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DETERMINATION OF THE CALCIUM ANTAGONIST FLUNARIZINE IN BIOLOGICAL FLUIDS BY GAS–LIQUID CHROMATOGRAPHY

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SUMMARY

A method is described for the quantitation of flunarizine in biological fluids including plasma, urine, milk, fecal and tissue homogenates using the analogue cinnarizine as the internal standard. As little as 1.5 ng of flunarizine per ml of plasma can be accurately quantitated, this being achieved by the combination of a selective extraction procedure and a nitrogen detector. The method has been used to determine the concentration of flunarizine in biological fluids in support of human and animal pharmacokinetic studies.

INTRODUCTION

Flunarizine, 1-cinnamyl-4[bis(*p*-fluorophenyl)methyl] piperazine (I, Fig. 1) is a new member of the calcium channel blocker family of compounds [1] synthesized and marketed by Janssen Pharmaceutica in Europe and currently

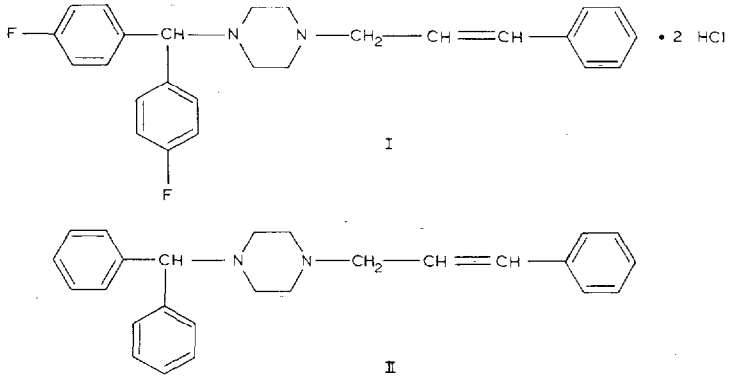

Fig. 1. Chemical structures of flunarizine (I) and cinnarizine (II).

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under clinical investigation by Ortho Pharmaceutical. In order to determine its pharmacokinetics and disposition, a sensitive and specific gas chromatographic assay was developed to quantitate flunarizine in plasma, urine, milk, fecal and tissue homogenate samples using the analogue cinnarizine (1-cinnamyl-4-diphenylmethylpiperazine) (II, Fig. 1) as the internal standard.

EXPERIMENTAL

Gas chromatography

A Varian Model 3700 gas chromatograph equipped with two thermionic specific detectors and two Varian auto samplers was used for analyses (Varian Assoc., Palo Alto, CA, U.S.A.). The 1.8 m × 2 mm I.D. glass column was packed with 3% OV-17 on 80—100 mesh Gas-Chrom Q (Supelco, Bellefonte, PA, U.S.A.). The column and injector port temperatures were maintained at 300°C and the detectors at 330°C. Flow-rates were 30—50 ml/min for the helium carrier gas, 100—170 ml/min for the air and 1—2 ml/min for the hydrogen. Bead current adjust was maintained between 550—650 and bias voltage between 5 and 6 for proper sensitivity. Chromatograms were traced on a strip-chart recorder (Houston Instuments, Austin, TX, U.S.A.). Peak heights were measured by a Model 3354 B computer interfaced with the detector by a Model 18652A A/D converter (Hewlett-Packard, Avondale, PA, U.S.A.).

Materials

Flunarizine dihydrochloride and cinnarizine were obtained from Janssen Pharmaceutica (Beerse, Belgium). PTFE-lined, screw-cap, 12-ml culture tubes were used for the extraction of the biological fluids. Kimax 3-ml conical tubes used for evaporation of the organic extract prior to injection were neutralized by immersion into a 10 *M* sodium hydroxide solution for 20 min, successive rinses with water and methanol and drying at 100°C in the oven. Hexane, methanol, ethyl acetate and toluene were glass-distilled (Burdick and Jackson Labs., Muskegon, MI, U.S.A.) and all aqueous solutions were made with glass-distilled water. The sodium carbonate—sodium bicarbonate pH 10.8 buffer [2] was stored at room temperature and replaced as needed.

Standards

A dilute (7 ng/μl) solution of flunarizine free base was prepared by weighing ten times the appropriate amount of the dihydrochloride salt and dissolving it in methanol. An aliquot (10 ml) of this solution was diluted with methanol in a 100-ml volumetric flask and used as assay standard as needed. A dilute (9 ng/μl) solution of cinnarizine was prepared and used in a similar way. Standards were kept in the freezer and checked periodically for concentration.

Analysis of biological fluids

Biological samples were stored frozen at −20°C and defrosted at the time of analysis. Milk samples were diluted 1:20 with distilled water prior to analysis. Fecal and tissue homogenates were obtained with water—methanol (50:50, v/v) in a Waring blender. Fat biopsy samples were homogenized in a mixture of hexane—ethyl acetate (3:1, v/v) in a tissue homogenizer (Akman).

Prior to analysis of samples, a drug-free biological fluid was analyzed and a calibration curve constructed for each biological fluid by adding the appropriate amounts of flunarizine (expected range) and internal standard (176 ng) to the appropriate aliquots. For analysis of unknowns, the appropiated sized aliquot (1, 4, 2, 0.5, and 20 ml, respectively, of plasma, urine, fecal and tissue homogenates and fat homogenates) was measured into a culture tube and 20 μl (176 ng) of the internal standard solution was added. All samples were analyzed in duplicate and a mini-calibration curve consisting of three concentrations (four replicates at the midpoint and two replicates each for the high and low points) covering the expected range of the unknowns, was analyzed with each set of unknowns. After addition of the internal standard, the tubes were vortexed vigorously and concentrated ammonium hydroxide (0.5 ml) and sodium carbonate—sodium bicarbonate buffer (1 *M*, pH 10.8, 1.0 ml) were added. The mixture was vortexed and hexane—ethyl acetate (3:1, v/v, 4 ml) was added. The mixture was shaken for 4 min and then centrifuged at 1500 *g* for 5 min. The organic layer was removed and the extraction repeated with another 4 ml hexane—ethyl acetate (3:1). The organic layers were combined and back extracted with hydrochloric acid (1 *N*, 6 ml). After shaking and centrifugation, the organic layer was aspirated under vacuum and discarded. The aqueous layer was then alkalinized with sodium hydroxide (10 *M*, 1 ml), vortexed and again extracted twice with the hexane—ethyl acetate mixture. After centrifugation, the organic layer was evaporated to dryness under nitrogen in a 3-ml conical tube. The residue was then reconstituted in 100 μl of toluene and 2—5 μl injected into the gas chromatograph. The concentration of flunarizine in the unknowns was determined by interpolation of the calibration curve extracted and analyzed alongside each set of samples using the peak height ratios versus concentration technique. The stability of flunarizine in the frozen plasma was established by spiking a five-point calibration curve (four replicates per point) with amounts of flunarizine (5—190 ng/ml), storing in the freezer at −20°C for three weeks, and analyzing for flunarizine. Stability in plasma was also determined by spiking 1-ml aliquots of blank plasma with 63.10 ng of flunarizine, storing and analyzing at several intervals.

RESULTS AND DISCUSSION

Flunarizine (I) and cinnarizine (II) (Fig. 1) are disubstituted piperazine derivatives, fairly volatile and very sensitive to the nitrogen detector. As little as 10 pg of these compounds injected on column can be accurately quantitated, although the practical limits of detection are determined by the background contribution of the biological matrix. The limit of 1.5 ng flunarizine per ml of plasma represents the smallest amount we could spike into blank plasma and consistently and accurately measure taking a coefficient of variation (C.V.) of 20% as the acceptance criterion. By using the 2× signal-to-noise ratio measurement a limit of detection as low as 0.5 ng/ml could be stated (Fig. 2), but day-to-day fluctuations in the background noise of the detectors makes this approach somewhat meaningless and more recently the former approach to establish the limits of detection has been recommended [3].

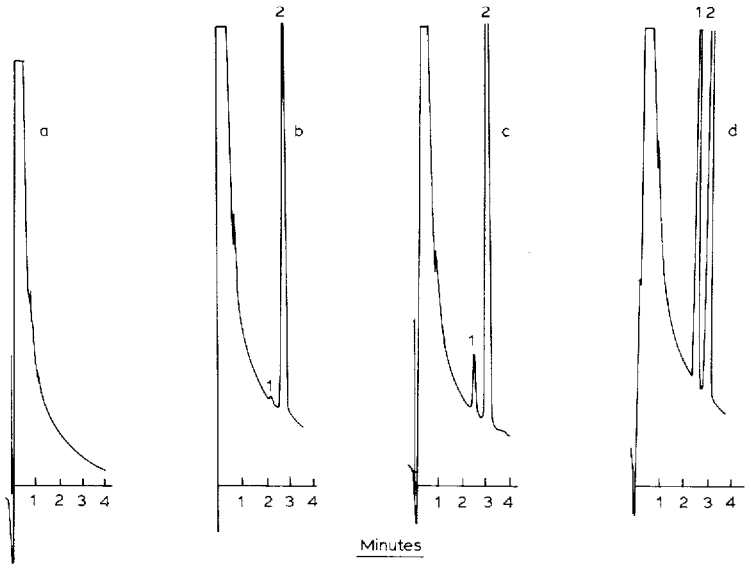

Fig. 2. Representative chromatograms of plasma samples containing: (a) no flunarizine and cinnarizine, (b) 1 ng/ml flunarizine and 176 ng/ml cinnarizine, (c) 10 ng/ml flunarizine and 176 ng/ml cinnarizine, and (d) 70 ng/ml flunarizine and 176 ng/ml cinnarizine. Peaks: 1 = flunarizine, 2 = cinnarizine.

Since flunarizine is in a very active stage of development, this methodology was required for several studies. Besides routine plasma analyses (pharmacokinetics and compliance), fecal homogenate and urinary analyses were useful to estimate absorption of the oral dose and excretion of unchanged compound. Milk analyses were used to study the extent of transport into the milk of the nursing female. Fat analyses investigated the possible accumulation into deep compartment tissues and tissue homogenate analyses were used for a variety of studies such as tissue distribution, passage through the placenta of the gestating female, etc.

The selectivity of the method was in each case demonstrated by the lack of interfering peaks in the analysis of the drug-free biological fluids. Furthermore, none of the known metabolites of flunarizine is eluted from the column under assay conditions. Linearity was demonstrated by the calibration curves constructed from each matrix (Table I). Correlation coefficients of peak height ratios versus flunarizine concentration of better than 0.990 were obtained in all cases except for the tissue homogenate matrix ($r = 0.93$). Accuracy and precision were established by running several replicates of each concentration. Except for the very low concentrations where the C.V. value was around 20%, most C.V. values were under 10% for the analysis of all matrices. The accuracy of the mean values was also good, with deviations from the true value generally staying under 10%, except for the lowest point in the milk curve where it was 20% (Table I).

A more extensive evaluation of the method was carried out for plasma analyses. Between-injection precision, established by repeated manual injections of the same sample extract showed a relatively high C.V. (17%) for the

TABLE I

PRECISION AND ACCURACY OF DETERMINATION OF FLUNARIZINE IN BIOLOGICAL FLUIDS

Biological matrix	Actual value (ng/ml)	Replicates (n)	$\bar{X}$	Accuracy*	C.V. (%)
Fat homogenates	28.8	6	28.3	−1.7	3.7
	46.2	6	43.4	−6.0	2.2
	124.5	6	133.7	+7.3	2.5
	249.1	6	247.6	0.6	2.9
	498.2	6	496.3	−0.4	0.9
	996.3	5	1008.7	+1.2	0.8
	1432.2	5	1472.2	+2.4	2.2
	1868.1	6	1903.4	+1.9	2.6
Urinary samples	5.8	6	5.8	0	5.1
	17.6	6	17.6	0	4.2
	29.2	6	29.2	0	2.1
	58.5	6	58.8	+0.5	1.4
	175.5	6	182.0	+3.7	2.9
Milk samples	16.6	3	12.8	−22.9	5.0
	53.2	2	53.2	0	1.5
	106.4	3	104.8	+1.5	3.8
	305.9	3	290.7	−5.0	0.6
Tissue homogenates	66.5	3	55.1	−17.1	13.0
	166.2	3	158.2	−4.8	1.8
	498.8	3	450.7	−9.6	9.9
	987.5	3	919.1	−6.9	5.1
Fecal homogenates	6.5	3	6.2	−9.4	11.6
	32.4	3	31.1	−4.0	2.9
	97.3	3	88.5	−9.0	2.3
	324.2	3	336.1	−3.7	12.1
	648.4	3	635.4	−2.0	5.9
	1296.7	3	1379.4	+7.8	5.8

*Accuracy $= \dfrac{\bar{X} - X_{\text{actual}}}{X_{\text{actual}}} \times 100.$

TABLE II

BETWEEN-INJECTION PRECISION OF DETERMINATION OF FLUNARIZINE IN PLASMA

Actual value (ng/ml)	Replicates (n)	$\bar{X}$	C.V. (%)
1.0	6	1.0	16.9
8.5	5	8.7	3.4
21.8	6	21.6	1.0
72.1	5	71.9	5.6
131.0	6	132.9	2.7
267.5	6	266.8	3.7

TABLE III

BETWEEN-REPLICATE PRECISION OF DETERMINATION OF FLUNARIZINE IN PLASMA

Replicates	$\bar{X}$	C.V. (%)
6	1.3	22.1
2	7.7	1.8
6	9.4	7.7
3	22.0	2.5
2	36.1	4.7
6	68.7	2.6

TABLE IV

BETWEEN-DAY PRECISION AND ACCURACY OF DETERMINATION OF FLUNARIZINE IN PLASMA

Day	Actual value (ng/ml)	Replicates (n)	$\bar{X}$	Accuracy*	C.V. (%)
1	1.5	4	1.4	−6.7	19.3
	7.3	4	6.8	−6.8	12.6
	22.0	4	21.5	−2.3	3.5
	73.4	4	79.9	+8.9	5.0
	146.8	4	171.1	+16.6	7.6
	293.6	4	300.2	+2.2	9.6
2	1.5	4	1.2	−20.0	13.9
	7.3	4	8.2	+11.7	12.4
	22.0	4	22.6	+2.7	5.6
	73.4	4	71.6	+2.4	2.8
	146.8	4	127.8	−12.9	3.1
	293.6	4	260.0	+11.4	0.8
3	1.5	4	1.3	−13.3	18.9
	7.3	4	6.9	−5.5	2.7
	22.0	4	22.0	0	2.5
	73.4	4	77.6	+5.7	2.3
	146.8	4	155.2	+5.7	2.4
	293.6	4	332.8	+13.3	3.0
1,2,3	1.5	3	1.3	−13.3	7.7
	7.3	3	7.3	0	10.7
	22.0	3	22.0	0	2.5
	73.4	3	76.4	+4.1	5.6
	146.8	3	151.4	+3.1	14.5
	293.6	3	297.7	−1.4	12.2

* $\text{Accuracy} = \dfrac{\bar{X} - X_{\text{actual}}}{X_{\text{actual}}} \times 100.$

lowest concentration, and much lower (1—6%) for the rest of the points (Table II). This indicates significant detector variability at low concentrations. Between-replicate precision, established by analyzing six replicates per concentration in our most common analytical range (0—70 ng/ml), showed very similar values to the between-injection precision indicating the reproducibility of the extraction procedure (Table III). Between-day precision and accuracy were established by the analysis of identical calibration curves on three different days and again, except for the lowest concentration, C.V. values were below 10% for most points and deviations from true values under 10% (Table IV).

The inherent variation in the absolute response of the nitrogen detector makes the use of an internal standard necessary and the evaluation of absolute recoveries somewhat difficult. Absolute recoveries, estimated by comparison of the peak heights obtained after injection of methanolic solutions of the compounds and after injection of known amounts of spiked and extracted samples were around 80% for both flunarizine and cinnarizine. Initially, losses of both compounds due to absorption to the glass during the final evaporation step were observed. This was eliminated by the strong base wash of the tubes described in the Experimental section. Siliconization, which also eliminated the problem, was found to interfere at times with the nitrogen detector. Flunarizine is also unstable in very dilute (< 5 ng/μl) solutions, therefore our lowest concentration solution was kept around 7 ng/μl and checked periodically for concentration. Flunarizine, however, was found to be very stable in frozen plasma. Analysis of a calibration curve stored for 3 weeks at —20°C and of plasma-spiked samples (63.10 ng/ml) stored for a period of over 1 year showed values for the precision and accuracy comparable to those obtained for the freshly extracted plasma samples (Table V). Only plasma samples were stored for significant amounts of time in the freezer.

TABLE V

FLUNARIZINE STABILITY IN FROZEN PLASMA

Days after preparation	Actual value (ng/ml)	$\bar{X}$	n	C.V. (%)
27	63.1	60.8	2	2.0
41	63.1	67.1	2	11.0
484	63.1	66.3	4	3.6
21	4.6	4.8	4	5.2
21	9.1	8.6	4	6.6
21	30.4	32.4	4	4.1
21	76.0	70.3	4	7.5
21	190.0	181.9	4	1.7

As previously stated, this methodology has been used among other applications, for pharmacokinetic studies. The pharmacokinetics of flunarizine after administration of a single oral dose (30 mg) to eight human volunteers were determined. Peak values at 2—3 h of around 100 ng/ml were observed and

residual levels of 2—3 ng/ml were still observed 7 days after administration (Figs. 3 and 4). To estimate the extent of absorption of the dose, fecal samples from this same study were also analyzed, as were urinary samples. The rest of the applications so far have been involved with animal studies.

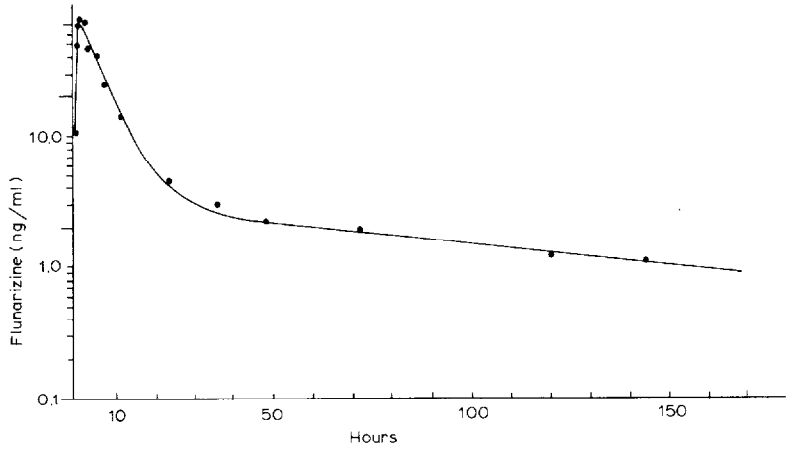

Fig. 3. Flunarizine plasma concentration versus time profile after single dose administration of 30 mg to man.

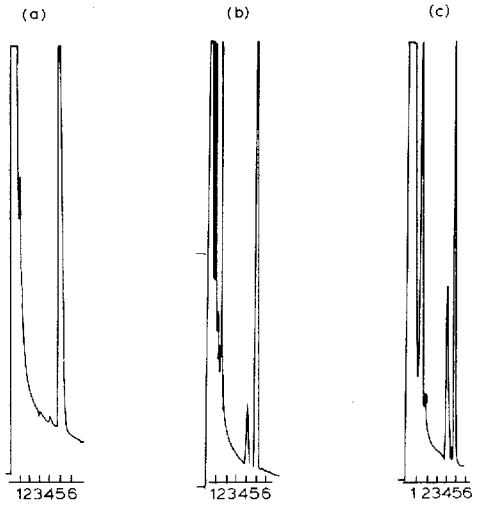

Fig. 4. Chromatograms of (a) 96-h, (b) 12-h and (c) 4-h human plasma samples obtained after administration of a single 30-mg dose of flunarizine. Concentrations found are 2, 22 and 70 ng/ml, respectively. Internal standard, cinnarizine (176 ng) was added to all the samples.

CONCLUSION

The methodology described is suitable for the determination of flunarizine in biological matrices and is sensitive enough for pharmacokinetic studies.

This sensitivity was achieved by the use of a nitrogen detector and a selective extraction procedure. The method is selective for flunarizine with no interferences from the known metabolites or endogenous materials observed.

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