	45.57	16.54
T_{max} (h)	3.35	1.25	3.6	1.35
$t_{½}$ (h)	6.47	3.34	9.41	5.5

half-life times ($t_{½}$) determined after logarithmic/linear regression, differ markedly: 6.5 h for formulation A and 9.4 h for formulation B.

The area under the curve (AUC^3, AUC^2, AUC^1) was calculated by the trapezoidal rule. (AUC^1 = area under the curve to the last measured value; AUC^3 = area under the curve extrapolated to infinity; AUC^2 = AUC^3 — AUC^1.) The ratio AUC formulation A/AUC formulation B gave the following results: AUC^1 88.14% and AUC^3 85.5%. In the ratio of the geometric means, formulation A has 85.5% of the area of formulation B. The Westlake confidence interval of 68—147% clearly exceeds the limits of 80—125%.

A major difference between both formulation is in the AUC^2, where A has a portion of 7.6%, whereas B has 11.3%. The differences between both

formulations, therefore, are in the period 24 h after drug administration, with plasma levels near, at or below the LLQ.

The method presented for quantitation of flunarizine in human plasma is sensitive, selective, precise and accurate for monitoring the pharmacokinetic profile of the analyte after low single doses. Application of the method on a clinical study with ten healthy subjects gave useful results for several pharmacokinetic parameters of two different drug formulations.

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