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Note

High-performance liquid chromatographic separation and quantitation of benzisoquinolinedione (Nafidimide) from biological fluids

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Nafidimide, 5-amino-2-[2-(dimethylamino)ethyl]-1*H*-benz[*de*]isoquinoline-1,3(2H)-dione (NSC-308847) (Fig. 1), is one of a series of imide derivatives of 1,8-naphthalic acid synthesized by Braña et al. [1] as experimental antitumor compounds. Based on its antitumor efficacy against experimental animal tumor models, Nafidimide has been selected by the National Cancer Institute to undergo Phase 1 investigational clinical trials at several institutions including M.D. Anderson Hospital and Tumor Institute. In order to evaluate the pharmacokinetic parameters of Nafidimide and its principal metabolites in humans and animals, an analytical procedure for the routine quantitation of drug from biological fluids was developed.

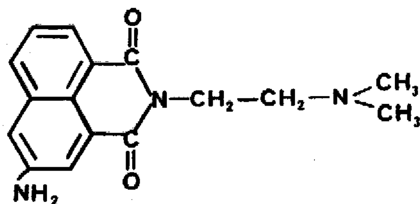

Fig. 1. Structure of Nafidimide.

EXPERIMENTAL

Standards and reagents

HPLC-grade acetonitrile and methanol were purchased from Burdick & Jackson Labs. (Muskegon, MI, U.S.A.). Nafidimide (benzisoquinolinedione,

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NSC-308847) was kindly supplied by the Developmental Therapeutics Program, Division of Cancer Treatment, National Institutes of Health (Bethesda, MD, U.S.A.). Stock solutions of Nafidimide were prepared by adding 4 ml of sterile water to 100 mg of lyophilized drug to yield a final Nafidimide concentration of 25 mg/ml. All other chemicals were of analytical-reagent grade and were obtained from regular commercial suppliers.

Chromatographic apparatus

All analyses were performed on a Waters Assoc. (Milford, MA, U.S.A.) high-performance liquid chromatograph equipped with a Model M6000A pump, U6K injector and Schoeffel Model SF-970 fluorescence detector (Kratos, Westwood, NJ, U.S.A.) with the excitation wavelength set at 350 nm and emission set at 550 nm. Separation was achieved on an analytical reversed-phase μBondapak C_{18} column (Water Assoc.) (30 cm × 4 mm, 10 μm particle size) using 0.02 *M* acetate buffer in 15% acetonitrile (pH 4.0) as eluent at a flow-rate of 2.0 ml/min at room temperature. Retention time was determined by use of a Shimadzu Chromatopac C-R1B integrator and data processor (Kyoto, Japan). Quantitative analysis was based on peak areas which were computed using a preset integration program.

Drug administration and sample collection

Dogs were administered 5 mg/kg Nafidimide intravenously as a bolus injection. Blood was drawn into heparinized tubes and centrifuged (12 000 *g*, 10 min) to obtain plasma. An indwelling Foley catheter was utilized for urine collection. Cerebrospinal fluid (CSF) was sampled by cisternal puncture and the bile was sampled by cannulation of the common bile duct. All bile samples were diluted 1:10 with saline prior to extraction.

Sample preparation and drug analysis

A C_{18} Sep-Pak cartridge (Waters Assoc.) was used as a minichromatographic column to prepare the plasma, urine and CSF samples according to a slight modification of the method of Robert [2]. The cartridge was first washed with 4 ml of methanol, followed sequentially by 4 ml of methanol—water (1:1), 4 ml of 0.02 *M* acetate buffer (pH 4.0), 1 ml of biological sample (urine, plasma, CSF or diluted bile), and then 4 ml of 0.02 *M* acetate buffer. The above eluates were discarded. The column was then washed with 6 ml of chloroform—methanol (1:1, v/v), and the eluent collected in a drying cup and evaporated to dryness under a stream of nitrogen in a Brinkmann Model SC/48 evaporator (Brinkmann Instruments, Westbury, NY, U.S.A.). Samples were reconstituted with 250 μl of 0.02 *M* acetate buffer, pH 4.0, in 70% acetonitrile instead of 15% acetonitrile since the latter would give a cloudy suspension, yet there was no significant difference in retention time or integration units in either of the constituents. Samples of 200 μl were then injected for high-performance liquid chromatographic (HPLC) analyses. Those urine samples that remained cloudy after reconstitution were recentrifuged at 12 000 *g* for 15 min and the supernatant was collected again for injection into the HPLC system.

RESULTS AND DISCUSSION

Using the conditions described above, Nafidimide was found to elute on a typical chromatogram of plasma-, bile- or CSF-extracted samples at 9.5 min (Fig. 2). Elution of the drug from extracted urine samples, however, often occurred at 9.7—10.0 min due, presumably, to the relatively high content of non-drug urinary constituents which eluted near the column void volume (Fig. 2). The C_{18} Sep-Pak extraction procedure along with the procedures of evaporation, centrifugation and transfer of samples resulted in a recovery of 87.1 ± 7.4% (n = 7) of the parent compound from plasma and 80.6 ± 4.8% (n = 5) from urine. Because of the minor differences in extraction efficiencies in recovering drug from either urine or plasma, standard calibration curves of Nafidimide were prepared on a daily basis using control samples of each biological fluid. Inter-assay coefficients of variation (C.V.) of 2.7% (n = 9) utilizing plasma samples and 2.9% (n = 12) utilizing urine samples were routinely obtained.

Tables I and II demonstrate the standard curves and the accuracy at each concentration for Nafidimide extracted from plasma and urine. The extracted drug concentration is linear over a range of 150—10 000 ng/ml in plasma and

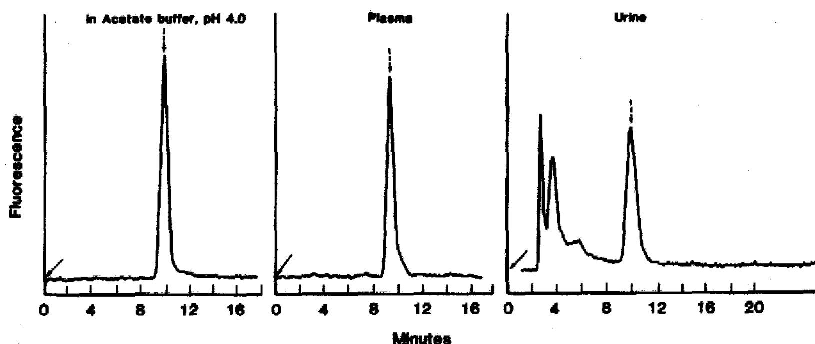

Fig. 2. HPLC elution profiles of Nafidimide ($1.8 \cdot 10^{-4}$ μM) in 0.02 M acetate buffer pH 4.0 (left), in extracted dog plasma (center) and in extracted dog urine (right). Solid arrow indicates time of sample injection; dashed arrow indicates Nafidimide peak.

TABLE I

STANDARD CURVE AND ACCURACY OF NAFIDIMIDE IN DOG PLASMA

$y = -26.96 + 0.06x$; $r^2 = 0.99$.

Concentration (ng/ml)	Peak area (mean ± S.D., n = 5)	Coefficient of variation (%)
156.25	2.65 ± 0.4	15.1
312.5	5.65 ± 0.3	5.3
625.0	10.6 ± 1.0	9.4
1250.0	23.0 ± 3.0	13.04
2500.0	87.0 ± 9.0	10.34
5000.0	270.0 ± 27.0	10.0
10 000.0	551.0 ± 27.0	4.9

TABLE II

STANDARD CURVE AND ACCURACY OF NAFIDIMIDE IN DOG URINE

$y = -59.95 + 51.54x$; $r^2 = 0.97$.

Concentration (μg/ml)	Peak area (mean ± S.D., $n = 4$)	Coefficient of variation (%)
0.625	18.3 ± 1.3	7.1
1.25	33.15 ± 0.4	1.1
2.50	84.5 ± 11.5	13.6
5.00	192.0 ± 18.0	9.4
10.00	481.0 ± 13.5	2.8
20.00	887.0 ± 80.0	9.0
40.00	2173.0 ± 258.0	11.9
80.00	3363.0 ± 170.0	5.1
100.00	5600.0 ± 160.0	2.9

0.6–100 μg/ml in urine. The average correlation coefficient for plasma is 0.99 ($n = 5$) and 0.97 ($n = 4$) for urine. The lower limit of detection of Nafidimide was 30.0 ng/ml at a detector range setting of 0.01 a.u.f.s. The signal-to-noise ratio was 3 or greater under these conditions.

To establish preliminary pharmacokinetic parameters, dogs were administered Nafidimide as single intravenous bolus injections (5 mg/kg). Fig. 3 shows representative HPLC chromatograms of Nafidimide as extractions from plasma, CSF, urine and bile. Immediately following injection of drug (i.e. zero time), only the parent drug was detected in plasma (Fig. 3). At 30 min post-

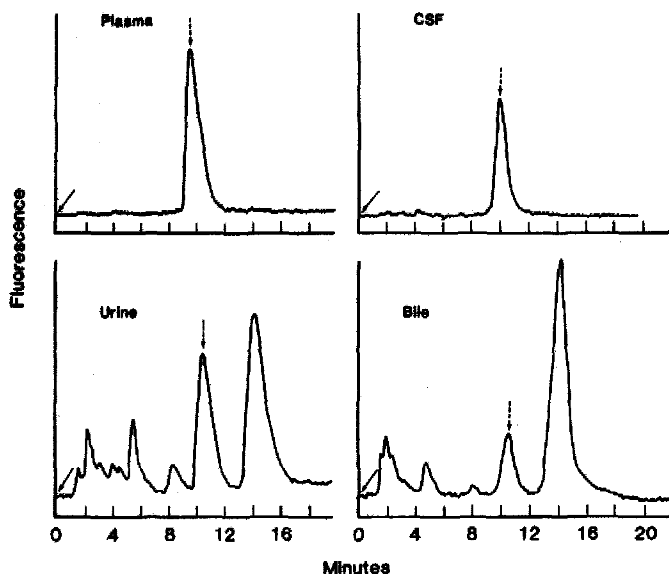

Fig. 3. Representative chromatograms of Nafidimide and metabolites from extracted dog plasma at 0 min ($2.8 \cdot 10^{-4}$ μM), cerebrospinal fluid (CSF) at 30 min ($5.0 \cdot 10^{-4}$ μM), urine at 2 min ($2.6 \cdot 10^{-3}$ μM) and bile at 1 min ($6.1 \cdot 10^{-5}$ μM) samples. Times are those following administration of Nafidimide. Solid arrows indicate time of sample injection, dashed arrows indicate Nafidimide peak.

injection, parent drug was also observed in the CSF indicating relatively rapid uptake into this compartment. Metabolism of Nafidimide was apparent as soon as 1 h with at least three major metabolites appearing in both urine and bile (Fig. 3). The method described in this paper is currently being used to separate, collect and aid in the identification of the structure and antitumor efficacy of the principal urinary metabolite.

The methodology presented in this paper has proved to be rapid, sensitive and reproducible providing easy detection of the parent drug and major metabolites in biological fluids such as plasma, urine and CSF. This assay is currently being used to study the pharmacology of Nafidimide and its metabolism in clinical Phase 1 trials at our institution.

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