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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF NAFIMIDONE AND ITS MAJOR METABOLITE NAFIMIDONE ALCOHOL IN HUMAN PLASMA AND URINE

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SUMMARY

A rapid, sensitive and selective method for the determination in plasma and urine of nafimidone, a new antiepileptic drug, and its major metabolite, nafimidone alcohol, has been developed which uses a high-performance liquid chromatographic system and a fluorescence detector for nafimidone or ultraviolet detector for nafimidone alcohol. The detection limits for nafimidone and nafimidone alcohol are 5.0 and 12.5 ng/ml, respectively.

INTRODUCTION

Nafimidone, 1-(2-naphthoylmethyl)imidazole hydrochloride (Fig. 1), is a new antiepileptic drug which may be effective in the treatment of partial onset seizures [1]. The major metabolite of the drug is nafimidone alcohol, 1-[2-hydroxy-2-(2-naphthyl)ethyl]imidazole, which is also pharmacologically active [2]. Graham et al. [3] have described a liquid chromatographic method for simultaneous determination of both compounds in the plasma of dog and monkey. Following administration of the drug to rats, dogs and monkeys, nafimidone is rapidly reduced to nafimidone alcohol in the plasma and the parent drug is barely detectable after 1–2 h. We describe here a modification of the method of Graham et al. [3] which we have used for the quantification of nafimidone and nafimidone alcohol in human plasma and urine. We developed this method in order to investigate the pharmacokinetics of nafimidone and nafimidone alcohol [4], to study nafimidone interaction with other anti-convulsant agents in patients [5], such

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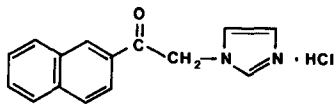
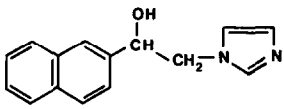


NAFIMIDONE



NAFIMIDONE ALCOHOL

Fig. 1. Structure of nafimidone and nafimidone alcohol.

as that which has been reported by Kapetanovic and Kupferberg [6] in rat microsomes, and for analyses of human plasma and urine samples during clinical trials.

EXPERIMENTAL

Materials

Nafimidone, nafimidone alcohol and the internal standards methylnafimidone {1-[6-methyl-(2-naphthoyl)methyl]imidazole} and methoxynafimidone {1-[2-methoxy-2-(2-naphthoyl)ethyl]imidazole} were supplied as the hydrochloride salts by Syntex Research (Palo Alto, CA, U.S.A.). HPLC-grade hexane, ethyl acetate, methanol and water were purchased from J.T. Baker (Phillipsburg, NJ, U.S.A.). β-Glucuronidase (from Helix pomatia, Type H-2) was from Sigma (St. Louis, MO, U.S.A.). Ammonium phosphate monobasic, sodium phosphate dibasic, potassium hydroxide and phosphoric acid were from Mallinckrodt (Paris, KY, U.S.A.). Potassium phosphate monobasic was from Allied Chemical (Morristown, NJ, U.S.A.) and sodium dodecylsulfate (SDS) was obtained from Aldrich (Milwaukee, WI, U.S.A.).

Preparation of standard solutions

Stock solutions of nafimidone, nafimidone alcohol, methylnafimidone and methoxynafimidone were made up in double-distilled water (1 mg/ml) and stored at 4°C in amber bottles. The final working concentration of each compound was as follows: nafimidone, 10 ng/μl; nafimidone alcohol, 20 ng/μl; the internal standards methylnafimidone, 10 ng/μl; and methoxynafimidone, 5 ng/μl.

Apparatus and chromatographic conditions

An Altex (Altex Scientific, Berkeley, CA, U.S.A.), high performance liquid chromatographic (HPLC) system which consisted of two Model 110 A pumps, a Model 421 controller and a C-R1A integrator was used. Two detection methods (UV and fluorescence detection) were employed for the quantification of nafimidone and its metabolite nafimidone alcohol. For the parent drug, nafimidone, a Kratos FS 970 fluorometer operated at λ_{ex} 245 nm and λ_{em} 456 nm, was used. For the metabolite, nafimidone alcohol, a Schoeffel SF 770 variable-wavelength

detector set at 225 nm was used (both from Kratos, Westwood, NJ, U.S.A.). The separation in both procedures was carried out in a reversed-phase system with Whatman Partisil 10 ODS-3 (Whatman, Clifton, NJ, U.S.A.) as the stationary phase (250 mm×4.6 mm I.D., particle size 10 μm) and methanol–SDS buffer (64:36, v/v) as the mobile phase. The SDS buffer was prepared by adding 1.44 g of SDS and 5.75 g of $NH_4H_2PO_4$ to 1 l water, followed by 3.38 ml of phosphoric acid. The flow-rate of the mobile phase was 1.5 ml/min for nafimidone and 1.7 ml/min for the nafimidone alcohol assay. A Rheodyne 7125 injection valve (Rheodyne, Cotati, CA, U.S.A.) with a 20-μl loop was used. The column was heated to 37°C by a Bio-Rad column heater (Bio-Rad Labs., Richmond, CA, U.S.A.).

Sample preparation and standard curve

Venous blood was withdrawn from patients being treated with nafimidone into 15-ml heparinized tubes. The plasma was separated by centrifugation and stored at −20°C until used. For the plasma assay, 30 ng methylnafimidone or 50 ng methoxynafimidone as the internal standards, 0.5 ml distilled water, 1.0 ml of 1/15 *M* phosphate buffer, pH 8.0 (1/15 *M* Na_2HPO_4 adjusted to pH 8.0 by adding 1/15 *M* KH_2PO_4), and 8.0 ml hexane–ethyl acetate (50:50, v/v) were added to 0.5 ml of patient plasma in a 15-ml screw top culture tube. The samples were then vortexed for 30 s, shaken for 5 min (Eberbach, Ann Arbor, MI, U.S.A.), followed by centrifugation for 5 min at 800 *g*. The organic phase (upper layer) was transferred to a clean new 15-ml screw-top tube and extracted with 1.0 ml of 0.1 *M* hydrochloric acid. After removal of the organic phase, 0.4 ml of 0.5 *M* potassium hydroxide were added to the aqueous fraction and extracted with 4.0 ml hexane–ethyl acetate (50:50, v/v). The top layer was transferred to a 15-ml conical test tube which was subsequently placed in a water bath at 20°C. The organic phase was evaporated to dryness under a gentle stream of nitrogen. The dried residue was reconstituted in 80 μl of mobile phase and 20 μl were injected into the chromatograph.

For the drug assay in urine, a 100-μl aliquot of a pooled 24-h urine collection was diluted to 1.0 ml with distilled water and transferred to a 15-ml screw-top culture tube. A 1.0-ml volume of 0.5 *M* acetate buffer pH 5.0 and 10 μl of β-glucuronidase (1100 Fishman units) were added, and the solution was incubated aerobically at 37°C for 17 h. Thereafter, 50 ng of the internal standard methoxynafimidone, 0.2 ml 0.5 *M* potassium hydroxide, 1.0 ml of 1/15 *M* phosphate buffer pH 8.0 and 8.0 ml hexane–ethyl acetate (50:50, v/v) were added to the incubation mixture and the extraction procedure as described for plasma samples was followed.

Standard curves for nafimidone and nafimidone alcohol were determined in a similar manner using drug-free plasma and urine to which known amounts of nafimidone (10–100 ng) and nafimidone alcohol (50–1000 ng) were added. Concentrations of nafimidone and nafimidone alcohol were determined by the ratio of the peak areas of each compound to the peak area of the internal standard plotted against the known concentrations of the standards.

TABLE I

WITHIN-DAY PRECISION OF THE DETERMINATION OF NAFIMIDONE AND NAFIMIDONE ALCOHOL PLASMA CONCENTRATIONS

Concentration (ng/ml)	Peak-area ratio (mean ± S.D.)	Coefficient of variation (%)
Nafimidone assay (n=5)		
20	0.1305 ± 0.0085	6.5
40	0.2563 ± 0.0031	1.2
100	0.6576 ± 0.0088	1.3
150	0.9129 ± 0.0302	3.3
200	1.2838 ± 0.0331	2.6
Slope	0.0126 ± 0.0003	2.4
Nafimidone alcohol assay (n=5)		
100	0.1523 ± 0.0027	1.7
200	0.3018 ± 0.0035	1.2
400	0.5977 ± 0.0026	0.4
1000	1.4897 ± 0.0208	1.4
2000	2.8941 ± 0.0417	1.4
Slope	0.0029 ± 0.0001	3.4

RESULTS

Linearity and precision

The standard curves for nafimidone and nafimidone alcohol exhibited good linearity over the range 20–200 ng/ml for the parent drug (regression equation, $y=0.0126x+0.0104$, $r=0.9995$) and 100–2000 ng/ml for the metabolite (regression equation, $y=0.0029x+0.0033$, $r=0.9999$). Mean values for each standard concentration were determined by pentaplicate runs on the same day (Table I). Precision between runs was determined from standard curves that were extracted and injected on five different days (Table II). The coefficient of variation for the individual data points and standard curve slopes was less than 7.0% in all cases. Reproducibility of retention times for both nafimidone and nafimidone alcohol during analysis of 30 different injections were found to vary by only a few percent.

Accuracy

The accuracy of the method was determined by preparing pools of plasma spiked with nafimidone or nafimidone alcohol at different concentrations. For each concentration eight analyses were performed using the pooled plasma, the results of which are given in Table III.

Selectivity

Fig. 2 shows typical chromatograms obtained from (A) drug-free plasma spiked with the internal standard methylnafimidone, (B) drug-free plasma spiked with nafimidone and internal standard methylnafimidone and (C) patient plasma.

TABLE II

BETWEEN-DAY PRECISION OF THE DETERMINATION OF NAFIMIDONE AND NAFIMIDONE ALCOHOL PLASMA CONCENTRATIONS

Concentration (ng/ml)	Peak-area ratio (mean ± S.D.)	Coefficient of variation (%)
Nafimidone assay (n=5)		
20	0.1225 ± 0.0041	3.3
40	0.2600 ± 0.0135	5.2
100	0.6350 ± 0.0241	3.8
150	0.9186 ± 0.0154	1.7
200	1.2742 ± 0.0260	2.0
Slope	0.0126 ± 0.0002	1.5
Nafimidone alcohol assay (n=5)		
100	0.1550 ± 0.0028	1.8
200	0.2954 ± 0.0089	3.0
400	0.5965 ± 0.0029	0.5
1000	1.4839 ± 0.0345	2.3
2000	2.8437 ± 0.0920	3.2
Slope	0.0028 ± 0.0001	3.6

Figs. 3 and 4 represent the chromatograms of the metabolite, nafimidone alcohol, extracted from plasma and urine samples. It can be seen from the chromatograms that no endogenous plasma or urine components interfere with the assay. Table IV lists the retention times of various antiepileptic drugs detected by these two chromatographic methods. None of the compounds interfered with nafimidone or nafimidone alcohol analysis.

TABLE III

ACCURACY OF THE DETERMINATION OF NAFIMIDONE AND NAFIMIDONE ALCOHOL IN PLASMA

Actual concentration (ng/ml)	Measured concentration (mean ± S.D.) (ng/ml)	Deviation from actual concentration (%)
Nafimidone assay (n=8)		
25	26.38 ± 0.69	+5.5
50	52.08 ± 4.31	+4.2
125	115.20 ± 3.52	−7.8
Nafimidone alcohol assay (n=8)		
50	48.15 ± 2.70	−3.7
250	272.89 ± 6.54	+9.2
1000	1078.05 ± 22.21	+7.8

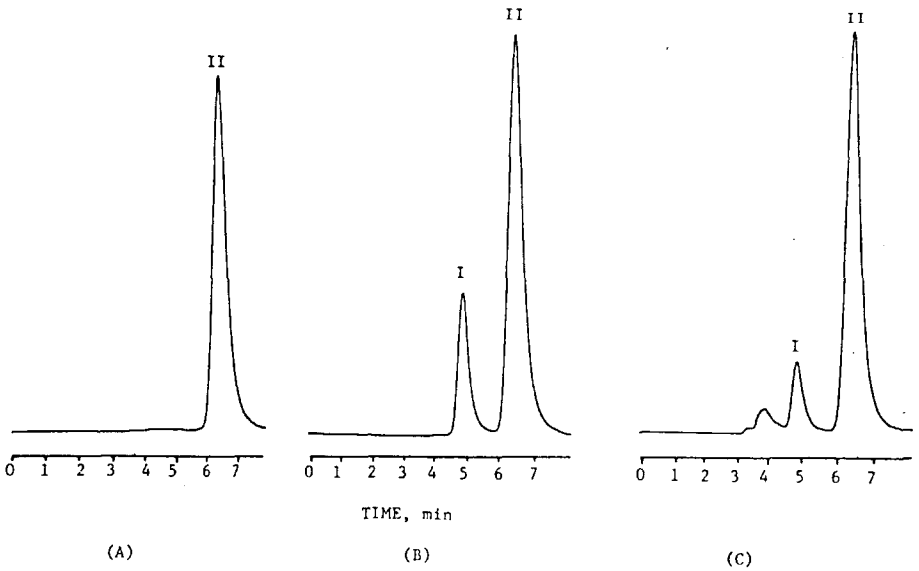


Fig. 2. Chromatograms showing (A) extracted blank plasma with internal standard methylnafimidone, (B) extracted spiked plasma (nafimidone 40 ng/ml) and (C) patient plasma. Peaks: I = nafimidone; II = internal standard methylnafimidone. Column: 10-μm Partisil ODS-3, 250 × 4.6 mm I.D.; mobile phase: methanol-SDS buffer (64:36, v/v); flow-rate: 1.5 ml/min; detector: fluorescence, $\lambda_{ex} = 245$ nm, $\lambda_{em} = 456$ nm.

Recovery

The extractability of nafimidone and nafimidone alcohol was determined by comparing the peak-area ratios measured in extracts of plasma containing 40 and 100 ng/ml nafimidone or 200 and 400 ng/ml nafimidone alcohol with those peak-area ratios resulting from unextracted samples supplemented with the same amounts of nafimidone or nafimidone alcohol. The internal standard was added to the sample just before injection into the chromatograph. The extraction recoveries determined in this manner were found to be 80 and 83% for nafimidone and nafimidone alcohol, respectively.

Sensitivity

The detection limit (detector response four times the noise level) for nafimidone was 5.0 ng/ml, and 12.5 ng/ml for the major metabolite nafimidone alcohol when the detector was set at 0.1 a.u.f.s. with a recording scale of 2 mV. The limits are based on extraction of 0.5 ml plasma. If a plasma sample of 1.0 ml or more was used, it was possible to detect 2–3 ng/ml of nafimidone.

Plasma concentration of nafimidone and nafimidone alcohol

After administration of a single 100-mg dose of nafimidone to subject D.A., the plasma concentration of the major metabolite nafimidone alcohol reached a maximum level of 1225 ng/ml within 1 h after intake of the dose, and decreased thereafter with an elimination half-life of 2.4 h. In contrast, the plasma concentration

of the parent drug nafimidone was much less than the major metabolite and only barely detectable after 4 h post-dose. The half-life was 1.5 h. Plasma concentration profiles of nafimidone and nafimidone alcohol are shown in Table V.

TABLE IV

RETENTION TIMES FOR SELECTED ANTIEPILEPTIC DRUGS AND METABOLITES

Antiepileptic drug	Retention time (min)	
	UV detection	Fluorescence detection
Nafimidone	5.17	4.85
Nafimidone alcohol	4.04	No peak
Methylnafimidone (internal standard)	7.01	6.60
Methoxynafimidone (internal standard)	6.32	No peak
Carbamazepine	3.36	No peak
Carbamazepine-10,11-epoxide	2.60	3.38
Phenytoin	2.89	No peak
5-Hydroxyphenylphenylhydantoin	2.20	No peak
Primidone	2.33	No peak
Phenobarbital	2.46	No peak
Lorazepam	3.90	No peak
Diazepam	5.97	No peak

TABLE V

PLASMA CONCENTRATIONS OF NAFIMIDONE AND NAFIMIDONE ALCOHOL AFTER A SINGLE 100-mg DOSE IN ONE SUBJECT

The linear regression coefficients of the plasma concentrations from 1 h on were 0.973 and 0.996 for nafimidone and nafimidone alcohol, respectively; the elimination half-lives were 1.5 and 2.4 h, respectively

Time (h)	Nafimidone (ng/ml)	Nafimidone alcohol (ng/ml)
0	–	–
0.5	–	124.6
1	22.0	1224.3
2	10.2	957.0
3	6.7	652.3
4	3.6*	543.1
6	2.3*	349.9
8	–	184.0
12	–	45.5

*A 1-ml volume of plasma was used for these determinations.

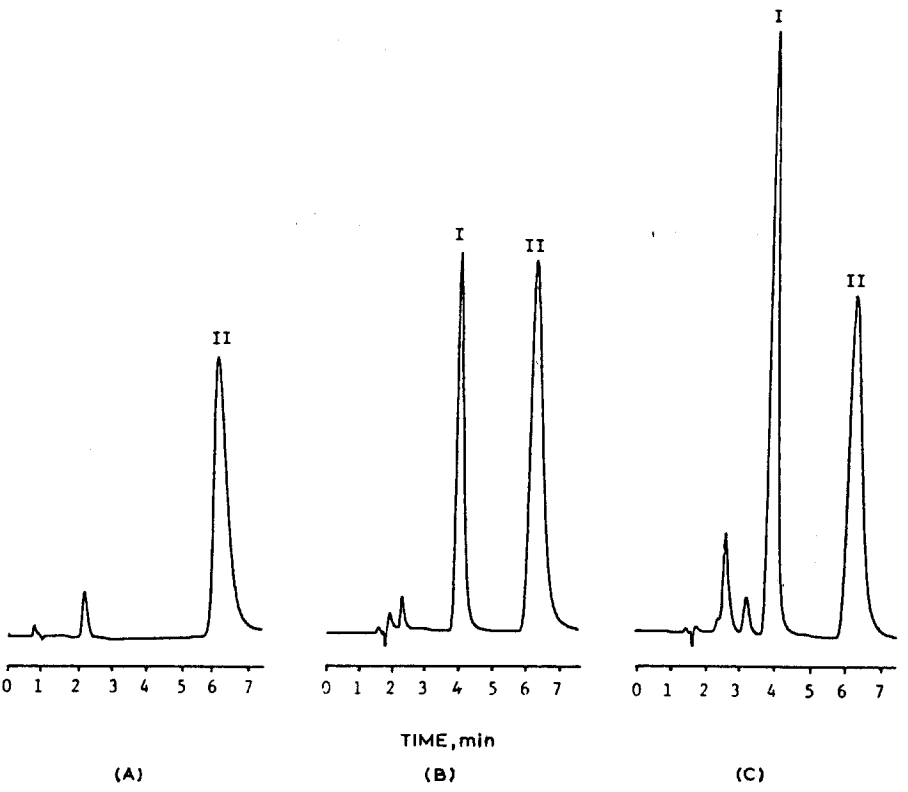


Fig. 3. Chromatograms showing (A) extracted blank plasma with internal standard methoxynafimidone, (B) extracted spiked plasma (nafimidone alcohol 400 ng/ml) and (C) patient plasma. Peaks: I = nafimidone alcohol; II = internal standard methoxynafimidone. Column: 10-μm Partisil ODS-3, 250 × 4.6 mm I.D.; mobile phase: methanol–SDS buffer (64:36, v/v); flow-rate: 1.7 ml/min; detector: UV, $\lambda_{ab} = 225$ nm.

DISCUSSION

The HPLC methods described in this report for nafimidone and nafimidone alcohol determination in human plasma samples are selective, sensitive and accurate. Possible interference of commonly used antiepileptic drugs with the assay was evaluated. None of the compounds listed in Table IV showed potential interference with the analysis. This assay therefore can be used in epileptic patients being treated with other antiepileptic drugs as well as with nafimidone. Although nafimidone can be measured using a UV detector set at 248 nm, use of the fluorescence detector increases the sensitivity from 10 to 3 ng/ml [3]. The same assay procedures can be used for the quantification of nafimidone and nafimidone alcohol in urine, except that the sample is incubated with β-glucuronidase in order to measure both the free and glucuronidated compounds. Nafimidone can be seen in spiked urine samples. We have not detected nafimidone in patient urine samples but have been able to measure nafimidone alcohol. Nafimidone.

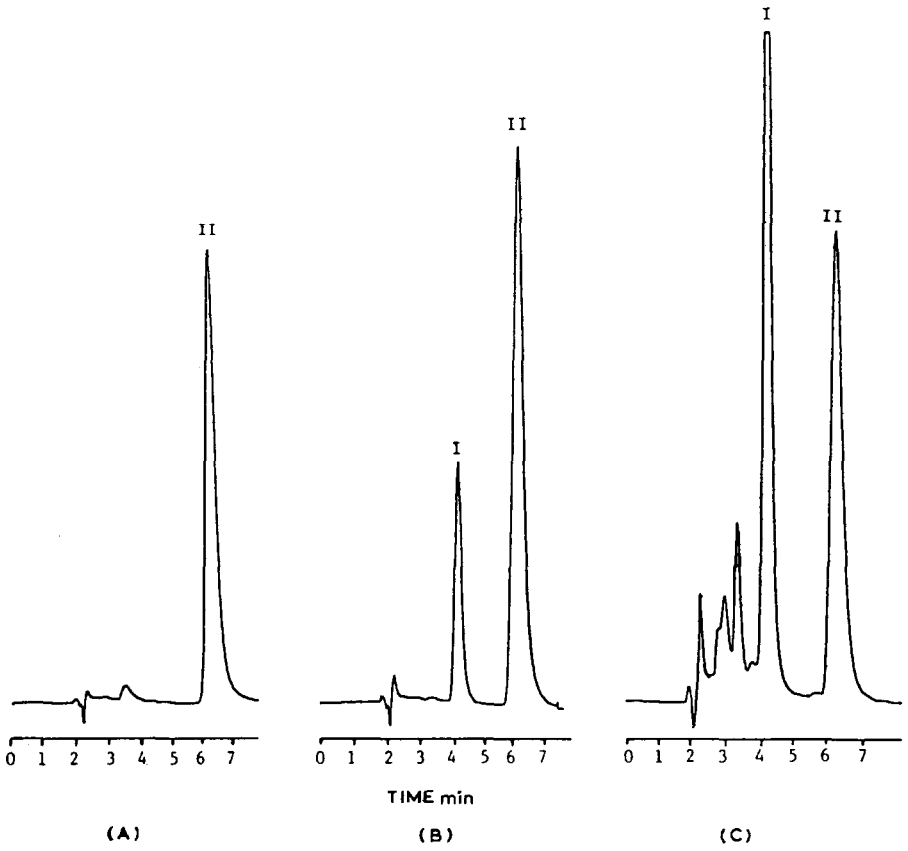


Fig. 4. Chromatograms showing (A) extracted blank urine with internal standard methoxynafimidone, (B) extracted spiked urine (nafimidone alcohol 200 ng/ml) and (C) patient urine. Peaks: I = nafimidone alcohol; II = internal standard methoxynafimidone. Column: 10-μm Partisil ODS-3, 250 × 4.6 mm I.D.; mobile phase: methanol–SDS buffer (64:36, v/v); flow-rate: 1.7 ml/min; detector: UV, λ_{ab} = 225 nm.

however, is present in spiked samples. This suggests that the assay is capable of detecting nafimidone in urine if it is there. However, it appears that nafimidone is so rapidly metabolized that none of the parent drug appears in the urine.

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