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Note

Automated capillary gas chromatographic determination of flunarizine

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Flunarizine, a difluorinated piperazine derivative, is an effective calcium channel blocker. It has antihistaminic, antiarrhythmic, and anticonvulsant activity. Its pharmacokinetic and pharmacodynamic properties were recently reviewed [1]. The Antiepileptic Drug Development Program of the National Institute of Neurological and Communicative Disorders and Stroke is currently conducting clinical trials of flunarizine in epileptic patients treated concomitantly with phenytoin and/or carbamazepine. Carbamazepine and phenytoin are known to induce the metabolic disposition of other drugs and thereby lower their plasma levels [2]. Therefore, a sensitive method for flunarizine determination in plasma from epileptic patients was needed for pharmacokinetic studies. Previous gas chromatographic (GC) methods, using packed columns [3, 4], had to quantitate a very small flunarizine peak eluting on the tail of a very large solvent front. In addition, the lower limit of detection for these methods was inadequate for the anticipated needs. Therefore, a capillary GC method was developed, allowing quantitation of flunarizine at concentrations as low as 0.25 ng/ml in plasma samples from epileptic patients on comedication.

EXPERIMENTAL

Chemicals

Analytical-grade samples of flunarizine hydrochloride, (*E*)-1-[bis(4-fluorophenyl)methyl]-4-(3-phenyl-2-propenyl)piperazine hydrochloride, and the internal standard (I.S.), (*E*)-1-[(4-chlorophenyl-4-fluorophenyl)methyl]-4-(3-

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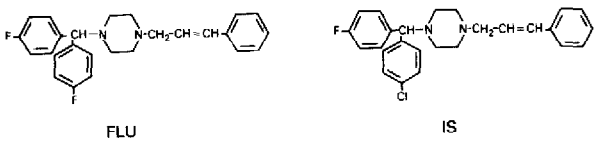

Fig. 1. Chemical structures of flunarizine (FLU) and internal standard (IS).

phenyl-2-propenyl)piperazine dihydrochloride, 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.

Extraction of flunarizine

Plasma was added to disposable 125 mm × 16 mm glass culture tubes with polypropylene screw caps. Each tube contained 1 ml of plasma. When the plasma volume was reduced because of a small sample or a flunarizine concentration greater than 100 ng/ml, blank plasma was added to the sample to make a total volume of 1 ml. Outdated human plasma (NIH Blood Bank, Bethesda, MD, U.S.A.) was used as blank plasma. Standard curve samples were spiked with 25 μl methanolic solution containing an appropriate amount of flunarizine. Methanolic solution (25 μl) of I.S. was added to all samples. All samples were made to contain a total of 50 μl of methanol. The resultant mixture was mixed briefly by vortexing. After the addition of 1 ml of 0.0125 *M* sodium borate buffer, pH 8.5, and vortex-mixing, samples were extracted twice with 4 ml of pentane—isopropanol (98:2, v/v) for 5 min using a double-reciprocating shaker (Eberbach, Ann Arbor, MI, U.S.A.) at low speed and centrifuging at 1400 *g* for 5 min at 4°C (Sorvall Refrigerated RT6000B centrifuge, Dupont, Newtown, CT, U.S.A.). The combined organic phase was placed into silylated (Glas-Treet, Regis, Morton Grove, IL, U.S.A.) disposable 125 mm × 16 mm culture tubes and extracted into 6 ml of 1 *M* hydrochloric acid using the double-reciprocating shaker. After centrifugation, the organic phase was aspirated off. Samples were made alkaline with 1 ml of 10 *M* sodium hydroxide and extracted twice with pentane—isopropanol (98:2, v/v). The combined organic phase was placed into 5-ml disposable glass conical centrifuge tubes and evaporated to dryness under nitrogen at about 35°C (Meyer N-EVAP analytical evaporator, Organomation Assoc., South Berlin, MA, U.S.A.). The residue was dissolved in 75 or 30 μl of toluene—methanol (90:10, v/v) and transferred to a 100-μl glass insert for a 1-ml glass injector vial (SunBrokers, Wilmington, NC, U.S.A.). The vials were sealed with 11-mm PTFE-lined septa (Supelco, Bellefonte, PA, U.S.A.).

Gas chromatography

A Model 5880A gas chromatograph (Hewlett-Packard, Avondale, PA, U.S.A.) was equipped with a Model 7672A automatic sampler, splitless injector, and nitrogen-selective detector. A 15 m × 0.32 mm I.D. cross-linked and bonded 100% dimethylpolysiloxane (0.25-μm film) DB-1+ fused-silica capillary column (J&W Scientific, Rancho Cordova, CA, U.S.A.) was used for

chromatographic separation. The carrier gas was ultra-high-purity helium adjusted to a flow-rate of 4.5 ml/min. The injector and detector temperatures were maintained at 300°C. The oven temperature was maintained at 90°C for 1 min and then programmed at 30°C/min to 265°C and held for 4 min. An additional 2-min bake-off period at 290°C was also included. The splitless injector inlet purge delay was set at 1 min. Quartz glass splitless injector liners were used after pretreatment. They were acid-washed, rinsed with water, then methanol, and oven-dried. This was followed by Col-Treet (Regis Chemical) treatment for 10 min, a hexane wash, and final drying at 150°C. After this initial silylation procedure, the liners were cleaned with a pipe cleaner and hexane after each series of analyses.

The GC terminal was programmed to control the gas chromatograph and automatic sampler for unattended overnight operation. The automatic sampler was loaded with alternating sample vials and wash vials containing 1 ml of toluene—methanol (80:20, v/v). The sampler was programmed to rinse the syringe fifteen times with 10 μl of the wash solution each time, and then to flush itself three times with 1 μl of the sample to be injected before injecting 1 μl of the analyte. Syringe washes between sample injections were necessary to eliminate sample carry-over. Standard curve samples were injected at the beginning and the end of each run, and the rest of the samples were injected randomly.

Quantitation

For each analysis, a standard curve was generated by adding known, varying amounts of flunarizine and a constant amount of I.S. to blank plasma. Two standard curves were used to bracket the range of experimental values: 0.25—7.0 and 7.0—100.0 ng/ml flunarizine. The amount of I.S. used for the low and high standard curves was 2.5 and 50.0 ng per sample, respectively. Each standard curve consisted of six or seven samples. Quantitation was achieved by using peak-height ratios of flunarizine to I.S. Linear least-squares regression analysis was performed to obtain the best fit for the standard curve data and to calculate the experimental values.

RESULTS AND DISCUSSION

The overall extraction efficiency of the method was determined to be 83.5%. However, judicious selection of solvents was found to be critical. The pentane—isopropanol mixture was selected because both solvents are commercially available in high purity, they minimize emulsion formation, and they evaporate easily. The ratio of pentane to isopropanol was determined empirically on the basis of maximum extraction efficiency. The acid step was also problematic. The use of sulfuric acid led to apparently irreversible loss of flunarizine. Hydrochloric acid was found acceptable if silylated tubes were used and if the time of contact of flunarizine with the acid was minimized. The use of methyl ethyl ketone for dissolving the final residue and for injecting flunarizine led to apparent decomposition as judged by a loss of the flunarizine peak and the appearance of another peak. The use of neat toluene was also unacceptable because of adsorption and/or solubility problems. This was corrected by using a toluene—methanol mixture.

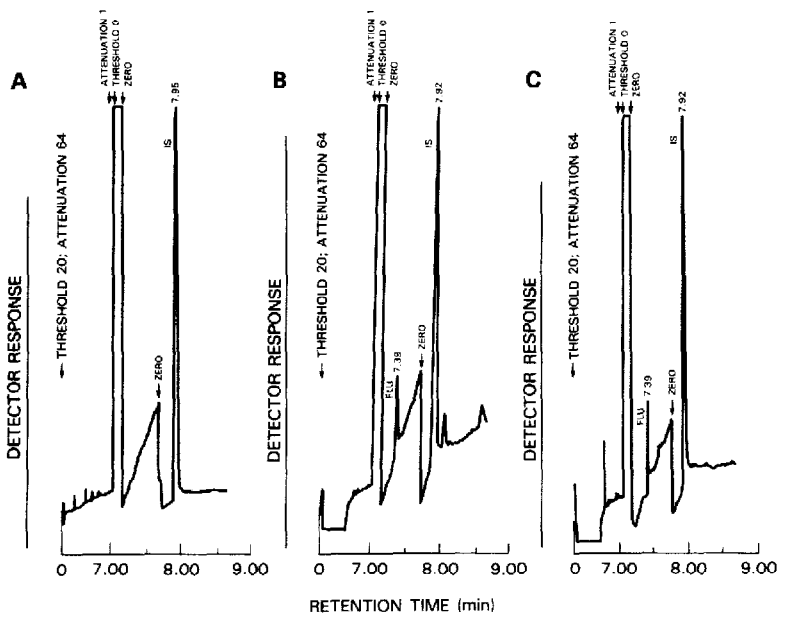

Fig. 2. Typical chromatograms of extracts from (A) plasma of an epileptic patient before administration of flunarizine, (B) plasma of an epileptic patient containing 0.32 ng/ml flunarizine, and (C) blank plasma spiked with 0.25 ng/ml flunarizine. Peaks: FLU = flunarizine; IS = internal standard.

Representative chromatograms of extracts from blank plasma, spiked plasma, and patient plasma (Fig. 2) show that sensitivity and clean sample criteria were met. They also show sharp, well defined, and easily quantifiable peaks resulting from subnanogram concentrations of flunarizine. This constitutes a marked chromatographic improvement over previously reported methods [3, 4], which used a packed column for separation. With the packed column, as opposed to the capillary column, the flunarizine peak was markedly broader, which resulted in much smaller peak height. In addition, this small peak eluted on the tail of a large solvent front. These limitations of packed-column separation hinder reliable quantitation of flunarizine at less than 1 ng per sample.

In order to cover a large concentration range (0.25—100.0 ng/ml) without sacrificing accuracy or precision, it was advisable to use two standard curves. The low standard curve was designed for 0.25—7.0 ng/ml and the high curve for 7.0—100.0 ng/ml flunarizine. Each standard curve consisted of at least six points covering the anticipated assay range. Excellent linearity and a negligible y-intercept were found routinely. Using a least-squares linear regression analysis, representative equations of the line and the regression coefficients (r^2) for the low and high standard curves were: $y = 1.307x + 0.035$, $r^2 = 0.999$ and $y = 0.059x + 0.008$, $r^2 = 0.999$, where y is the peak-height ratio of flunarizine/I.S. and x is the concentration of flunarizine.

The method was validated by using spiked plasma. The results showed good accuracy and precision throughout the concentration ranges tested (Table I).

A concentration—time profile for flunarizine after the administration of a

TABLE I

PRECISION AND ACCURACY OF AUTOMATED CAPILLARY GC METHOD FOR DETERMINATION OF FLUNARIZINE IN PLASMA

Spiked unknowns were bracketed by a standard curve ranging from 0.25 to 7.0 or 7.0 to 100.0 ng/ml. Each value represents four replicate samples. (See text for additional details.)

Amount flunarizine added (ng)	Amount flunarizine measured (mean ± S.D.) (ng)
0.30	0.31 ± 0.02
1.20	1.11 ± 0.07
2.40	2.31 ± 0.15
5.25	4.89 ± 0.17
7.50	7.30 ± 0.26
20.00	20.70 ± 0.70
35.60	34.80 ± 3.00
75.00	72.70 ± 7.78

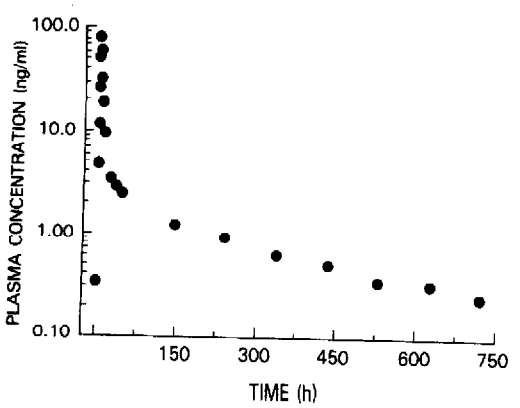

Fig. 3. Plasma concentration versus time curve for flunarizine following the administration of a single 30-mg oral dose of flunarizine to an epileptic patient, who was also receiving 340 mg of phenytoin per day.

single 30-mg oral dose of flunarizine to an epileptic patient (Fig. 3) shows that the concentrations of flunarizine during the slow elimination phase were mostly below 1 ng/ml. This elimination phase determines the elimination half-life, which is very long for flunarizine (150—450 h), and it also contributes extensively to the total area under the concentration—time curve. Therefore, pharmacokinetic studies following a single oral dose of flunarizine in epileptic patients require a more sensitive method than previously described. The analytical method described here provides the necessary sensitivity and is applicable to clinical pharmacokinetic studies. In addition, the use of the automatic sampler allows operator-free chromatographic analysis, thereby improving the method's efficiency.

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