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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF NAFAZATROM IN HUMAN PLASMA USING FLUORESCENCE DETECTION

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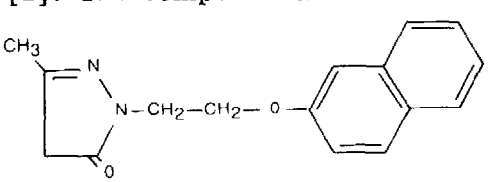
SUMMARY

A rapid, sensitive, and selective high-performance liquid chromatographic assay was developed for determination of the pyrazole derivative nafazatrom (Bay g 6575, NFZ) in human plasma. Separation was obtained using a normal-phase Si-60 column and a mobile phase of methylene chloride—methanol (90:10, v/v) containing 0.25% water. The fluorescence of NFZ was monitored at excitation and emission wavelengths of 232 and 362 nm, respectively. The recovery of NFZ extracted from plasma with methylene chloride was 109 ± 5% (mean ± S.D.) in the concentration range from 5.0 to 500 ng/ml.

The assay was applied to the determination of plasma concentrations of NFZ following administration of the compound to patients in a Phase I clinical trial.

INTRODUCTION

Nafazatrom (NFZ) {3-methyl-1-[2-(2-napthyloxy)-ethyl]-2-pyrazolin-5-one} (Fig. 1) was synthesized by Bayer (Wuppertal-Elberfeld, F.R.G.). NFZ has shown significant antithrombotic and thrombolytic activity in animal models [1]. The compound also has been reported to possess antitumor and anti-



3-Methyl-1-[2-(2-Naphthyloxy)-ethyl]-2-Pyrazolin-5-one

Fig. 1. Chemical name and structure of NFZ

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metastatic activities [2], and it is currently being investigated in a Phase I clinical trial at Wayne State University (Detroit, MI, U.S.A.). An analytical method was needed to determine NFZ in plasma to characterize its pharmacokinetic behavior. A method using high-performance thin-layer chromatography (HPTLC) reported recently by Ritter [3] has 5.0 ng/ml sensitivity but requires post-chromatographic derivatization, the use of an autospotter optimized for high performance, and densitometric detection.

This report describes a high-performance liquid chromatographic (HPLC) assay with a one-step extraction procedure and fluorescence detection for quantitative determination of NFZ at clinically achievable plasma concentrations.

EXPERIMENTAL

Chemicals and reagents

NFZ was supplied by Miles Pharmaceuticals (West Haven, CT, U.S.A.). Citric acid and disodium phosphate were received from Fischer (Fair Lawn, NJ, U.S.A.). Distilled in glass methanol and methylene chloride were received from Burdick and Jackson Labs. (Muskegon, MI, U.S.A.). L-Cysteine hydrochloride (monohydrate) was obtained from Sigma (St. Louis, MO, U.S.A.). Human plasma was obtained from Harper Hospital Blood Bank (Detroit, MI, U.S.A.). Deionized water was used for preparation of all buffers.

Chromatography conditions

The HPLC system consisted of a Model M-45 solvent pump, a Model 710-B WISP automatic injector, and a Model M-730 Data Module (Waters Assoc., Milford, MA, U.S.A.). Separation was obtained using a Hibar-II, LiChrosorb Si 60, 10-μm 250 mm × 4.6 mm column (Anspec Company, Ann Arbor, MI, U.S.A.). The mobile phase consisted of methylene chloride—methanol (90:10) with 0.25% water. A Perkin-Elmer Model 650-10M fluorescence spectrophotometer or Model LC-75 variable-wavelength UV detector (Perkin-Elmer, Norwalk, CT, U.S.A.) was used for quantification. For fluorescence the excitation wavelength was 232 nm, and the emission wavelength was 362 nm. Slit widths of 7 nm, range of 30, and normal gain, response, and mode were used. A wavelength of 280 nm was monitored for UV detection. The chromatographic system was operated at ambient temperature with a flow-rate of 1.0 ml/min. Under these conditions the retention time for NFZ was approximately 4.5 min.

Standard solutions

NFZ standard solutions were prepared by adding an accurately weighed amount of NFZ to methylene chloride and diluting with additional methylene chloride to the working range of the method, 5.0—500 ng/ml. These solutions were injected directly.

NFZ spiking solutions were prepared by adding accurately weighed amounts of NFZ to methanol to yield concentrations from $5.0 \cdot 10^3$ to $5.0 \cdot 10^6$ ng/ml. These solutions were used to prepare plasma standards.

NFZ plasma standards were prepared by adding 10 μl of the appropriate methanol standard solution to 10 ml of plasma to yield plasma concentrations

from 5.0 to 500 ng/ml. After adding 50 μl of a 1 g/ml (w/v) solution of cysteine, the samples were vortexed for 30 sec. The plasma standards were used to establish a calibration curve for the quantification of the concentration of NFZ in plasma from patients.

All standard solutions, spiking solutions, plasma standards, and cysteine solutions were prepared the same day as samples were assayed.

Stability studies

The effect of cysteine on the stability of NFZ in human plasma was evaluated at 21°C as a function of time. Two 10-ml aliquots of plasma were spiked with 10 μl of a $0.9 \cdot 10^6$ ng/ml NFZ standard in methanol. To one of these plasma standards were added 50 μl of a 1 g/ml solution of cysteine in water. Three 1-ml aliquots of each plasma standard were analyzed immediately and at selected times thereafter. Plasma was obtained either from the hospital blood bank (three weeks old) or from a healthy volunteer for immediate use.

One plasma sample (containing added cysteine) from each of four different patients who received NFZ was analyzed as described and then stored at 0°C. The samples were again analyzed ten days later for stability.

The influence of pH on the stability of NFZ was studied between pH 2.0 and 10.0 using 0.1 *M* citric acid buffer at 21°C. To 10 ml of the various buffer solutions were added 10 μl of a $0.9 \cdot 10^6$ ng/ml NFZ standard in methanol. Three 1-ml aliquots of each solution were analyzed immediately and at selected times thereafter.

Plasma samples from a cancer patient given NFZ were analyzed by the above HPLC method and immediately frozen. These samples were packed in dry ice and shipped as soon as possible to Miles Pharmaceuticals. Ten days later the samples were analyzed using an HPTLC method [3].

Sample handling

Because of the potential instability of NFZ in plasma (see results), blood samples were centrifuged immediately after collection to separate plasma from blood cells, and 5 μl of a 1 g/ml cysteine solution were added per ml of plasma. The plasma samples were stored in an ice bath if the analysis was to be performed the same day or frozen at 0°C.

Extraction procedure

Plasma (1 ml), 1 ml of a 0.1 *M* citric acid buffer, pH 7, and 1 ml of methylene chloride were added to a 10-ml centrifuge tube and capped. The test tubes were shaken for 10 min and centrifuged for 10 min at 600 *g* at room temperature. The aqueous phase was discarded and 10—50 μl of the organic phase were chromatographed depending on the concentration expected. All patient samples with expected concentrations higher than 500 ng/ml were diluted (prior to extraction) into the working range 5.0—500 ng/ml with 0.1 *M* citric acid buffer, pH 7.

Preparation of patient samples

Cancer patients were given a twice-daily oral dose of 4000 mg/m^2 of NFZ in tablet form. Blood samples were collected at various time points for three

days via intravenous catheter and transferred to heparinized test tubes.

Duplicate plasma samples were extracted and chromatographed according to the above procedure.

Calculations

The recovery of NFZ in the extraction procedure was calculated by comparison of peak heights of NFZ in the standard solutions with peak heights of the extract of the spiked human plasma. The concentration of NFZ in ng/ml of plasma in an unknown sample was determined by interpolation from calibrations curves of standards processed along with the unknowns.

RESULTS

Sample chromatograms of methylene chloride extracts of control plasma (A), spiked plasma (B), and patient plasma (C) are shown in Fig. 2. No interfering peaks were seen in control plasma obtained from the blood bank or in patient plasma obtained before administration of NFZ.

The linearity, accuracy, and precision of the method were evaluated in plasma over a concentration range from 5.0 to 500 ng/ml NFZ, Table I. Triplicate samples at each of nine concentrations were extracted and chromatographed as described. The least-squares linear regression analysis of the data shown in Table I gives a correlation coefficient of 0.999. The mean coefficient of variation was 2.9%. The percent recovery of NFZ from human plasma was 109 ± 5%, and the limit of detection was 5.0 ng/ml in plasma.

The effect of cysteine on the stability of NFZ in fresh or stored human plasma can be seen in Table II. In fresh plasma NFZ was apparently stable for

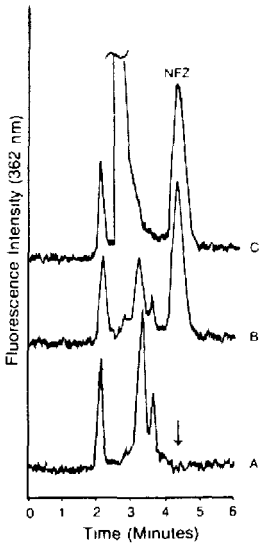

Fig. 2. Chromatograms of plasma extracts of (A) control plasma, (B) control plasma containing 54 ng/ml NFZ and (C) patient plasma 8 h after the second oral dose of 4000 mg NFZ per m^2 body surface area. Column: Hibar-II, LiChrosorb Si 60, 10-μm, 250 mm × 4.6 4.6 mm; solvent: methylene chloride—methanol (90:10, v/v) with 0.25% water; flow-rate: 1.0 ml/min.

TABLE I

LINEARITY AND PRECISION OF THE HPLC ASSAY OF NFZ IN PLASMA

NFZ concentration (ng/ml)		Coefficient of variation (%)	Recovery*** (%)
Theoretical*	Found** (mean ± S.D.)		
4.9	5.0 ± 0.0	0.1	100
9.7	10.2 ± 0 8	7.8	110
16.2	17 1 ± 0.7	4.1	114
26.8	27.3 ± 0.8	2.9	103
54.0	57.0 ± 0.7	1.2	114
108.0	114.7 ± 3.3	2.9	114
180.0	180.2 ± 5.0	2.8	106
300.0	303.9 ± 4.0	1.3	107
501.0	496.3 ± 16.4	3.3	109
	Mean ± S.D.	2.9	109 ± 5

*Theoretical concentrations were based on the amount of NFZ in methanol added to plasma.
**The concentration found was calculated from the calibration curve for each of three plasma samples per concentration. The mean, standard deviation, and coefficient of variation (n = 3) are tabulated
***The percent recovery was calculated from peak heights of plasma standards divided by peak heights of standard solutions in methylene chloride containing the corresponding concentrations.

TABLE II

STABILITY OF NFZ SPIKED IN FRESH OR AGED PLASMA WITH OR WITHOUT ADDED CYSTEINE

Time (h)	Fresh plasma*		Aged plasma**	
	With cysteine	Without cysteine	With cysteine	Without cysteine
0	96, 100***	106, 101	106	105
1	106, 105	103, 105	103	94
2	114, 101	89, 104	98	84
3	104, 101	110, 101	99	77
4	106, 99	97, 103	97	74
5	101, 101	103, 100	105	66

*Obtained from a healthy volunteer immediately before use.
**Obtained from blood bank after three weeks of storage.
***Percent of added 90 ng NFZ per ml plasma remaining.

5 h whether cysteine was added or not. However, in stored plasma from the hospital blood bank (three weeks old) the NFZ concentration decreased 34% in 5 h if cysteine were not added.

The stability of NFZ in frozen plasma samples with added cysteine was investigated. The recovery after ten days based on the concentration found in the initial assay was 100.3 ± 2.9% (mean ± S.D., n = 4).

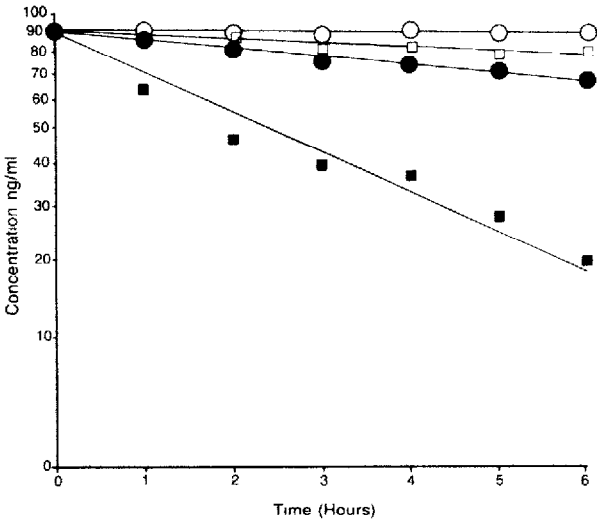

Fig. 3. The effect of pH on the stability of NFZ (90 ng/ml) in 0.1 *M* citric acid buffer. Key: (○) pH 7.0; (□) pH 4.0; (●) pH 2.0; (■) pH 10.0.

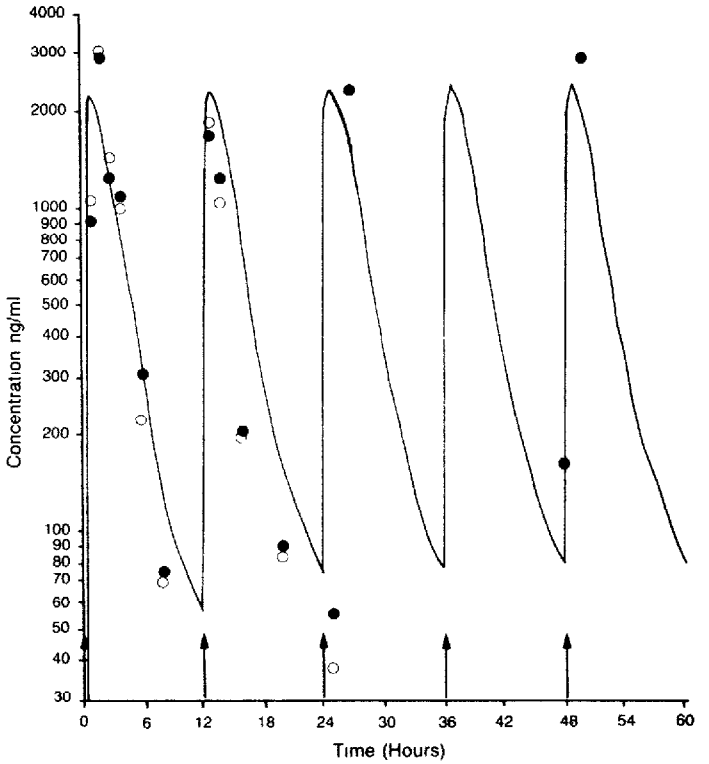

Fig. 4. Plasma concentration—time curve in cancer patient after repeated oral doses of 4000 mg NFZ/m² per 12 h. Key: (●) HPLC results described herein; (○) HPTLC results performed at Miles Pharmaceuticals Laboratory; arrows represent time of dose; curve is a computer simulation.

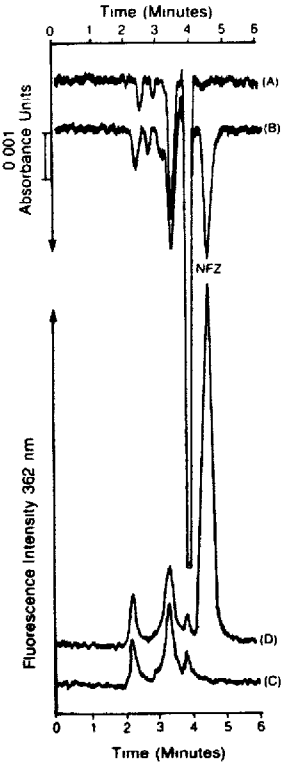

Fig. 5. Chromatograms of (A) blank methylene chloride, and (B) 56 ng/ml NFZ standard in methylene chloride by UV detection compared to (C) blank methylene chloride and (D) 56 ng/ml NFZ standard in methylene chloride by fluorescence detection. UV detector: LC-75 (Perkin-Elmer) 228 nm, 0.01 a.u.f.s.; fluorescence detector: 650-10M (Perkin-Elmer); excitation 232 nm, emission 362 nm, range 30.

The influence of pH on the stability of NFZ is shown in Fig. 3. The apparent linear degradation is much more rapid at extreme acidic or basic conditions. For this reason plasma samples were diluted 1:1 with a 0.1 *M* citric acid buffer, pH 7.0, before extraction.

Fig. 4 shows the concentration versus time curve for samples assayed both by the HPLC procedure described herein, and the HPTLC method performed at Miles Pharmaceuticals Laboratory. The ratio of the concentrations determined by HPLC to those by HPTLC was 1.09 ± 0.21 (mean ± S.D., n = 11).

Fig. 5 shows a direct comparison of UV absorption and fluorescence intensity with both instruments set at maximum sensitivity. A 56 ng/ml NFZ standard in methylene chloride was analyzed, and both UV (optimized at 228 nm) and fluorescence were monitored simultaneously. The fluorescence intensity is approximately three times greater than UV absorbance.

DISCUSSION

Due to the influence of the naphthylene ring on the chemical properties of NFZ, it can be extracted easily from an aqueous phase with methylene chloride. Chloroform also extracted approximately 100% of the NFZ from plasma, but a small co-extracted peak limited the sensitivity of the assay. Hexane or heptane did not extract NFZ from plasma.

The method described herein has a sensitivity limit of 5.0 ng/ml NFZ in plasma. Therefore, a concentration step was unnecessary. This low level of detection can be attributed partially to the excellent fluorescence properties of the drug.

The addition of cysteine to stored plasma samples enhances the stability of NFZ. Since fresh human plasma contains about 4 mg L-cysteine per l [4], the protective effect of adding more cysteine is not apparent. However, in stored plasma it may be speculated that L-cysteine becomes oxidized, leaving the pyrazolinone ring unprotected from oxidation. Thus, adding cysteine to standards prepared in stored plasma has a protective effect (Table II), and as a safeguard a large excess of cysteine is added to all plasma samples.

NFZ was found to be most stable near physiological pH. It is known that NFZ exists in either enol or enol-tautomeric forms due to the acidic hydrogens on the pyrazolinone ring [5]. NFZ has a pK_a of 8.24 determined by the —OH group on the pyrazolinone ring. Therefore, at physiological pH the majority of the drug will be in the keto form. Our studies show that the drug is most stable in solutions buffered at a pH which favors this form.

The correlation coefficient of 0.999 indicates a high degree of linearity in the concentration range from 5.0 to 500 ng/ml. The mean coefficient of variation of 2.9% demonstrates excellent precision. The percent recovery of NFZ from human plasma was 109 ± 5% and the limit of detection was 5.0 ng/ml in plasma.

The validity of the quantitative method has been confirmed by comparing results from samples analyzed by this HPLC method and a HPTLC method with good correlation.

The developed method is both specific and sensitive enough to measure the disappearance of NFZ from human plasma after drug administration [6]. It is a simple one-step extraction and analysis that requires basic HPLC equipment.

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