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Note

High-performance liquid chromatographic assay of flunarizine, (*E*)-1-[bis(4-fluorophenyl)methyl]-4-(3-phenyl-2-propenyl)piperazine, in plasma of epileptic patients

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The Antiepileptic Drug Development Program of the National Institute of Neurological and Communicative Disorders and Stroke is currently conducting clinical trials of flunarizine (FLU) in epileptic patients. A sensitive capillary gas chromatographic (GC) method [1] was developed for the analysis of single-dose plasma samples, but for chronic dosing it was desirable to develop a simpler chromatographic method.

Previously published high-performance liquid chromatographic (HPLC) procedures [2–4] used a single-step extraction which gave us samples that were discolored and sometimes cloudy. These samples led to rapid degradation of chromatographic conditions, occluding filters, increasing the pressure of the system, and loss of column efficiency. In addition, those methods were not cross-validated.

This paper reports the development of a new HPLC method with UV detection. The method allows the separation of apparent FLU metabolites which appeared in patient samples while on chronic dosing schedules. Using previously cited HPLC methods [2–4] we were unable to separate the apparent metabolites from FLU. The FLU values determined using this HPLC method were consistent with values determined by the GC method.

EXPERIMENTAL

Chemicals

Analytical-grade samples of flunarizine hydrochloride, (*E*)-1-[bis(4-fluorophenyl)methyl]-4-(3-phenyl-2-propenyl)piperazine hydrochloride, and the in-

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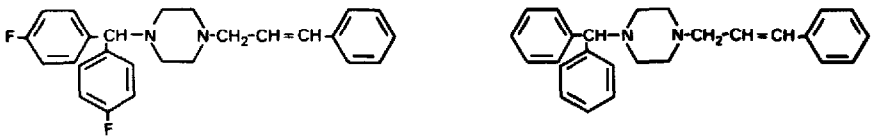

FLUNARIZINE CINNARIZINE

Fig. 1. Chemical structures of flunarizine and cinnarizine (internal standard).

ternal standard (I.S.), cinnarizine, 1-(diphenylmethyl)-4-(3-phenyl-2-propenyl)piperazine, were obtained from Janssen Pharmaceutica (Beerse, Belgium). The chemical structures of these compounds are shown in Fig. 1. Reagent-grade chemicals were purchased from Fisher Scientific (Fair Lawn, NJ, U.S.A.) and organic solvents from Burdick & Jackson Labs. (Muskegon, MI, U.S.A.). Other chemicals were of the best commercially available grade.

Subjects

Patients in this study received chronic dosing with FLU, doses ranging from 5 to 180 mg/day. These doses gave steady state values from 12.58 to 331.4 ng/ml.

Extraction of flunarizine

The extraction is as described previously [1]. Utilization of a different internal standard and modification of GC conditions previously reported further improved the precision of the assay.

Disposable PTFE-lined screw-cap culture tubes (16×125 mm), used for the acid back-extraction step, were silylated (Glas-Treet, Regis, Morton Grove, IL, U.S.A.) to prevent adsorption of FLU and the I.S., cinnarizine, to the glass.

Standard curve samples were prepared by spiking blank plasma with 25 μl of methanolic solution containing FLU to cover the range of the curve: 7.0–100.0 ng/ml. Methanolic solution (50 ng per 25 μl) of I.S. was added to all samples. Patient samples had 25 μl of methanol added in addition to the I.S., so that all samples contained 50 μl of methanol.

For the extraction, 1 ml of plasma and 1 ml of 0.0125 *M* sodium borate buffer (pH 8.5) were mixed by vortexing. The samples were double extracted with 4 ml of pentane–isopropanol (98:2) for 5 min using a double reciprocating shaker at low speed followed by centrifugation at 1400 *g* for 5 min at 4°C. The combined organic phases were transferred to silylated culture tubes and extracted into 6 ml of 1 *M* hydrochloric acid. Since we previously noticed an irreversible loss of FLU and I.S. under certain acidic conditions, the time the compounds were in acid was kept to a minimum. After centrifugation, the organic phase was aspirated off, the samples were made alkaline with 1 ml of 10 *M* sodium hydroxide, and then double extracted with pentane–isopropanol (98:2). The combined organic phases were evaporated to dryness under nitrogen at about 35°C.

The residue was dissolved in 200 μl of mobile phase with 50 μl used for HPLC analysis. When the same extract was to be analyzed by both HPLC and GC, the residue was first dissolved in 100 μl toluene–methanol (90:10), and 1 μl was used for GC analysis. The residual toluene–methanol was then transferred back to the

original test tube, reevaporated to dryness under nitrogen, and 150 μl mobile phase were added for the HPLC analysis.

To monitor inter-day variability, quality control samples were prepared by spiking blank plasma with one of three concentrations of methanolic FLU solution (80, 40, and 10 ng per 25 μl). Individual 1-ml aliquots were stored frozen and one set of samples included in every extraction and analysis run.

High-performance liquid chromatography

A Gilson (Gilson France, Villiers Le Bel, France) Model 116 UV detector set at 254 nm and a sensitivity of 0.005 a.u.f.s. was used for peak detection. Peak heights were quantitated using a Shimadzu C-R3A integrator (Shimadzu, Kyoto, Japan). Chromatographic separation was achieved using a Axxi-chrom C_8, 3 μm, 10 cm $\times$ 4.6 mm column run at ambient temperature (Cole Scientific, Calabasas, CA, U.S.A.).

Mobile phase was run isocratically and consisted of: (A) 79% 0.05 *M* $NH_4H_2PO_4$ + 0.025 *M* H_3PO_4 in water–methanol (50:50, v/v); (B) 21% water–acetonitrile–methanol (20:40:40, v/v/v). Total flow-rate using Gilson Model 302 pumps was 1.0 ml/min.

RESULTS AND DISCUSSION

Representative chromatograms of extracts from blank plasma, spiked plasma, and patient plasma show that sensitivity and clean sample criteria were met (Fig. 2).

The spiked quality control samples showed that this procedure gave good precision and accuracy (Table I).

The extracts from patient samples under chronic FLU therapy showed extra peaks in both GC and HPLC methods. These peaks were present only in patient samples, being most prominent during chronic treatment and increasing in size with increasing dose. Since they were absent from all blank plasmas tested and

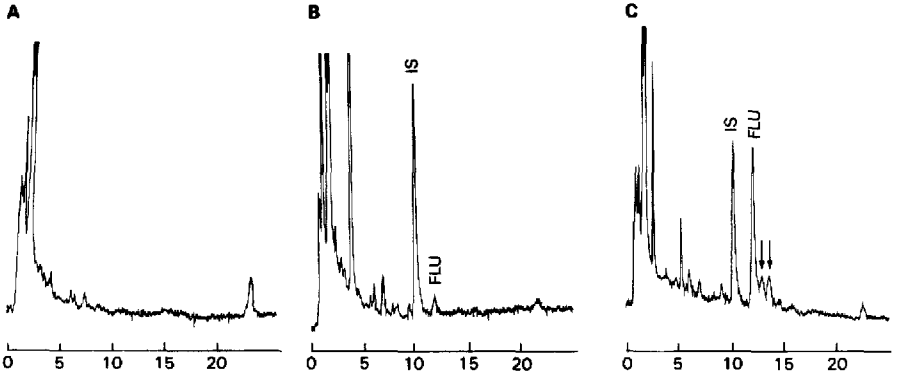

Fig. 2. Chromatograms of extracts from (A) blank plasma, (B) blank plasma spiked with 7.0 ng/ml FLU, and (C) plasma of an epileptic patient containing 78.6 ng/ml FLU. Arrows indicate putative metabolites. The *x*-axis represents time in minutes.

TABLE I

PRECISION AND ACCURACY OF THE HPLC METHOD FOR DETERMINATION OF FLUNARIZINE IN PLASMA

FLU added (ng)	FLU measured (mean ± S.D.) (ng)	Coefficient of variation (%)	*n*
80.00	81.22 ± 3.68	4.53	4
40.00	40.04 ± 2.13	5.32	4
10.00	10.63 ± 0.47	4.43	5

from control plasmas of patients obtained prior to administration of FLU, these peaks appear to be unidentified metabolites of FLU. They did not co-elute with several known FLU metabolites that were available to us: 4-hydroxyflunarizine, bis(4-fluorophenyl)methanol, 1-[bis(4-fluorophenyl)methyl]piperazine, flunarizine N-oxide, 4-hydroxyflunarizine N-oxide cinnamyl piperazine.

In addition, using conditions specified in previously published HPLC methods, we were unable to separate the apparent metabolites and FLU. It was impossible to see that there were two peaks as the FLU and apparent metabolite totally coemerged. These conditions lead to erroneously high FLU levels. In fact, high FLU levels in patient samples, not quality control samples, run concurrently on HPLC and then compared to GC alerted us to the separation problem (Table II). Discovery of the unresolved peaks demonstrates the advantage of having a totally independent method for validating a new analytical assay, as there was no indication that these peaks were superimposed.

This method was cross-validated by comparing the results from 24 patient samples ranging from 13.5 to 305.9 ng/ml with values obtained by modified previously cited capillary GC [1]. Each sample was analyzed in duplicate on different days

TABLE II

HPLC VERSUS GC DETERMINATION OF PATIENT SAMPLES

Representative patient samples were extracted and the same sample was determined by GC, HPLC, and HPLC.

GC	HPLC*	HPLC**
105.2	106.4	134.6
73.2	71.2	74.0
93.2	90.0	108.2
139.9	141.2	143.9
160 0	150.1	178.1
130.6	125.7	170.3
55.8	62.7	75.5
50.5	50.2	66.2

*Optimized HPLC conditions reported in this paper.

**HPLC conditions with unresolved interfering compounds (see paper for details).

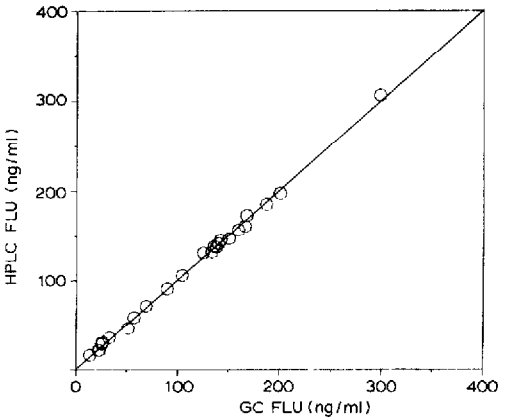

Fig. 3. Comparison of mean FLU concentrations using HPLC and GC. Least-squares linear regression analysis of FLU concentrations as determined by HPLC and capillary GC revealed $y = 0.999x + 1.26$ ($r^2 = 0.998$). Steady state plasma samples of 24 patients receiving FLU were run in duplicate and the mean values were used for comparison.

and the mean values were compared using least-squares linear regression analysis. There is good agreement between the two methods throughout the concentration range (Fig. 3).

The reported HPLC method is well suited for routine quantitation of FLU in plasma. It has been successfully cross-validated to GC results. HPLC is a simpler and more common method than capillary GC and gives equivalent results in the concentration range 7.0–100.0 ng/ml.

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