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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF INDALPINE, A NEW NON-TRICYCLIC ANTIDEPRESSANT, IN HUMAN PLASMA

IDENTIFICATION AND SIMULTANEOUS MEASUREMENT OF ITS MAJOR PLASMA METABOLITE

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SUMMARY

Indalpine or 4-[2-(3-indolyl)ethyl]piperidine, a selective inhibitor of 5-hydroxytryptamine uptake in central monoamine neurons, has proved to be an effective agent in the treatment of chronically ill depressed patients. We have developed a rapid high-performance liquid chromatographic method for the simultaneous determination of indalpine and its major metabolite in human plasma. Isolation by high-performance liquid chromatography and identification of this metabolite by mass spectrometry are also described.

INTRODUCTION

Indalpine (LM 5008), or 4-[2-(3-indolyl)ethyl]piperidine, is a potent and specific inhibitor of 5-hydroxytryptamine uptake, active orally as an antidepressant agent [1—4]. In order to correlate human doses with pharmacokinetic parameters a method for plasma determination has been improved, based on the fact that indalpine can be detected with suitable sensitivity by fluorescence. In the course of preliminary investigation on plasma from volunteers receiving a single dose of indalpine, a metabolite was detected in relatively high quantities. Thus, a procedure for isolation and identification of this metabolite was imperative in order to measure simultaneously its plasma concentrations and to compare its activity to that of indalpine.

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EXPERIMENTAL

Materials

Chromatography was performed on a component system consisting of a Waters Assoc. Model 6000A delivery system, Model U6K injector, Model 440 dual channel absorbance detection operated at 254 nm, and a Schoeffel FS 970 fluorescence LC detector. The outputs of the detectors were connected to a 10-mV linear recorder B 5000 Omniscribe (Houston Instruments). A μBondapak C_{18} (10 μm particle size) column (30 cm × 3.9 mm I.D.) was used with a Bondapak C_{18}/Corasil guard column (2 cm × 3 mm I.D.) in series (Waters Assoc.).

Thin-layer chromatography (TLC) was carried out on HP-KF Whatman precoated plates (200 μm) prewashed with methanol. Radioactive compounds were located using a TLC scanner. Low-resolution electron impact/chemical ionization (EI/CI) mass spectra were obtained using a VG Micromass Model 70-70F double focusing mass spectrometer operating at 70 eV. Both direct insertion probe and combined gas chromatography—mass spectrometry (GC—MS) were used for identifying the metabolite. A Hewlett-Packard gas chromatograph (Model 5710A) was equipped with an OV-101 coated glass capillary column (20 m × 2.5 mm I.D.). High-resolution data were obtained by the peak-matching method using the same instrument operating at a resolving power of 10,000 with heptacosaperfluorotributylamine as the reference compound. The elemental composition of the molecular ion for which accurate mass measurement was obtained was determined using computer program.

Reagents and chemicals

Methanol and methylene chloride were fluorometry grade (Merck, Darmstadt, G.F.R.). Acetic acid, dipotassium hydrogen phosphate, sodium hydroxide, and ammonia were analytical grade (Merck). Reagents were used without further purification; water was doubly distilled in glass. [Indole-2-^{14}C]-indalpine (specific activity 91.3 μCi/mg) and [^{3}H]indalpine (15.35 mCi/mg) were prepared at the Radiochemical Centre, Amersham, Great Britain. Indalpine, PK 10157 or 4[2-(3-indolyl)ethyl]-2-piperidinone, and derivatives were synthesized in our chemical department.

Internal standard

Another indole-4-piperidine derivative, 4[(5-methoxy-3-indolyl)methyl]-piperidine (PK 26042), structurally related to indalpine, was used as internal standard. Stock solution of PK 26042 (10 mg/l) was prepared in methanol and stored in glass at 4°C. Dilutions were made to bring the final volume added to the sample to 100 ng per 100 μl.

Extraction procedure

To a 15-ml glass stoppered centrifuge tube were added 2 ml of plasma, 100 μl of the internal standard solution and 0.2 ml of 5 *N* sodium hydroxide. The mixture was vortexed for 1 min and 4 ml of dichloromethane were added. The tube was sealed and shaken for 15 min. After centrifugation (3000 *g*, 10 min) the organic layer was transferred to a second tube and the extraction

was repeated under the same conditions.

The organic layers were pooled and evaporated to dryness at 38°C with a nitrogen stream. The residue was dissolved in 50 μl of mobile phase, Vortex mixed and centrifuged at 3000 *g* for 5 min.

Chromatography analysis

Ten microlitres of the extract were chromatographed (Fig. 1) on a μBondapak C_{18} column. Components were eluted isocratically at a flow-rate of 1 ml/min with a mobile phase consisting of methanol—0.01 *M* aqueous K_2HPO_4—acetic acid (50:50:1, v/v). The solution was degasified before use in an ultrasonic bath. Detection was carried out with a Schoeffel FS 970 fluorescence LC detector with the excitation monochromator set at 220 nm and emission filter cut-off at 370 nm.

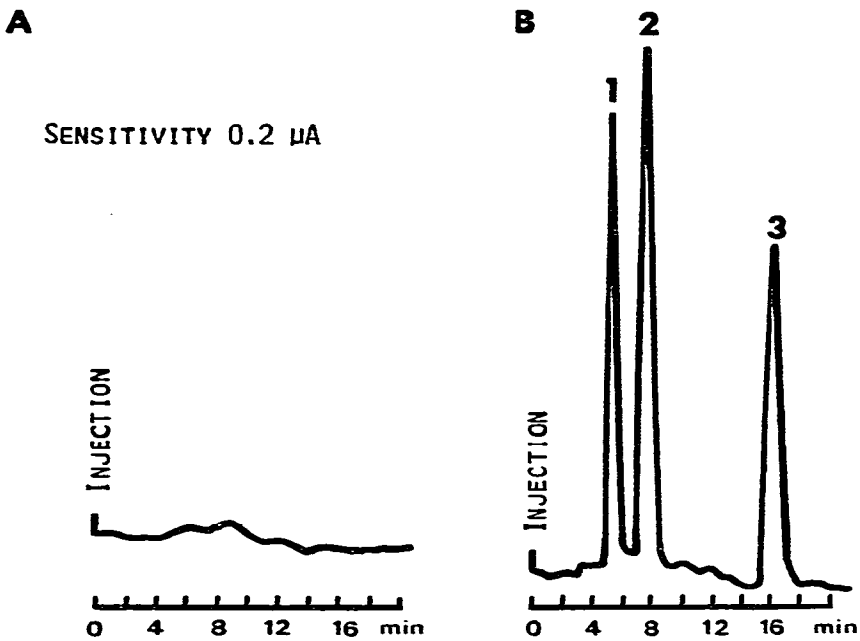

Fig. 1. (A) Chromatogram of extract of blank human plasma sample (2 ml). (B) Chromatogram of plasma sample extract 2 h after a single oral dose of 25 mg of indalpine. 1 = Internal standard; 2 = indalpine; 3 = metabolite. Chromatographic conditions are as given in the text.

RESULTS

Calibration curve

Known quantities of indalpine and PK 10157 (dissolved in 50 μl of methanol) ranging from 5 to 200 ng were added to 2 ml of blank plasma containing internal standard. The samples were extracted and chromatographed as outlined above. Calibration curves were constructed by calculating the ratio of the peak height of each compound to that of the internal standard and plotting the ratio

against the amount of compound added to the sample. The curves were linear for both indalpine, $Y_1 = 0.012X_1 + 0.023$ ($r = 0.998$), and its metabolite $Y_2 = 0.112X_2 - 0.008$ ($r = 0.998$).

Recovery

To estimate the recovery of indalpine a stock solution in methanol (65 pCi/μl) of [^{3}H]indalpine obtained by reduction with tritium gas of 4[2-(3-indolyl)-ethyl]pyridine was prepared. Ten microlitres of this solution, which correspond to 5 ng of indalpine, were added to 2 ml of plasma and extracted with dichloromethane in the same way as described above: 80.4 ± 3.6% (mean ± S.D., $n = 5$) of the radioactivity was recovered.

Analytical recovery of the compounds was also measured by comparing the peak heights of analyzed samples containing known amounts of indalpine, its metabolite and the internal standard to the respective peak height obtained by injecting equal amounts directly into the chromatograph. The recovery of all compounds from plasma was 75—80% when approximately 90% of the dichloromethane phase was available for evaporation. The recovery from plasma samples spiked with these compounds in the concentration range 25—100 ng was 75 ± 3.7% for internal standard, 79.5 ± 3.5% for indalpine and 80.2 ± 3.2% for PK 10157 (mean ± S.D., $n = 5$).

Accuracy

An accuracy study of plasma samples spiked with 5, 25 and 50 ng/ml indalpine and PK 10157 is reported in Table I. The coefficient of variation (C.V.) ranged from 9.9 to 2.7% for indalpine and 9.8 to 2.3% for PK 10157.

TABLE I

ACCURACY AND PRECISION TEST

Five assays in each case.

Indalpine			PK 10157		
Added (ng/ml)	Recovered (ng/ml)	C.V.(%)	Added (ng/ml)	Recovered (ng/ml)	C.V.(%)
5	4.5	9.9	5	4.7	9.8
25	24.7	3.5	25	24.4	3.7
50	49.8	2.7	50	49.9	2.3

Application of the method

Ten healthy volunteers received a single oral dose of 25 mg of indalpine. Venous blood samples were collected in heparinized bottles at 15, 30, 60, 120, 180 and 360 min after administration. The blood was centrifuged immediately and plasma was stored at −20°C until analysis. Plasma concentration—time curves for indalpine and its metabolite determined by the above procedure are shown in Fig. 2.

Indalpine and PK 10157 concentrations increased progressively in all subjects during the first hour following tablet ingestion, with a maximum value between 90 and 120 min. Mean peak concentration at 120 min was 44.5 ±

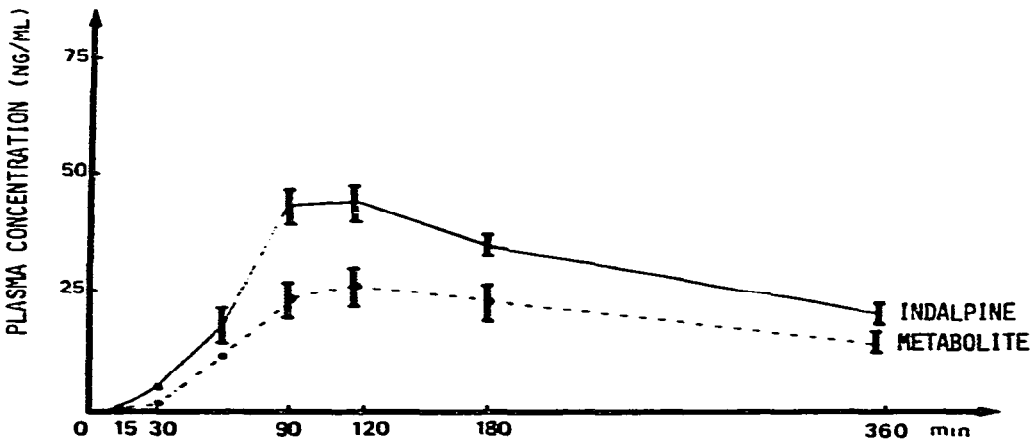

Fig. 2. Plasma concentrations of indalpine and its metabolite after oral administration of 25 mg of indalpine to ten subjects (mean ± S.E.M.).

3.0 (S.E.M.) ng/ml for indalpine and 23.3 ± 3.8 (S.E.M.) ng/ml for its metabolite. Six hours after ingestion mean plasma levels were 21.8 ± 2.4 (S.E.M.) ng/ml for indalpine and 14.6 ± 1.7 (S.E.M.) ng/ml for PK 10157. These results suggest that the clearance time is slower than the absorption time for indalpine and show similar plasma profile curves for indalpine and its metabolite.

Isolation and purification of the metabolite

Ninety millilitres of selected plasma samples were adjusted to pH 13 with 5 *N* sodium hydroxide and extracted with 360 ml of dichloromethane. The organic phase was evaporated to dryness and reconstituted in methanol (100 µl). This extract was injected into a µBondapak C_{18} column (60 cm × 3.9 mm I.D.) (Fig. 3) under the chromatographic conditions described above. The fractions isolated by HPLC were re-extracted with dichloromethane from the alkalinized mobile phase, concentrated in methanol and spotted on a silica gel TLC plate. The mobile phase used was chloroform—methanol—ammonia (75:23:2, v/v). Localization of the metabolite was made by examining the plate under UV light at 254 nm and by reference with the R_F of an identical ^{14}C-labelled metabolite obtained from a plasma extract of a monkey fed with [^{14}C]indalpine. The area of the silica gel TLC plate corresponding to the metabolite was then scraped off and extracted with methanol; a second purification using HPLC achieved good purification of the metabolite (Fig. 4).

Identification of the metabolite

EI 70-eV low-resolution mass spectra of the purified metabolite obtained by direct insertion probe or GC—MS show an abundant molecular ion at *m/z* 242 and a characteristic quinolinium fragment ion at *m/z* 130 (Fig. 5A).

CI low-resolution mass spectra obtained using ammonia or isobutane as reagent gas confirm the value determined for the molecular mass. Precise mass measurement of molecular ion by EI high-resolution mass spectrometry gives an elemental composition of $C_{15}H_{18}N_2O$, which shows an oxidation process of indalpine.

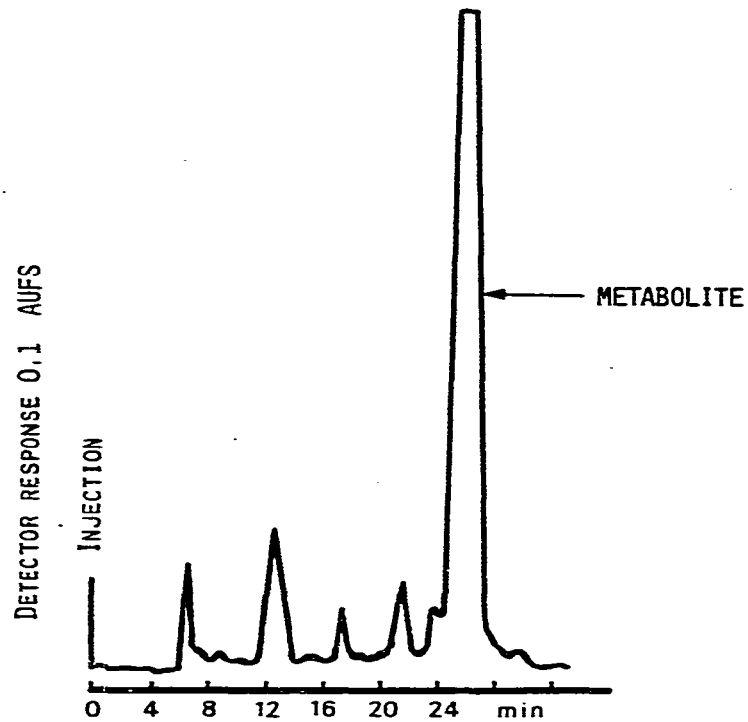

Fig. 3. Chromatogram of a reversed-phase separation of 90 ml of plasma extract using a UV detector at 254 nm.

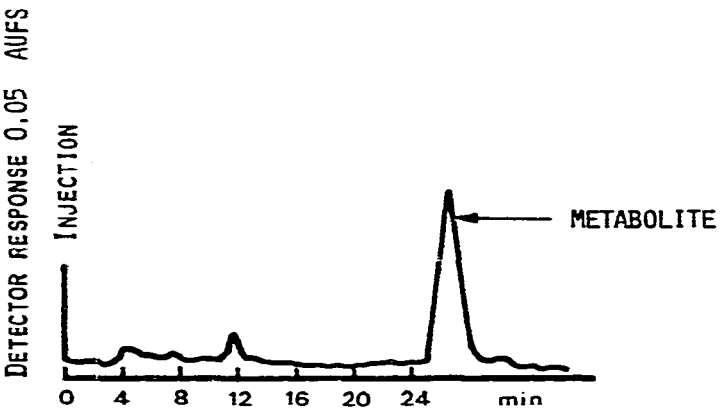

Fig. 4. Chromatogram of the purified metabolite.

Determination of the structure can be made by comparison of these results with EI mass spectra of indalpine, 4[2-(1-methyl-3-indolyl)ethyl] piperidine and 4[2-(5-methoxy-3-indolyl)ethyl] piperidine reproduced in Figs. 6—8. These spectra show abundant characteristic ions at *m/z* 85, 84 and 82, which arise from piperidine ring loss of these fragments in the metabolite spectra, suggesting oxidation of piperidine to lactam, which agrees with the observed physical properties and mass spectra results.

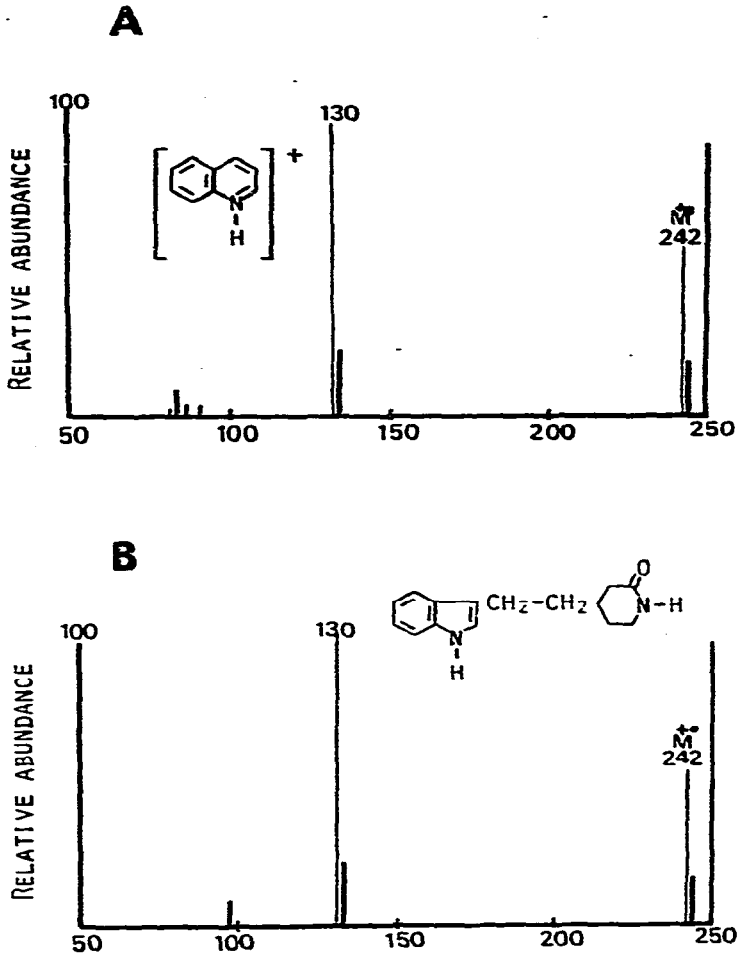

Fig. 5. (A) EI mass spectrum of metabolite isolated from plasma with chromatographic inlet. (B) EI mass spectrum of 4[2-(3-indolyl)ethyl]-2-piperidinone.

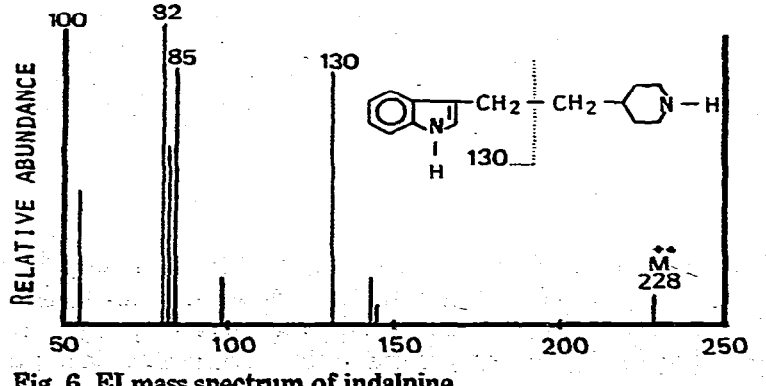

Fig. 6. EI mass spectrum of indalpine.

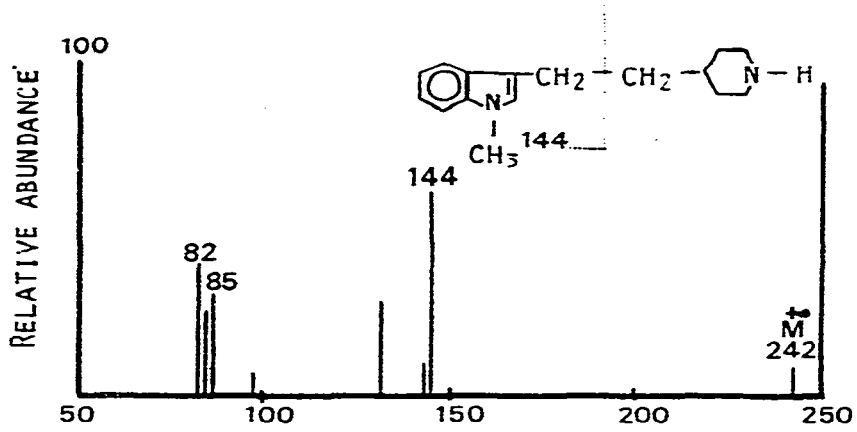

Fig. 7. EI mass spectrum of 4-[2-(1-methyl-3-indolyl)ethyl]piperidine.

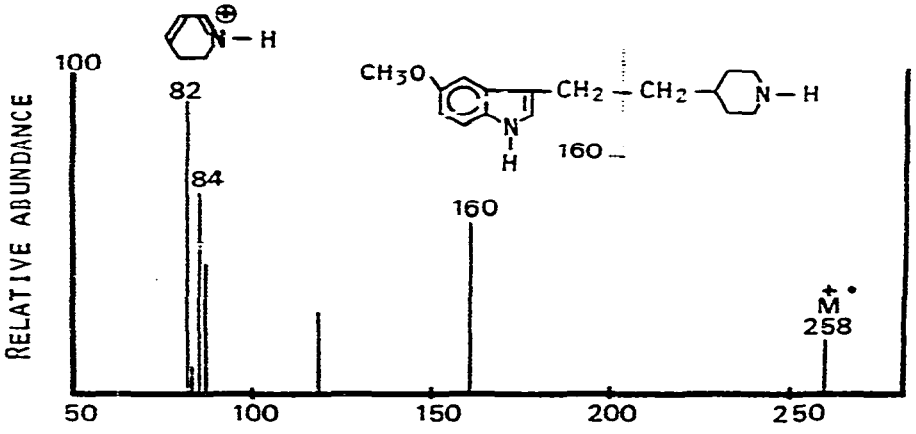

Fig. 8. EI mass spectrum of 4-[2-(5-methoxy-3-indolyl)ethyl]piperidine.

Verification

Corroborative evidence for the proposed structure was provided by synthesis of the lactam. Results obtained with HPLC and TLC using the chromatographic conditions described above show identical retention times for the metabolite and 4[2-(3-indolyl)ethyl]-2-piperidinone (PK 10157) chemically synthesized. The mass spectra were identical (Fig. 5A and B).

DISCUSSION

The extraction procedure described yields a clean extract (Fig. 1A) allowing measurement of indalpine and PK 10157 at the highest detector sensitivity. Under these conditions the lower limit of indalpine detection in plasma is about 2 ng/ml. This sensitivity fulfils the requirement for a pharmacokinetic study of indalpine and its metabolite at lower therapeutic doses (25 mg).

Lactam metabolite detected in human plasma was also present in plasma of other species such as rat, rabbit, guinea pig and monkey. Thus the biotransformation of indalpine into 4[2-(3-indolyl)ethyl]-2-piperidinone seems a general and important metabolic pathway for this drug. The biotransformation may be described by a two-step process [5], giving first the 2-hydroxypiperidine derivative, then the lactam derivative.

The kinetic curves observed for indalpine and its metabolite show that indalpine is rapidly metabolized into 4[2-(3-indolyl)ethyl]-2-piperidinone and that the metabolite level is a function of its parent drug level in the blood. This relationship led to investigations into the biochemical and pharmacological properties of PK 10157 which revealed that the lactam derivative is inactive in antidepressant tests.

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