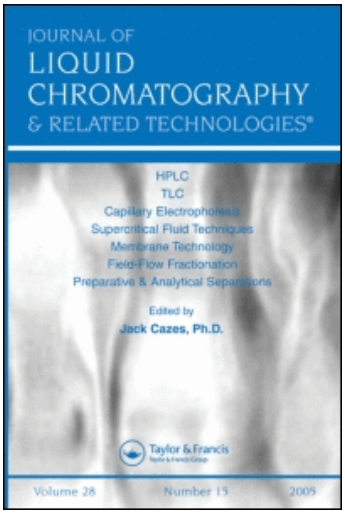

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DETERMINATION OF FLUNARIZINE IN RAT'S BRAIN BY LIQUID CHROMATOGRAPHY WITH UV/VISIBLE DETECTION

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ABSTRACT

This paper describes a newly developed method to determine Flunarizine (FZ) in rat's brain by high performance liquid chromatography (HPLC) with UV/Vis detection.

FZ was extracted by acetonitrile/water mixture and then analyzed by LC assay. Good extraction recovery (>90%) and linearity (r^2=0.9999) were determined. The precision of this method was adequate for our application. The application of this newly developed method was demonstrated by examining the pharmacokinetics of FZ in rats.

INTRODUCTION

Flunarizine (FZ), [trans-1-cinnamyl-4-(4,4-difluorobenzenhydryl) piperazine dihydrochloride], is one of the piperazine derivatives with antihistamine properties and calcium channel blocking activity. FZ is now widely used in the treatment of cerebral and peripheral vascular insufficiency.[1,2]

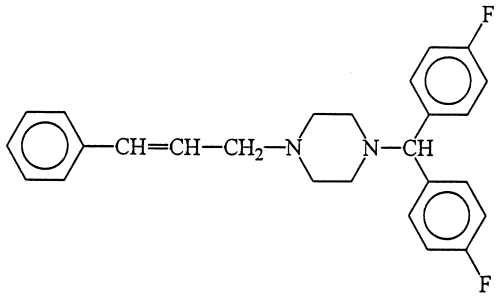

Figure 1. Chemical structure of flunarizine.

Figure 1 shows the structural formula of FZ. FZ's high lipophilic property enables it easily to penetrate the blood-brain barrier and reach the brain target. The half-life of FZ in male rats is 42 hr and in female rats is 77 hr in plasma.[3] The concentration of FZ in the brain is about 2 to3 folds higher than its plasma concentration.[4,5]

Most of the previous studies focused on the concentrations of FZ in the plasma and few reports monitored the concentrations of FZ in the brain. A number of gas chromatography (GC) and high performance liquid chromatography (HPLC) methods for the determination of FZ were also reported.[6-10] The reported GC methods required multiple-step extraction procedure prior to GC analysis. Some of previous reported HPLC methods also required complex sample preparation to extract FZ from plasma; furthermore, some of them utilized complex mobile phase at elevated temperature. Since relative poor precision of the reported method makes it unsuitable for our application, a highly sensitive HPLC method was then developed to determine FZ in rat's brain in order to study the pharmacokinetics of FZ in the brain rather than in the plasma. This newly developed HPLC method is rapid, sensitive, and precise.

EXPERIMENTAL

Chemicals

HPLC grade acetonitrile (Malinckrodt Baker, Inc., Paris, Kentucky, USA) and HPLC grade water (Labscan Limited, Dublin, Ireland) were used throughout the experiment. Ammonia solution (28%) was purchased from Nacalai Tesque, Inc. (Kyoto, Japan). Flunarizine dihydrochloride was from Sigma Chemical Company (St. Louis, MO, USA).

HPLC System

HP1100 LC system which consists of a quaternary pump, an on-line degaser, an autosampler and a UV/visible detector (Hewlett-Packard Co., Palo Alto, CA, USA) was used. A LiChroCART RP-18e column (Purospher, 125 x 3 mm, 5 μm, Merck KGaA, Germany) with an LiChroCART 4-4 on-line guard column was used for separation. The mobile phase was the mixture of 0.14% of ammonia water (pH 10.4) and acetonitrile (20/80, v/v). HPLC flow rate was 0.5mL/min. and operated at room temperature. The UV detector was set at 254 nm. The injection volume for HPLC analysis was 50 μL.

Standard Solution

For the examination of chromatography elution conditions, linearity, and detection limit associated with this method, 0.01 mM of FZ in 80% acetonitrile and 20% water (v/v) solution was prepared and stored at 4°C in the dark. This stock solution and the working solution that diluted with acetonitrile/water were prepared weekly. For interday and intraday precision studies, the stock and working solution were prepared daily. Standard solutions for the pharmacokinetic study were directly diluted from an injection solution, which was given to rats i.p., by our HPLC mobile phase. This could eliminate the preparing errors between standard solutions and injection solution.

Pharmacokinetic Study

Male Sprague-Dawley rats (250-350 g weight; Animal Center, National Science Council, Taiwan, R.O.C.) were used. On the experimental day, the rats received 60 mg/kg i.p. of FZ. The injection solution was prepared by first weighing 30 mg FZ and then dissolving in 3 drops Tween 80 solution. Finally, we added, gradually, by saline to it to 1 mL. At 20, 40, 60, 80, 100, 120, 140, 160, and 180 min. post FZ administration, the rats were decapitated. The brain was quickly removed and weighed. The brain tissues were then homogenized with 5 mL HPLC mobile phase for 1 min (Ultra-turrax T25, Janke & Kunkel Gmbh Co., Germany). Thereafter, samples were centrifuged at 4°C for 10 min. (8,000 rpm) and the supernatants were collected. The sample was filtered through a microporous syringe filter (0.2 μm, Lida Manufacturing Corp., Wisconsin, USA) before HPLC analysis. The data were expressed as μg FZ/g tissue.

RESULTS AND DISCUSSION

Figure 2 shows the typical chromatograms of a FZ standard solution and supernatant from rat brain extract (before and after FZ administration). FZ was

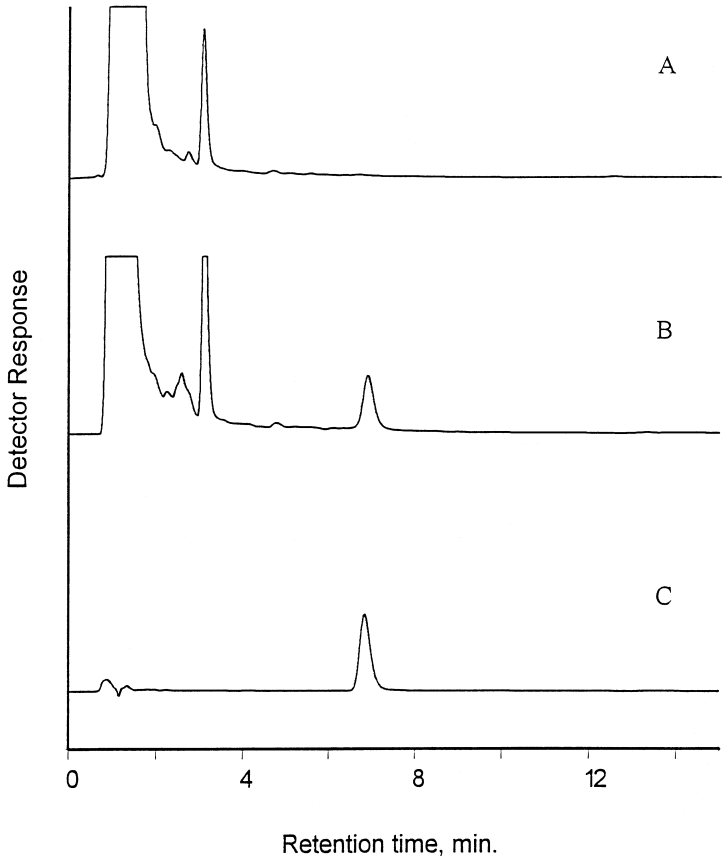

Figure 2. Chromatograms of (A) a rat brain (without FZ administration) extraction sample; (B) a rat brain (after FZ administration) extraction sample; (C) FZ in acetonitrile/water (80/20, v/v).

eluted at 6.9 minutes. Although there are many substances in rat brain extraction sample, none of them interfered with FZ. Ammonia solution was added in mobile phase to improve the separation of FZ in brain extract. Severe band-broadening of FZ chromatographic peak occurred in rat brain extraction sample without adding ammonia in mobile phase.

A series of FZ standards were prepared and injected into HPLC for analysis. Each standard was analyzed three times. A calibration curve for FZ standards was constructed and the linearity was good from 0.13 to 12.5 μM (r^2=0.9999).

Table 1

Recovery of Solvent Extraction

Added Concn. μM	Measured Conc.[a] μM	Recovery[a] %
6.20	5.76	92.9
6.20	5.51	88.9
3.10	2.87	92.6
3.10	2.82	91.0
0.78	0.73	93.6
0.78	0.75	96.2

[a] Average of three measurements.

Table 2

Recovery of Filtration

Added Conc. μM	Measured Conc.[a] μM	Recovery[a] %
6.20	6.28	101.3
6.20	6.29	101.4
3.10	3.04	98.1
3.10	3.17	102.2
0.78	0.76	97.4
0.78	0.77	98.7

[a] Average of three measurements.

The detection limit (DL) based on a signal-to-noise ratio of 3 and the limit of quantitation (LOQ) of this newly developed method were approximately 0.02 and 0.13 μM, respectively. The extraction efficiency of FZ from rat brain by extracting solvent was examined. FZ was spiked in rat brain, extracted by acetonitrile/water (80/20, v/v) solution, and then determined by LC analysis. The results are summarized in Table 1. Good recoveries ranging from 88.9 to 96.2 % were determined.

In addition to centrifuge, microporous filter was used to pre-treat extracted brain sample prior to LC analysis. This procedure is used to remove the residue and other large molecules in brain extract so as to prolong the lifetime of column. We have found some residual compounds from microporous filter that

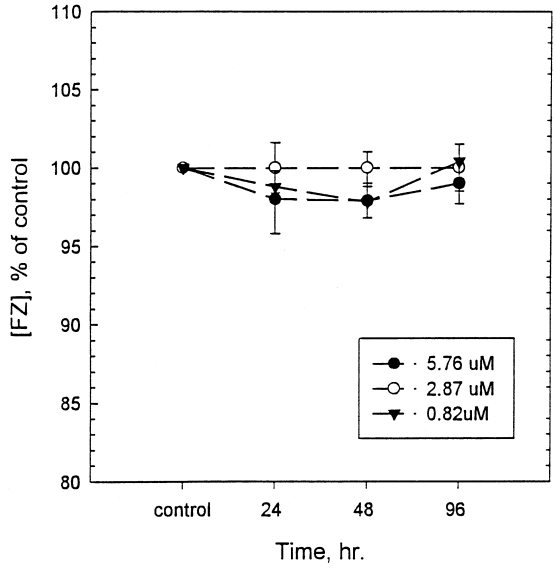

Figure 3. Stability of FZ samples versus time. Samples were stored in a 4°C refrigerator. Values are represented as the percentile changes of control (mean ± S.E.M., n=3).

would interfere with FZ analysis. However, These interfering components from microporous filter could be effectively removed by acetonitrile/water mixture. In addition, we examined the recovery of this filtration procedure to ensure the accuracy of test.

Various FZ standard solutions were filtered and then evaluated by LC. Table 2 is the summary of this study. Good recoveries ranging from 98.1 to 102.2% were measured.

The effect of sample storage condition was then evaluated. Some of the samples used for extraction efficiency were stored in a refrigerator (4°C) and then determined by HPLC after 24 and 48 hours of storage. The results are shown in Figure 3. The 0 hour sample served as a control. The difference between the results of 24 and 48 hours and the control was examined by one-tailed student-t test, and $P<0.05$ was taken to indicate statistical significance.

There was no significant decrease in response after storage in the refrigerator. In order to minimize the deterioration of sample, all samples were measured by HPLC immediately after extraction and pretreatment.

Table 3

Interday and Intraday Precision of Flunarizine in Extraction Solvent

Added Conc. •M		Intraday Measured Conc. •M	Interday Measured Conc. •M
6.19	Mean	6.19	6.13
	S.D.	0.100	0.130
	CV	1.62	2.12
3.10	Mean	3.09	3.07
	S.D.	0.060	0.050
	CV	1.94	1.63
1.55	Mean	1.55	1.55
	S.D.	0.030	0.033
	CV	1.93	2.13
0.77	Mean	0.78	0.78
	S.D.	0.010	0.016
	CV	1.28	2.05
0.52	Mean	0.53	0.53
	S.D.	0.006	0.011
	CV	1.13	2.08
0.26	Mean	0.27	0.27
	S.D.	0.003	0.010
	CV	1.11	3.70
0.13	Mean	0.14	0.14
	S.D.	0.002	0.006
	CV	1.43	4.28

Interday and intraday precision of this method was evaluated by replicated analysis of FZ spiked samples. Calibration standards were prepared and analyzed each day. A total of three series of samples were analyzed over a week period and each sample was measured in triplicate.

The results of precision study are summarized in Table 3. The intraday and interday precision showed a coefficient variation (CV) ranging from 1.11% to 1.93% and 1.63% to 4.28%, respectively.

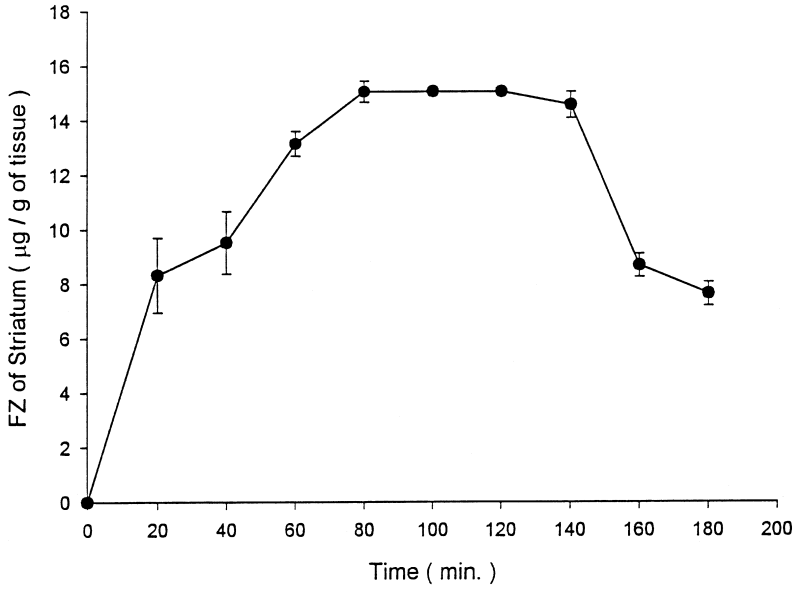

Figure 4. Time course of FZ in rat brain (n=6) following 60 mg/kg FZ i.p. injection.

The time course of FZ concentration (n=6) in the brain tissue of rats that received 60 mg/kg i.p. is shown in Figure 4. Other metabolites discussed in previous reports were not evaluated in this study. FZ concentration quickly increased in the brain and reached a maximum concentration of 14.46 ± 0.14 µg/g tissue from 60 to 80 min. post drug administration. After 120 min, the FZ concentration started to decline.

A non-linear curve-fitting computer program, Minsq, written by MicroMath Scientific Software (Salt Lake City, Utah, U.S.A.) was used to fit an equation consisting of the difference of two first-order kinetic processes for the appearance and disappearance of FZ in the brain to experimental FZ data:

$$FZ = A\,(\exp(-k_1 t)-\exp(-k_2 t)), \text{ where } A = (k1/(k_1+k_2))C_0$$

Three parameters fit into the data: A, k_1, and k_2. A is a concentration and absorption efficiency factor, k_1 is the first-order rate constant for the appearance of FZ, and k_2 is the first-order rate constant for the disappearance of FZ. Both rate constants have the units of min^{-1}. After this non-linear fit, the k_1 was 0.0119 and k_2 was 0.0162. The value obtained for the concentration factor, A, was 123.0 ug/g of brain tissue.

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