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DETERMINATION OF ELLIPTICINE IN BIOLOGICAL SAMPLES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

Ellipticine, a plant alkaloid effective against murine leukemias and solid tumors, is presently undergoing toxicological assessment prior to clinical trial. A rapid, sensitive, reversed-phase high-performance liquid chromatographic method employing an internal standard was developed for the detection of ellipticine and its principal metabolite 9-hydroxyellipticine after extraction from biological samples. The method was successfully applied to the quantitation of ellipticine in mouse blood and tissues after intravenous administration of ellipticine and to mouse blood levels of drug after oral administration. Similar success was achieved in determinations of ellipticine and 9-hydroxyellipticine in samples of spiked human blood and plasma. Mouse blood ellipticine levels monitored over 3 h after the intravenous administration of drug demonstrated a biphasic decline with a terminal half-life of 52 min.

INTRODUCTION

Ellipticine (NSC-71795; 5,11-dimethyl-6H-pyrido[4,3-*b*]carbazole) (Fig. 1) is a natural product isolated from *Ochrosia elliptica* and several other species of *Ochrosia* and *Bleekeria vitiensis* [1]. Ellipticine has demonstrated a broad spectrum of antitumor activity in both murine leukemias and solid tumors [1]. Initial toxicological studies in animals found significant hemolysis when the drug was given intravenously [2]. Since the drug is orally active [3] this route of administration would seem to be a reasonable alternative to minimize hemolysis. Ellipticine is currently undergoing toxicological evaluation in animals by the oral route at the National Cancer Institute. Pending a satisfactory completion of these studies, clinical trials will be initiated in man.

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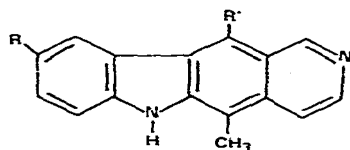

Ellipticine	R = H, R' = CH_3
9-Hydroxyellipticine	R = OH, R' = CH_3
11-Demethylellipticine	R = H, R' = H

Fig. 1. Structures of the ellipticines.

Ellipticine has been determined in various media by several methods. In a biological matrix ellipticine and metabolites have been determined radiochemically after thin-layer chromatographic separation of radiolabeled drug [4]. Also ellipticine equivalents have been measured by ultraviolet spectroscopy after extraction from tissues and fluids [3, 5]. High-performance liquid chromatography (HPLC) has been used in the preparative mode to collect synthetic and in vivo metabolic products of ellipticine [6]. HPLC with fluorescence detection has been employed to separate ellipticine and a number of analogues in non-biological samples [7]. However, we are aware of no reports describing the quantitation of ellipticine and metabolites in tissue and biological fluids by HPLC.

The objective of this work was to develop a simple and reliable reversed-phase HPLC procedure for the determination of ellipticine and its principal metabolite, 9-hydroxyellipticine, in biological materials. Such a method would be valuable in future pharmacological and bioavailability studies of ellipticine.

EXPERIMENTAL

Chromatographic apparatus and conditions

A modular high-performance liquid chromatograph (Model 3500B, Spectra-Physics, Santa Clara, CA, U.S.A.), including a reciprocating piston pump with flow feedback control, delivered mobile phase at a flow-rate of 1.4 ml/min to a stainless-steel column (300 × 4.0 mm) packed with fully porous 10-μm silica particles bonded with a monomolecular layer of octadecylsilane (μBondapak C_{18}, Waters Assoc., Milford, MA, U.S.A.). A stainless-steel guard column (50 × 4.6 mm) filled with 40-μm pellicular C_{18} packing material (Supelco, Bellefonte, PA, U.S.A.) preceded the analytical column. Samples were introduced to the column with a manual injection valve equipped with a 100-μl sample loop (Model CV-6-UHP-a-N60, Valco, Houston, TX, U.S.A.). The eluted compounds were detected with a variable-wavelength ultraviolet detector (Model 770, Spectra-Physics) set at 300 nm and/or a filter fluorometer equipped with a 70-μl flow cell (Fluoro-Microphotometer, American Instrument Co., Silver Spring, MD, U.S.A.), a narrow pass 360-nm filter for excitation and a sharp cut 455-nm emission filter. Detector output signals were

recorded with a variable input strip-chart recorder (Model B5116-1, Omniscribe, Houston Instruments, Austin, TX, U.S.A.).

The mobile phase used when ellipticine alone was quantitated consisted of acetonitrile—0.01 *M* NaH_2PO_4 (36:64, v/v) adjusted to pH 3.5 with 2 *N* phosphoric acid (System A). When ellipticine and 9-hydroxyellipticine were determined simultaneously, the mobile phase consisted of acetonitrile—0.01 *M* NaH_2PO_4 (25:75, v/v) adjusted to pH 3.5 with 2 *N* phosphoric acid (System B). All separations were effected isocratically at ambient temperature.

The compounds were quantitated using an internal standard method. Standard curves constructed from the ratio of peak heights of ellipticine and 9-hydroxyellipticine to the internal standard (I.S.), 11-demethylellipticine, versus concentration were linear ($r > 0.999$) in the range of 20—500 ng/ml.

Reagents

Ellipiticine (NSC-71795), 9-hydroxyellipticine (NSC-210717) and 11-demethylellipticine (NSC-87206) were supplied by the Division of Cancer Treatment, National Cancer Institute (Bethesda, MD, U.S.A.). Acetonitrile, HPLC grade (Fisher Scientific, Fair Lawn, NJ, U.S.A.) and distilled water were filtered through 0.5-μm and 0.8-μm solvent resistant filters respectively (Millipore, Bedford, MA, U.S.A.). All other chemicals were reagent grade and were used as received.

Stock solutions of ellipticine hydrochloride, 9-hydroxyellipticine and 11-demethylellipticine were prepared (100 μg/ml in 0.01 *N* hydrochloric acid) and used as chromatographic standards after appropriate dilutions.

Biological studies

Ellipticine hydrochloride (0.15 mg/ml in 5% Dextrose Injection, U.S.P.) was administered to male BDF_1 mice (20—28 g) at 3.0 mg/kg via a lateral tail vein in a volume equivalent to 2% of body weight. Mice were housed in conventional cages with no access to food or water after drug treatment. Blood was collected (0.3—0.7 ml) with heparinized pasteur pipettes from an orbital sinus cavity at 5, 15, 30, 60, 120 and 180 min after drug administration. Blood was transferred to test tubes and was immediately frozen (—20°C). For tissue distribution studies, mice were sacrificed by cervical dislocation at 4 h after the administration of ellipticine. Liver, spleen, kidney, brain and heart were rapidly excised, rinsed in a cold 0.9% sodium chloride solution, lightly blotted with a lint-free towel, and immediately frozen (—20°C).

Prior to oral administration of drug, the male BDF_1 mice were fasted for 16 h. Ellipticine hydrochloride (1.5 mg/ml in Sterile Water for Injection, U.S.P.) was administered directly into the stomach at 30 mg/kg via oral intubation in a volume equivalent to 2% body weight. Blood was collected 2, 3 or 4 h post administration by the method described above.

Extraction

The extraction of ellipticine, 9-hydroxyellipticine and the internal standard 11-demethylellipticine was accomplished using a modification of a procedure described previously [5]. Blood and tissue samples (ca. 0.5 g) were weighed and subsequently homogenized in the presence of 1.5 ml of 0.05 *M* sodium

phosphate buffer (pH 7.4) containing internal standard using a manual all-glass homogenizer. The homogenates were extracted with water-saturated ethyl acetate (3.0 ml) by vigorous mixing on a mechanical mixer (Vortex Genie Mixer, Scientific Instruments, Bohemia, NY, U.S.A.) for 2 min. After centrifugation at approximately 1300 *g* and 4°C for 30 min (Centra-7R, International Equipment Co., Needham Heights, MA, U.S.A.) a 2.0-ml aliquot of the organic layer was removed and added to 0.5 or 1.0 ml of 0.01 *N* hydrochloric acid. The contents were mixed for 30 sec after which the layers separated upon standing. The aqueous layer was removed and a 100-µl aliquot was injected for HPLC analysis.

Extraction efficiency

Extraction efficiencies for ellipticine, 9-hydroxyellipticine and internal standard were determined by extracting pooled mouse blood spiked with 50—500 ng of the three compounds. Extraction efficiencies from human plasma and blood were determined in a similar manner. The efficiencies of extraction of ellipticine from various murine tissue were determined by extracting homogenates spiked with 100 or 150 ng of drug.

RESULTS AND DISCUSSION

Chromatographic behavior

9-Hydroxyellipticine, 11-demethylellipticine and ellipticine eluted from the column in that order in either HPLC system. The retention volumes (V_R), capacity factors (k') and separation factors (α) for the three compounds were calculated using standard methods [8] and are listed in Table I. The time for the chromatographic separation in System B was about 15 min. A typical chromatogram used in the quantitation of ellipticine and 9-hydroxyellipticine is seen in Fig. 2. The slightly lower organic composition of this mobile phase was necessary to ensure adequate resolution of 9-hydroxyellipticine, internal standard, ellipticine and endogenous components from the biological matrix. Since 9-hydroxyellipticine did not fluoresce under the chromatographic conditions employed it was necessary to use a UV detector separately or in series with the fluorescence detector in order to accomplish the assay of 9-hydroxyellipticine and ellipticine simultaneously. Alternatively, it was possible to deter-

TABLE I

RETENTION VOLUMES (V_R), CAPACITY FACTORS (k') AND SEPARATION FACTORS (α) FOR CHROMATOGRAPHIC SYSTEMS A AND B

Component	V_R (ml)		k'		α	
	A	B	A	B	A	B
9-Hydroxyellipticine	—*	5.18	—*	2.70		2.22
11-Demethylellipticine	8.96	9.80	5.40	6.00	1.37	1.58
Ellipticine	11.76	14.70	7.40	9.50		

*9-Hydroxyellipticine was not detected using System A with fluorescence detection.

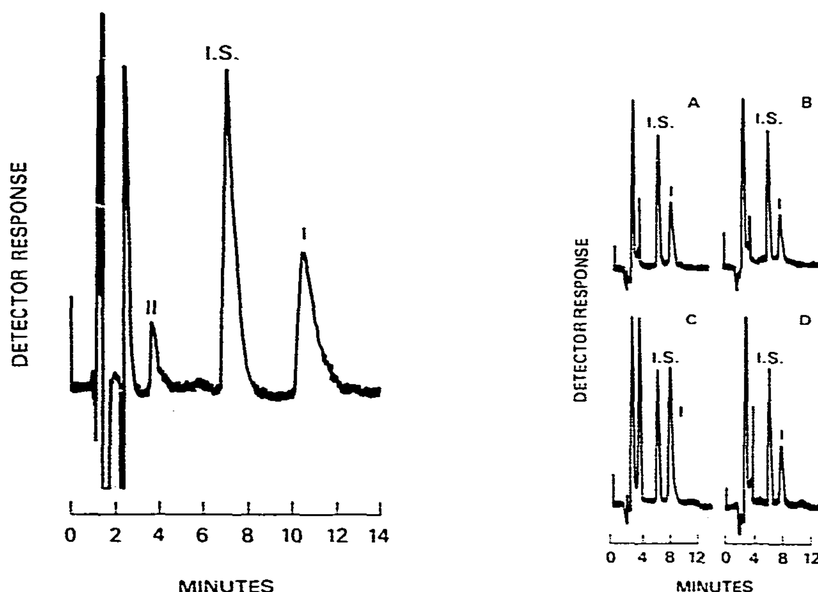

Fig. 2. Typical chromatogram showing the separation of ellipticine (I, 50 ng/ml), 9-hydroxy-ellipticine (II, 50 ng/ml) and the internal standard, 11-demethylellipticine (I.S., 150 ng/ml) after extraction from mouse blood (System B, UV detection).

Fig. 3. Typical chromatograms used for the quantitation of ellipticine (I) extracted from mouse kidney (A), spleen (B), liver (C), and heart (D), 4 h after the intravenous administration of 3 mg/kg of drug (System A, fluorescence detection).

mine ellipticine alone more rapidly using System A with fluorescence detection (9-hydroxyellipticine does not fluoresce and fewer early eluting endogenous components from the biological samples are encountered with fluorescence detection). The time for a complete chromatographic separation of internal standard and ellipticine in System A was about 10 min. Chromatograms illustrating the determination of ellipticine extracted from mouse blood and various tissues are seen in Fig. 3.

The detection limit for ellipticine using fluorescence detection was 5 ng/ml when a 0.5-ml blood sample was extracted. Using a UV detector the minimum detectable concentrations were 10 ng/ml and 25 ng/ml for ellipticine and 9-hydroxyellipticine respectively when extracted from a 0.5-ml blood or plasma sample.

Various proportions of methanol and 0.01 *M* sodium phosphate buffer (pH 3.5) were also evaluated as potential mobile phases. However, consistently sharper peaks were obtained using either of the two systems (A or B) containing acetonitrile. Variation of the pH in the aqueous component of the mobile phase demonstrated the apparent necessity of forming the protonated species to facilitate the elution of ellipticine from the column. When mobile phases containing water or buffers at >pH 7 were employed, the elution time for ellipticine at comparable flow-rates was more than 1 h. The reported pK_a for ellipticine is 5.8 [9].

TABLE II

EXTRACTION EFFICIENCY FROM BIOLOGICAL SAMPLES

Values are mean % ± S.D.

Drug	Mouse blood	Human blood	Human plasma	Mouse brain	Mouse heart	Mouse liver	Mouse kidney	Mouse spleen
Ellipticine	93.3 ± 4.7 (n = 19)	93.5 ± 5.6 (n = 25)	94.8 ± 3.7 (n = 30)	86.6 ± 7.2 (n = 6)	87.6 ± 3.0 (n = 6)	89.8 ± 3.7 (n = 6)	98.8 ± 3.1 (n = 6)	98.0 ± 6.5 (n = 6)
9-Hydroxy-ellipticine	92.4 ± 7.5 (n = 6)	90.7 ± 4.8 (n = 29)	94.7 ± 5.0 (n = 11)	—	—	—	—	—

Extraction efficiency

The extraction efficiencies for 9-hydroxyellipticine and ellipticine from spiked pooled mouse blood and from human blood and plasma are seen in Table II. Each value represents the mean and standard deviation of at least six separate extractions. The extraction efficiency for the recovery of ellipticine from various murine tissues is also presented in Table II. Each value reported is the mean ± standard deviation of at least six separate extractions. The internal standard was well extracted from all fluids and tissues (mean extraction efficiency overall was 93.0 ± 4.8%) with the exception of mouse liver. Extractions of 11-dimethylellipticine from liver homogenates were extremely variable and unpredictable. Therefore, when mouse liver samples were assayed for ellipticine, the internal standard was incorporated into the acid extract just prior to HPLC analysis.

Biological application

Blood ellipiticine levels in mice after a single intravenous administration at 3.0 mg/kg are seen in Fig. 4. Each plotted value represents the mean concentration obtained from measurements in at least four animals. The decline in blood levels appears to be biphasic. The mean blood concentration of ellipticine 5 min after administration was 696 ± 113 ng/g (mean ± S.E.). The blood levels declined rapidly during the first 60 min with a more gradual decline over the next 2 h. The half-life and rate constant calculated from the terminal phase were 52 min and 0.0132 min^{-1}, respectively. Table III presents representative mouse tissue levels after intravenous administration of drug at 3.0 mg/kg. Ellipticine was still detectable in various tissues 4 h after drug administration.

Blood levels of the 9-hydroxymetabolite of ellipticine have been shown to be relatively low after the intravenous administration of ellipticine [4]. The 9-hydroxymetabolite was not detected after intravenous administration of drug in the present study. However, analysis of blood samples after the oral administration of drug (30.0 mg/kg) showed significant levels of 9-hydroxyellipticine at 2, 3 and 4 h. Two hours post administration blood levels of 9-hydroxyellipticine and ellipticine were 164 ng/g and 590 ng/g respectively. Significant levels of 9-hydroxyellipticine were also detected 3 and 4 h after dosing (99.8 and 55.4 ng/g respectively). These relatively high concentrations of 9-hydroxy-

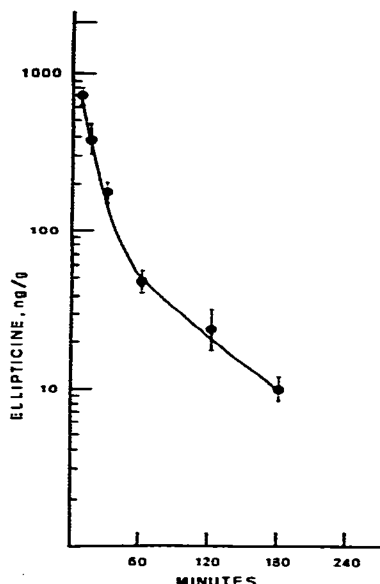

Fig. 4. Blood ellipticine levels in mice after the intravenous administration of drug at 3.0 mg/kg ($n \geq 4$).

TABLE III

TISSUE CONCENTRATIONS IN MICE AT 4 h AFTER INTRAVENOUS ADMINISTRATION OF ELLIPTICINE (3.0 mg/kg)

Each value represents the mean (± S.E.) of at least four animals.

Tissue	Concentration* (ng/g)
Brain	72.8 (13.2)
Liver	25.9 (5.6)
Kidney	126.8 (36.1)
Spleen	224.3 (38.8)

*Chromatographic System A, fluorescence detection.

ellipticine may indicate the existence of a significant first-pass metabolism of ellipticine when given orally. Since the drug will be administered orally to humans, this possibility should be given consideration in the design of protocols and pharmacological studies. Since 9-hydroxyellipticine possesses antitumor activity in animal models [10], the blood levels and elimination rates of both ellipticine and 9-hydroxyellipticine should be monitored to understand the proper dosing intervals and subsequent physiological effects.

In summary, a simple HPLC method for the determination of ellipticine and 9-hydroxyellipticine in biological samples was developed. The method has been successfully applied to the assay of mouse blood and tissue samples after the intravenous administration of ellipticine. Additionally, the method (System B) performed well in the determination of ellipticine and 9-hydroxyellipticine

in spiked human blood and plasma. Hopefully, the method should be useful in the assay of human plasma after the oral administration of ellipticine in clinical trials. Since relatively small sample volumes were extracted in this study, it should be possible to increase the sensitivity of the method by extraction of larger plasma samples during clinical studies.

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