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SIMULTANEOUS DETERMINATION OF FLUZINAMIDE AND THREE OF ITS ACTIVE METABOLITES IN PLASMA BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A sensitive and selective high-performance liquid chromatographic method has been developed for a new anticonvulsant, fluzinamide, and three of its active metabolites. This method requires only 0.5 ml of plasma, and it involves a single extraction with a mixture of hexane—dichloromethane—butanol (55:40:5). The plasma extract is chromatographed on a 10-μm, C_{18} reversed-phase column and quantitated by ultraviolet absorbance at 220 nm. The concentration—response curves for all four compounds are linear from 0.05 μg/ml to at least 10 μg/ml. The extraction efficiency of this method is greater than 90%. The accuracy and precision of the method were tested by analyzing spiked unknown samples that had been randomly distributed across the concentration range. The mean concentrations found were within ± 9% of the various amounts added with a standard deviation of ± 3.5%. This method has been successfully applied to the analysis of samples obtained from fluzinamide-dosed dogs, healthy unmedicated volunteers, and patients who were at steady state with phenytoin, carbamazepine, and fluzinamide.

INTRODUCTION

Fluzinamide, N-methyl-3-[3-(trifluoromethyl)phenoxy]-1-azetidine carboxamide, is a potential antiepileptic agent. It was shown to be effective in mice and rats in preventing convulsions induced by electrical and chemical stimuli. The profile of activity of fluzinamide most closely resembled those of phenobarbital and valproic acid [1].

Concentrations of anticonvulsants in the plasma of epileptic patients are routinely monitored in order to ensure that drug levels are within the therapeutic ranges. High-performance liquid chromatographic (HPLC) methods with either direct protein precipitation or simple solvent extraction techniques

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[2—7] are preferred because of their selectivity, speed, and sensitivity. Such a method for the determination of fluzinamide is especially important because of its poor solubility in water or aqueous buffer solutions and its potential for poor dosage form performance. During the early drug development process, plasma concentration data were needed to determine the bioavailability of this compound.

An HPLC method was initially developed [8] to monitor only the unchanged drug. This method involved a simple extraction with 40% dichloromethane in hexane, and the extract was chromatographed on a 10-μm, C_{18} reversed-phase column with 35% of acetonitrile in water as the mobile phase.

After the analysis of the first few samples, it was apparent that three metabolites were coextracted from plasma and chromatographed on the system described above. One of the metabolites was not completely resolved from fluzinamide.

In subsequent experiments, the three metabolites that circulated in the plasma along with parent compound were separated, identified, and synthesized [9]. They were all found to be active with structures similar to fluzinamide. The major metabolic pathway of fluzinamide was believed to be the formation, in sequence, of the N-hydroxymethyl derivative (I), followed by the N-formyl derivative (II) and then the 3-[3-(trifluoromethyl)phenoxy]-1-azetidine carboxamide (III), the N-desmethyl metabolite of the unchanged drug. Fig. 1 shows the chemical structures of fluzinamide, its active metabolites in the plasma, and the internal standard.

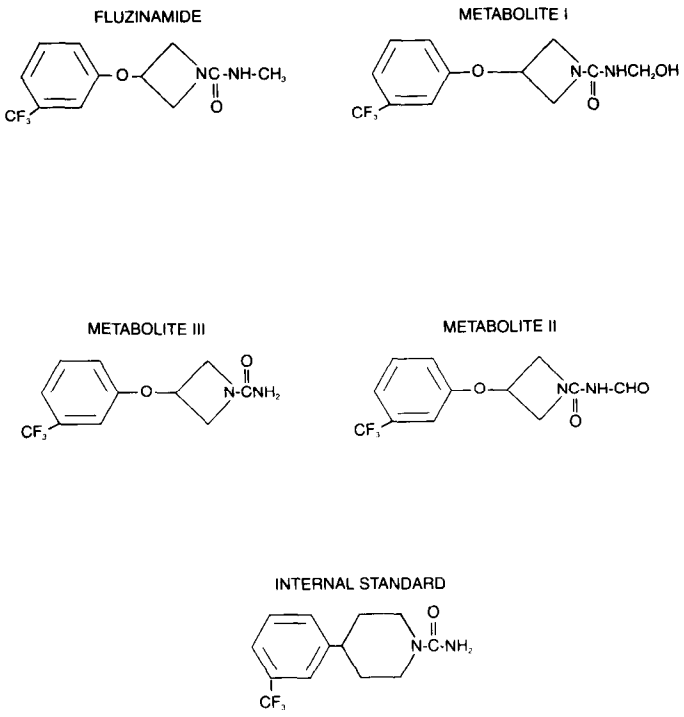

Fig. 1. Chemical structures of fluzinamide, three of its active metabolites, and the internal standard.

This paper is a description of a simple, selective, and sensitive method for the simultaneous quantitation of fluzinamide and its three active metabolites in plasma.

EXPERIMENTAL

Instruments and chromatographic conditions

The HPLC system consisted of a Waters Assoc. M6000A solvent delivery system, an autosampler (WISP Model 710B, Waters Assoc., Milford, MA, U.S.A.) and variable-wavelength detector (Schoeffel FS.770 LCUV, Schoeffel Instrument, Westwood, NJ, U.S.A.).

The chromatographic separation was achieved on a 10-μm, C_{18} reversed-phase Bondapak column. The mobile phase consisted of acetonitrile—tetrahydrofuran—0.025 *M* phosphate buffer, pH = 4.2 (30:5:65). It was delivered at a flow-rate of 1.5 ml/min and the compounds eluted were quantitated by their ultraviolet absorbance at 220 nm.

The output signal generated by the spectrophotometric detector was acquired by a computer-automated laboratory system (Computer Inquiry Systems, Waldwick, NJ, U.S.A.) with a Hewlett-Packard Model 1000 computer (Hewlett-Packard, Palo Alto, CA, U.S.A.). The chromatographic tracings were recorded on a 10-mV chart recorder (Hewlett Packard).

Chemicals and reagents

Acetonitrile, tetrahydrofuran, and monobasic sodium phosphate (analytical grade) were all purchased from J.T. Baker (Phillipsburg, NJ, U.S.A.). Hexane, butanol, and methylene chloride (analytical grade) were purchased from Burdick & Jackson Labs. (Muskegon, MI, U.S.A.). Fluzinamide, its three metabolites, and the internal standard were synthesized by A.H. Robins (Richmond, VA, U.S.A.).

Standard solutions

Stock solutions, corresponding to 100 μg/ml in acetonitrile—water solution (40:60), were prepared for fluzinamide, the internal standard, and metabolites I, II, and III.

Standard solutions ranging from 0.05 to 10 μg/ml were prepared by spiking drug-free plasma with the appropriate concentrations of all the compounds. To prepare the internal standard for spiking plasma samples, the stock solution was diluted to 50 μg/ml.

Extraction procedure

Samples (0.5 ml) of plasma, either standard or unknown, were transferred to 125 × 16 mm culture tubes. Internal standard (80 μl, 50 μg/ml) and 5 ml of extracting solvent that contained a mixture of hexane—methylene chloride—butanol (55:40:5, v/v/v) were added to each tube. The tube was vortexed for 15 sec and centrifuged for 5 min. The upper organic layer was pipetted to a clean tube and evaporated to dryness under a gentle stream of nitrogen in a water bath at 25°C. The residue was reconstituted in 200 μl of the mobile phase and 100 μl were injected into the HPLC system. All the samples and standards were run in duplicate.

Precision, reproducibility, and accuracy studies

To test the precision and reproducibility of this method, six individual standard curves of all compounds ranging in concentration from 0 to 10 μg/ml were run on consecutive days. The coefficient of variation values were determined for the peak height ratio at each concentration level. The slope, intercept, and correlation coefficient of the daily standard curves were calculated. The accuracy of the method was also determined by assaying thirty randomized samples spiked with various concentrations of the four compounds. The concentrations of these samples were unknown to the analyst at the time of analysis.

RESULTS AND DISCUSSION

Chromatograms of blank plasma spiked with the internal standard and blank plasma spiked with fluzinamide, three of its active metabolites, and the internal standard are shown in Figs. 2 and 3, respectively.

The chromatographic separation of all the compounds was accomplished on the column by the proper adjustment of the mobile phase. Tetrahydrofuran and the ratio of organic to aqueous fractions in the mobile phase appeared to be the two most critical factors. Tetrahydrofuran at 5—10% of the mobile phase composition was optimum for separating all five compounds. If the concentration of tetrahydrofuran exceeded 10%, metabolite II and the internal standard were not resolved; less than 5% tetrahydrofuran resulted in losing the resolution between metabolite II and fluzinamide. Changing the amount of

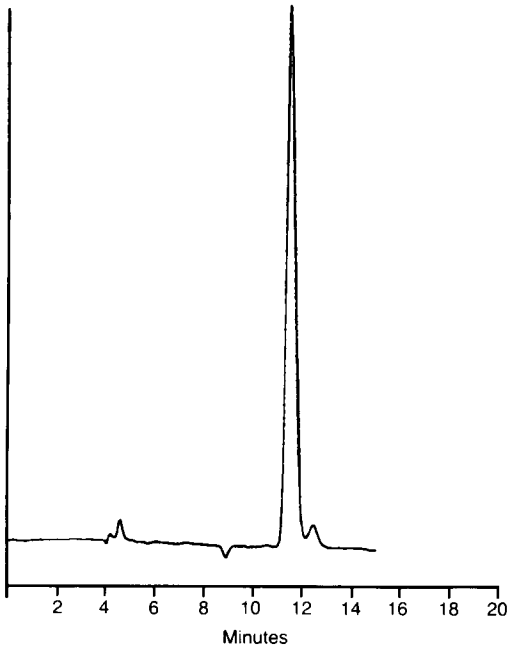

Fig. 2. Chromatogram of extracts from control plasma spiked with 4 μg/ml internal standard.

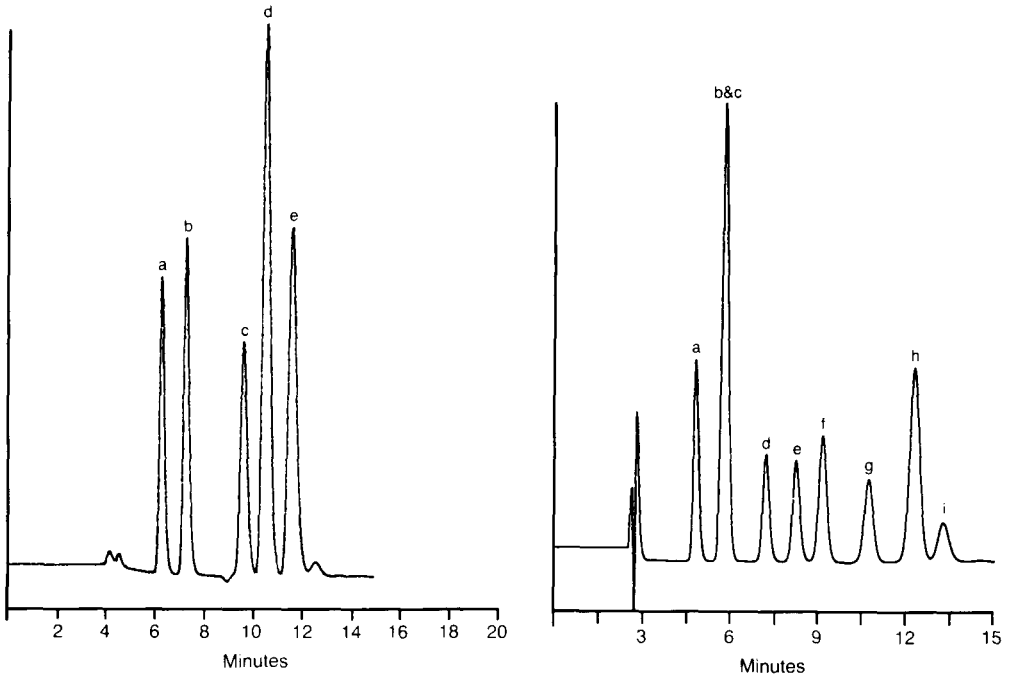

Fig. 3. Chromatogram of extracts from control plasma spiked with 5 μg/ml metabolite I (a), 5 μg/ml metabolite III (b), 5 μg/ml fluzinamide (c), 5 μg/ml metabolite II (d), and 4 μg/ml internal standard (e).

Fig. 4. Chromatogram of extracts from control plasma spiked with 10 μg/ml 5-(*p*-hydroxyphenyl)-5-phenylhydantoin (a), phenobarbital (b), carbamazepine (c), metabolite I (d), metabolite III (e), phenytoin (f), fluzinamide (g), metabolite II (h), and 4 μg/ml internal standard (i).

tetrahydrofuran in the mobile phase was necessary for the lot-to-lot change in column performance and the "aging" of analytical columns.

High plasma concentrations of the commonly used anticonvulsants such as phenytoin and carbamazepine are generally present in epileptic patients. The chromatography described in this method can separate these anticonvulsants from fluzinamide, its active metabolites, and the internal standard. Fig. 4 shows a typical chromatogram of extracts from plasma that had been spiked with 5-(*p*-hydroxyphenyl)-5-phenylhydantoin, phenobarbital, carbamazepine, fluzinamide, three of its active metabolites, phenytoin, and internal standard. The chromatogram shows good resolution for all compounds except between phenobarbital and carbamazepine. Therefore, the commonly used anticonvulsants do not interfere with the analysis of fluzinamide, its active metabolites, or the internal standard by the HPLC conditions described above.

Tetrahydrofuran at 8% appears to be the optimal concentration to be used in separating the large phenytoin peak from that of metabolite III by a 10-μm, C_{18} reversed-phase Bondapak column (Fig. 4). Slight variations in the retention times of all the compounds were observed between different batches of columns, but all the compounds and the internal standard should be eluted within 12 ± 2 min.

During the extraction and evaporation procedures, the temperature was kept

TABLE I

DETERMINATION OF UNKNOWN AMOUNTS OF FLUZINAMIDE AND METABOLITE I ADDED TO PLASMA

Fluzinamide added (μg/ml)	Number of samples	Concentration of fluzinamide found				I added (μg/ml)	Number of samples	Concentration of metabolite I found			
		Mean of set (μg/ml)	S.D. of set	C.V. of set (%)	Found (%)			Mean of set (μg/ml)	S.D. of set	C.V. of set (%)	Found (%)
0.00	4	BQL*	—	—	—	0.00	4	BQL	—	—	—
0.06	4	0.060	0.007	11.79	100	0.05	4	0.045	0.006	12.80	90
0.10	5	0.102	0.008	8.24	102	0.10	5	0.093	0.013	13.60	93
0.41	5	0.406	0.114	2.81	98	0.60	5	0.663	0.061	9.23	111
2.06	5	2.160	0.056	2.56	105	1.60	5	1.640	0.060	3.59	103
6.36	4	6.769	0.166	2.45	106	7.00	4	7.300	0.178	2.45	103
10.60	3	11.570	0.408	3.52	109	9.50	3	9.215	0.163	1.76	97

*BQL = below quantifiable limit, i.e. mean concentration is less than 0.05 μg/ml.

TABLE II

DETERMINATION OF UNKNOWN AMOUNTS OF METABOLITE II AND METABOLITE III ADDED TO PLASMA

II added (μg/ml)	Number of samples	Concentration of metabolite II found				III added (μg/ml)	Number of samples	Concentration of metabolite III found			
		Mean of set (μg/ml)	S.D. of set	C.V. of set (%)	Found (%)			Mean of set (μg/ml)	S.D. of set	C.V. of set (%)	Found (%)
0.00	4	BQL*	—	—	—	0.00	4	BQL	—	—	—
0.05	4	0.050	0.000	—	100	0.05	4	BQL	—	—	—
0.08	5	0.820	0.004	5.33	103	0.10	5	0.118	0.005	4.26	118
0.45	5	0.047	0.006	1.27	105	0.80	5	0.766	0.006	0.72	96
1.50	5	1.610	0.047	2.94	107	1.80	5	1.732	0.044	2.54	96
3.98	4	3.982	0.099	2.46	102	5.00	4	4.550	0.067	2.94	91
8.29	3	8.410	0.445	5.30	101	9.00	3	9.220	0.346	3.72	104

*BQL for metabolite III is 0.1 μg/ml and for metabolite II is 0.05 μg/ml.

at 25°C or below because the N-hydroxymethyl derivative of fluzinamide (metabolite I) is unstable at temperatures higher than 35°C. Preliminary studies on metabolite I showed that a significant amount of the compound was converted to metabolite III at temperatures over 35°C. However, fluzinamide and its active metabolites showed no significant degradation over a period of six months in spiked plasma samples stored at −20°C.

The maximum absorption wavelength for fluzinamide, its three metabolites, and the internal standard was around 220 ± 10 nm and the quantitative determination of all compounds was accomplished at 220 nm. Even though structurally very similar, the molar absorptivity of metabolite II is almost two times higher than fluzinamide or the other two metabolites at 220 nm. Generally, the results of six consecutive standard curves for fluzinamide and its active metabolites showed a linear range between 0 to at least 10 μg/ml. The mean slope values of the regression lines for fluzinamide and its three metabolites were 0.246 ± 0.01, 0.283 ± 0.02, 0.546 ± 0.01, and 0.348 ± 0.01 respectively. Good reproducibility was reflected by the tight correlation coefficients: r = 0.9994 ± 0.001. The intercept values of the regression lines were always negligible. The peak height for all compounds showed a coefficient of variation below 10% in the concentration range of 0.1—10 μg/ml.

Tables I and II show the recovery values of the thirty spiked samples. The percentages found were generally within ± 10% of the theoretical concentrations added. The lower limit of quantitation of fluzinamide, metabolite I, and metabolite II was 0.05 μg/ml; it was 0.1 μg/ml for metabolite III. The recovery by a single extraction step in this method was better than 90% for all the compounds.

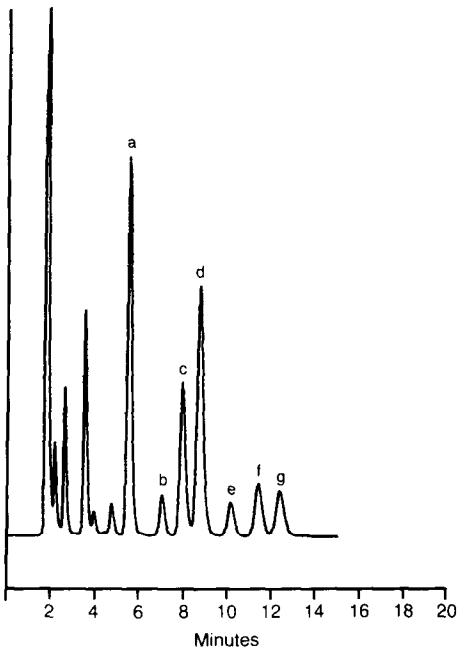

Fig. 5. Chromatogram of extracts from a plasma sample obtained from a patient at steady state of phenytoin, carbamazepine, and fluzinamide. Peaks: a = carbamazepine; b = metabolite I (4.64 μg/ml); c = metabolite III (6.14 μg/ml); d = phenytoin; e = fluzinamide (3.31 μg/ml); f = metabolite II (2.67 μg/ml); and g = internal standard (4 μg/ml).

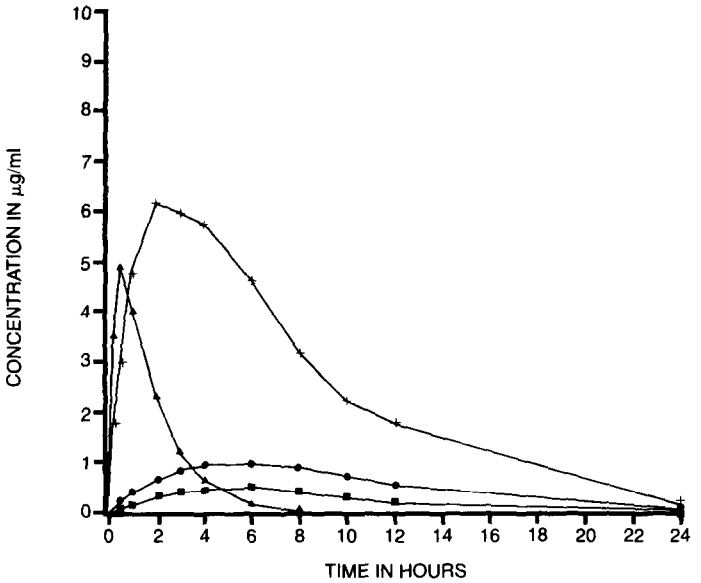

Fig. 6. Plasma concentration—time curve of fluzinamide and three of its active metabolites after a single 10 mg/kg oral dose of fluzinamide in a male dog. ▲, Fluzinamide; +, metabolite I; ■, metabolite II; ●, metabolite III.

This method was utilized in the analysis of plasma samples obtained from patients and dogs dosed with fluzinamide. Fig. 5 shows a chromatogram of extracts of a plasma sample obtained from a patient in which phenytoin, carbamazepine, and fluzinamide were at steady state. Fig. 6 shows the plasma concentration—time profile of fluzinamide and the three metabolites obtained after administration of a single oral dose (10 mg/kg) to a male dog.

CONCLUSION

A rapid, sensitive, and selective method has been developed for simultaneous determination of fluzinamide and those of its active metabolites at low microgram levels, with a linear range of 0.05—10 µg/ml, using 0.5 ml of plasma. The method exhibited a high degree of precision and accuracy, and it is well suited for routine analysis of plasma samples obtained from bioavailability and pharmacokinetic studies.

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