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Determination of flunarizine in plasma by a new high-performance liquid chromatography method. Application to a bioavailability study in the rat

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Abstract: A reversed-phase high performance liquid chromatographic (HPLC) method is described for the study of the pharmacokinetics of flunarizine. The method involves selective liquid–solid extraction of flunarizine and meclizine (as internal standard) from samples of rat plasma. The optimization of the extraction and HPLC separation parameters are discussed. Recoveries were satisfactory and the relative standard deviation for replicate assays was below 10%. The sensitivity of the method would allow the detection of flunarizine in plasma at 13 ng ml^{-1}. Kinetic parameters for a bioequivalency study between flunarizine and a liquid formulation (oral drops) have been evaluated; the relative bioavailability was 88%.

Keywords: *Flunarizine; rat plasma; pharmacokinetics; reversed-phase HPLC; solid phase extraction.*

Introduction

Flunarizine is (E)-1-[bis(4-fluorophenyl)methyl]-4-(3-phenyl-2-propenyl) piperazine dihydrochloride (FNZ), a fluorinated derivative of cinnarizine; it improves brain and peripheral blood circulation [1]. At a vascular level, it acts mainly by blockade of the inward flow of extracellular calcium ions [2]. In the peripheral circulation this effect is selective for vascular smooth muscle; myocardial tissue is not affected [3, 4]. Clinically, FNZ has been shown to be useful in occlusive peripheral vascular disease [5, 6] as well as in vertigo of central [7] or peripheral [8] origin and in the prophylaxis of migraine [9, 10]. Its long half-life (19 days) for plasma elimination [11] allows the administration of a single daily dose.

Few analytical methods have been reported for the determination of FNZ in plasma samples. The first assays were carried out by an HPLC procedure [12]. This has been followed more recently by two gas chromatographic (GC) methods using nitrogen selective detection [13, 14] and by two other HPLC methods [15, 16]. In all cases solvent extraction procedures were used to obtain samples suitable for GC or HPLC separation.

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A simplified liquid–solid extraction method and HPLC assay is reported here for the pharmacokinetic study of flunarizine dihydrochloride administered to rats either as the free base or in liquid form (as oral drops).

Experimental

Sep-Pak C-18 cartridges from Waters Assoc. (Milford, Mass. USA) were activated with 6 ml of methanol, 6 ml of water and dried by forcing 15 ml of air through the cartridges with a syringe. To 1 ml of plasma was added 50 μl of internal standard solution [160 mg meclizine (MCZ) dihydrochloride/100 ml of methanol]. The pH of the plasma sample was adjusted to pH 4.5 with 5 M hydrochloric acid and loaded into the cartridge which was then washed with 6 ml of water (pH 4.5) and 1 ml of water–methanol (50:50, v/v). Elution of retained flunarizine and meclizine was carried out with 5 ml of methanol. The eluate was evaporated to dryness and the residue redissolved in 200 μl of methanol. In all cases the volume injected into the HPLC column was 20 μl.

HPLC instrumentation

The HPLC system used was a Series 2 liquid chromatograph and an LC-75 spectrophotometric detector, both from Perkin–Elmer (Norwalk, PA, USA). Samples were injected through a Rheodyne valve injector model 7125 (Cotati, CA, USA) fitted with a 20-μl sample loop. Detection was carried out at 250 nm and detector output was recorded on a Hitachi Perkin–Elmer Recorder model 156.

The column was a 10-μm Spherisorb ODS-1 (250 × 4.6 mm i.d.) or a 5-μm Spherisorb ODS-2 (150 × 4.6 mm i.d.); both columns were packed by Tracer Analitica S.A. (Barcelona, Spain). Guard columns were packed by hand with 37–50 μm Bondapak (C18/Corasil). The eluent (pH 6.4) was methanol–water (84:16, v/v) with 0.5% (m/v) of sodium chloride. In some runs small amounts of diethylamine (DEA) were used as indicated in Fig. 1. The flow rate was 1.5 ml min^{-1} and the column was operated at ambient temperature.

Pharmacokinetic study

Male Wistar rats (100–200 g) were maintained in controlled conditions with free access to food and water. Each rat was given an oral (p.o.) dose of flunarizine dihydrochloride equivalent to 20 mg kg^{-1} of free base (as active principle or liquid formulation). In both cases, doses were administered via a gastric probe in distilled water at 10 ml kg^{-1}. Animals were fasted 18 h before administration of the drug. Three rats were used for each time point. The composition of the liquid formulation (oral drops) was: flunarizine dihydrochloride 1.178 g; trisodium citrate 0.470 g; citric acid 0.260 g; propylene glycol 88.770 g; orange essence, 0.050 g and distilled water to 100 ml.

Blood sampling was performed by sectioning the carotid arteries under ether anaesthesia at the following times after administration of the drug: 15 and 30 min, 1, 2, 4, 8, 24 and 48 h. Blood was collected in heparinized tubes and was immediately centrifuged at 1500 r.p.m. for 10 min to separate the plasma. Plasma samples were kept frozen at −20°C until analysis.

Calculation of pharmacokinetic parameters

Curve fitting was accomplished using the iterative polyexponential curve stripping program JANA [17]; the reciprocal of the squared plasma concentration was used as the

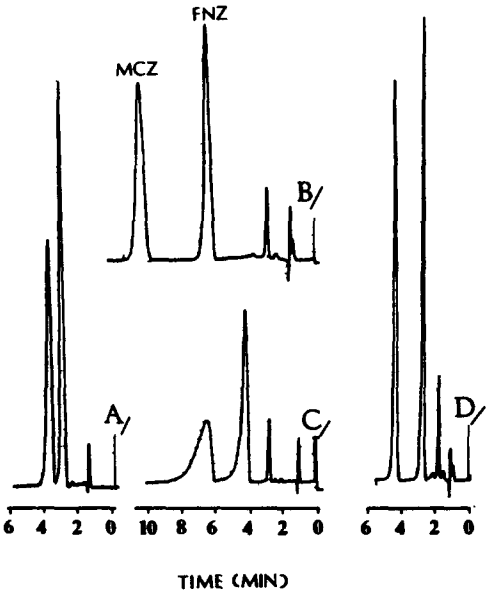

Figure 1
HPLC separation of authentic flunarizine (FNZ) and meclizine (MCZ) on (A) a reversed-phase 10-μm Spherisorb ODS-1 (250 × 4.6 mm i.d.) eluted with MeOH:H_2O (84:16, v/v) with 0.5% NaCl; detection at 250 nm. (B) same as (A) with 0.1% DEA in the mobile phase. (C) same as (A) except for the column which was a 5-μm Spherisorb ODS (150 × 4.6 mm i.d.). (D) same as (C) with 0.05% DEA in the mobile phase and 90% (v/v) MeOH.

weighting factor. The half-life of plasma elimination ($t_{1/2}\beta$) is obtained according to the equation: $t_{1/2}\beta = \ln 2/\beta$ where β is the slope of the elimination line.

The area under the curve for plasma values from zero to infinite (AUC_0^∞) was obtained from application of the trapezoidal rule to the last experimental value (C_t) and from this value to infinite by the equation: $AUC_t^\infty = C_t/\beta$.

Relative bioavailability was obtained by the equation: $B_R = A_p/A_s \times 100$ where A_p and A_s correspond to the areas under the curves for flunarizine in the liquid formulation and free flunarizine, respectively.

Results and Discussion

Flunarizine is a very hydrophobic compound which should interact strongly with apolar C18 reversed-phase stationary phases. This is indicated by the high content of organic modifier (84% v/v methanol) in the mobile phase necessary to achieve elution in a reasonable time.

Solvent extraction procedures from alkaline media can be lengthy and time consuming [14] or even unreliable in terms of reproducibility. In the authors' laboratory, the extraction method described in a recent report [15] did not yield satisfactory results. That method involved addition of sodium hydroxide to the plasma sample and extraction with methylene choloride. Clouding of the sample and emulsion formation occasionally hampered recoveries. In contrast, another recent report [16] described a similar extraction procedure except that the plasma was acidified to pH 1 before extraction into methylene chloride. Good recoveries were claimed for both methods although because of the pKa values of FNZ ($pKa_1 = 7.71$ and $pKa_2 = 4$ [18]) it appears that extraction from a basic medium into an organic solvent would give better results.

In the present paper a more direct liquid–solid method is reported in which reversed-phase sample enrichment cartridges are used to selectively retain FNZ and the internal standard MCZ. After passing the plasma samples through these cartridges, the two

compounds were eluted with methanol. Recoveries were 92.7 and 94.0% (mean of 3 determinations) for FNZ and MCZ, respectively.

Contrary to predictions based on its pKa_1 (7.71) and pKa_2 (4.0) values, samples containing FNZ were best loaded at acidic pH onto the Sep-Pak C18 cartridges for maximum recovery. Thus, recoveries of standard solutions of FNZ and the internal standard MCZ were high in the pH range 1.5–4.5 but decreased to approximately 50% at pH 10. This would indicate that although, in theory, selective retention on the C18 cartridges for sample clean-up purposes should be favoured at pH values higher than the pKa_1 value, the undissociated species would be too hydrophobic to be effectively displaced from the reversed-phase sorbent.

The separation of FNZ and MCZ on 10-μm Spherisorb ODS-1 (Fig. 1A) and on 5-μm Spherisorb ODS-2 (Fig. 1C) columns at pH 6.5 is illustrated in Fig. 1. Addition of 0.05% of diethylamine (DEA) prevents the tailing observed in the 5-μm column with the result shown in Fig. 1D. When adding DEA, the pH of the eluent can be controlled with phosphoric acid. On the other hand, DEA was not found to be necessary for the satisfactory elution of these two compounds in the 10-μm column; indeed 0.1% of DEA increased substantially both retention times as shown in Fig. 1B. This behaviour could be expected from the basic nature of the two compounds. In contrast, other authors have found that the addition of 0.15% of *n*-propylamine to an acetonitrile–water eluent was essential for the elution of FNZ from different reversed-phase columns [15]. The use of a similar acetonitrile–water eluent system with 1% of triethylamine had been reported previously for the determination of FNZ [18].

The pharmacokinetic study was carried on the 10-μm column. A typical separation is shown in Fig. 2. Retention time was effectively adjusted by pH control of the mobile phase within the range 6–7. Faster elution can be achieved at lower pH values. However,

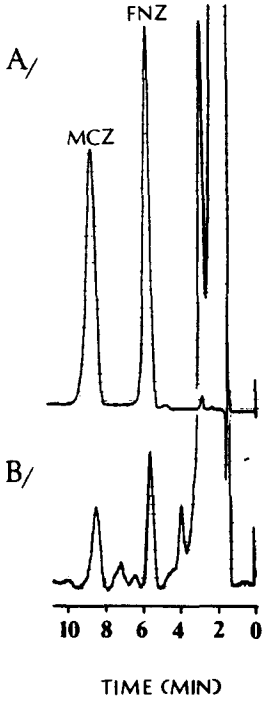

Figure 2
HPLC profile from a sample of rat plasma processed as described in the experimental section (bottom trace). The upper trace shows the separation corresponding to standard flunarizine and meclizine under the same conditions as in Fig. 1 A. Eluant pH adjusted with phosphoric acid to 6.5.

for precision and reliability of plasma determination both compounds should be preferentially eluted in a zone of the elution profile free from endogenous sources of interference or masking effects from the elution front.

Response was found to be linear both for flunarizine (2.6 ng–4.4 μg) and meclizine (50.9 ng–84.9 μg). The equation for the response (cm) vs concentration (ng) line was $y = 0.3900x - 13.6474$ and the correlation coefficient $r = 0.9994$. The absolute detection limit on the 5-μm ODS-2 column was 2.6 ng at a 3:1 signal to noise ratio. This would allow for the detection and quantification of a minimum concentration of 13 ng ml^{-1} in the plasma samples, as established by analyzing plasma aliquots supplemented with decreasing concentrations of FNZ. However, the actual working range for FNZ in the plasma samples assayed was 70 ng ml^{-1} to 1.8 μg ml^{-1}, as illustrated by the pharmacokinetic plot of Fig. 3. Thus there was no need to work near the detection limits in this case.

The precision and accuracy of HPLC determinations of flunarizine is illustrated in Table 1. The intra-assay reproducibility (relative standard deviation) ranges from 0.5 to 6.9% for different concentrations whereas the inter-assay value was 3.0%.

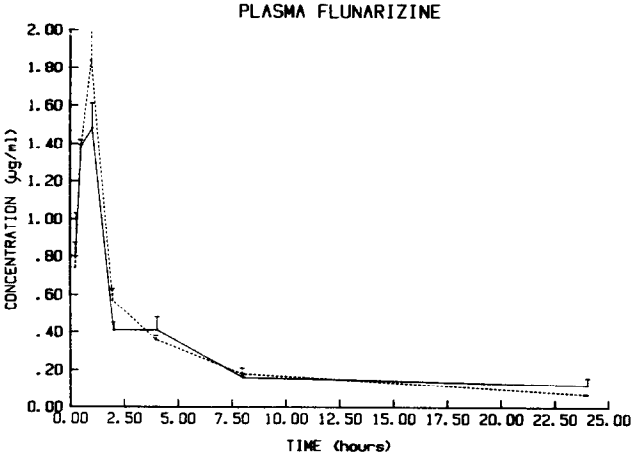

Figure 3
Plasma levels of flunarizine as active principle (solid line) and flunarizine as liquid preparation (dotted line) in male Wistar rats administered orally in a dose of 20 mg kg^{-1}. Each point at 15, 30 min, 1, 2, 4, 8 and 24 h on the time scale is the mean for 3 animals and vertical bars correspond to S.E.M. Not represented is the value of 0.03 ± 0.003 μg ml^{-1} active principle at 48 h. FNZ could not be detected 48 h after the administration of the liquid formulation.

Table 1
Precision and accuracy of determination of flunarizine in plasma

Day	*n*	Content (μg ml^{-1})	Found (μg ml^{-1})	Accuracy (%)	Relative standard deviation (%)
1	5	0.177	0.191	+7.9	6.9
	4	1.329	1.217	−8.4	5.3
	5	2.215	2.211	−0.1	0.5
3	5	1.329	1.202	−9.5	4.2
5	5	1.329	1.273	−4.2	1.6

From plots of the plasma levels against time, characteristic bicompartimental kinetic curves [19] were obtained as shown in Fig. 3. The best fitting equation, according to the JANA program [17] was found to correspond to a triexponential equation with lag time of the type:

$$C = A_1 e^{-\alpha(t-t_{lag})} + A_2 e^{-\beta(t-t_{lag})} + A_3 e^{-Ka(t-t_{lag})}$$

where one or both A_1 and A_3 values will be negative.

In this manner the following equations were obtained for free flunarizine:

$$C_{ff} = 11.0665e^{-2.6764(t-0.15)} + 0.3658e^{-0.0519(t-0.15)} - 11.4437e^{-3.5474(t-0.15)}$$

and for flunarizine oral drops:

$$C_{fd} = 2.1797e^{-0.7687(t-0.13)} + 0.2864e^{-0.0590(t-0.13)} - 2.4698e^{-4.0757(t-0.13)}$$

Kinetic parameters are given in Table 2. Michiels *et al.* [20] studied the absorption and tissue distribution of labelled FNZ in the rat, dog and pig. The present pharmacokinetic assay follows a similar approach, the plasma sampling time being the only experimental difference. A shorter sampling time of 48 h was used rather than 192 h used by Michiels *et al.* [20]. The plasma levels of FNZ, as well as the shape of the curve and most of the kinetic parameters obtained in the present assay, either after administration of the free base or the liquid formulation (see Table 2 and Fig. 3), are in agreement with previously published results [20]; this finding validates the efficiency of the present HPLC method. The values in Table 2 confirm the rapid absorption of FNZ in both cases (free base and liquid formulation) so that the peak concentration in plasma is attained 1 h after administration.

On the other hand, values were obtained for the plasma elimination half-life of 11.7–13.3 h indicating slow elimination kinetics. This agrees with results of studies showing that FNZ binds to adipose tissue and that the speed of distribution from this tissue is the factor determining the slow elimination [20]. However the present values are different from that of 54 h reported by Michiels *et al.* [20] although this could be explained by the shorter sampling period in line with similar observations described in clinical studies. A $t_{1/2}\beta$ of 4 days was reported in a study on 8 healthy volunteers who received a single oral dose of 30 mg FNZ where the sampling period was restricted to 48 h. A similar study on 6 volunteers carried out within a sampling period of 28 days resulted in a $t_{1/2}\beta$ of 18 days. The other kinetic parameters indicate a similar absorption

Table 2
Pharmacokinetic parameters

Treatment	Route	Dose mg kg^{-1}	Parameters t_{max} (h)	C_{max} (±S.E.M.) (μg ml^{-1})	AUC_0^∞ (μg ml^{-1}h)	$t_{1/2}\beta$ (h)
Flunarizine	p.o.	20	1	1.48 ± 0.13	8.63	13.3
Flunarizine (liquid formulation)	p.o.	20	1	1.84 ± 0.30	7.56	11.7

both for the oral drops and for the free drug [21]. By comparison of the AUC values it has been shown that the relative bioavailability of the liquid formulation is 88%.

The method has been found to be both practical and simple for sample preparation and HPLC separation and permits rapid determination of flunarizine in pharmacokinetic studies.

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