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DETERMINATION OF INDICINE N-OXIDE AND INDICINE IN PLASMA AND URINE BY ELECTRON-CAPTURE GAS–LIQUID CHROMATOGRAPHY

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SUMMARY

A sensitive and specific method is described for the quantitative analysis of indicine N-oxide and indicine in plasma and urine. Indicine N-oxide is reduced to the tertiary amine with zinc–acetic acid (plasma) or zinc–ammonium chloride (urine). The indicine is derivatized with pentafluoropropionic anhydride to provide electron-capture detection. Assay of rabbit and human plasma and urine samples can detect 100 ng/ml (plasma) and 200 ng/ml (urine), and the method was applied to the analysis of indicine N-oxide and indicine in rabbits and one patient following administration of indicine N-oxide.

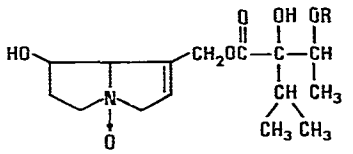
INTRODUCTION

Pyrrolizidine alkaloids are found in many plants in the form of the tertiary base or the N-oxide[1]. The ability of some alkaloids of the group to cause an unusual chronic liver disease when ingested by domestic animals[2] has prompted extensive study of their chemical and toxicologic properties[3]. They have been found to have antimitotic activity and to be mutagenic[4] and carcinogenic[5] in mammals. Several pyrrolizidine alkaloids exhibit antitumor activity but the acute and chronic toxicities associated with all the active agents has precluded their clinical use[1]. Indicine N-oxide, the major alkaloid in extracts of *Heliotropium indicum* Linn (Boraginaceae)[6], has limited toxicity but possesses significant antitumor activity in a variety of test systems[7] and has recently been placed in clinical trial at the Mayo Clinic.

Several methods have been used for the measurement of pyrrolizidine alkaloids. Qualitative analysis has been achieved with paper chromatography and visualization with iodine vapor[8] or platinum iodide[9]. Thin-layer chromatographic methods have included visualization by the use of Dragendorff's reagent[10], platinum iodide[9] and by a procedure which converts the tertiary bases to N-oxides with hydrogen peroxide and then to pyrroles with acetic anhydride, which are detected with Ehrlich's reagent[11]. The conversion to pyrroles and reaction of Ehrlich's reagent has been adopted for the spectrophotometric determination of pyrrolizidine alkaloids in plant extracts[12,13] and

animal tissues[14]. The thin-layer and paper chromatographic methods lack sensitivity while the spectrophotometric method is also relatively insensitive and nonspecific. Plant extracts have recently been analyzed for pyrrolizidine alkaloids by high-pressure liquid chromatographic[15], gas chromatographic (GC)[16] and gas chromatographic –mass spectrometric methods[16]. GC analysis was based on electron-capture detection of derivatives following base hydrolysis of all alkaloids to retronicine[16]. While affording sufficient sensitivity, this method has not been adapted to biological fluids and detects only the product of hydrolysis, retronicine.

We now report an assay for the sensitive and specific detection of indicine N-oxide (Fig. 1) and indicine in plasma and urine. The procedure is based on electron-capture GC detection of the pentafluoropropionyl derivative of indicine, either directly extracted from plasma or urine or extracted following an aqueous (plasma or urine) reduction of the N-oxide to indicine. Quantitative analysis is achieved with heliotrine and heliotrine N-oxide (Fig. 1) as internal standards. We also report the application of the assay to the determinations of indicine N-oxide and indicine in the plasma and urine of rabbits and one patient following administration of indicine N-oxide.



Indicine N-Oxide R=H
Heliotrine N-Oxide R=CH_3

Fig. 1. Structures of indicine N-oxide and heliotrine N-oxide.

MATERIALS AND METHODS

All solvents were glass-distilled and obtained from Burdick & Jackson Labs. (Muskegon, Mich., U.S.A.). Zinc dust was obtained from Pfaltz & Bauer (Stanford, Calif., U.S.A.) and pentafluoropropionic anhydride (PFPA) from either Pierce (Rockford, Ill., U.S.A.) or Pfaltz & Bauer. Heliotrine and heliotrine N-oxide were generously provided by J. P. Davignon (National Cancer Institute Bethesda, Md., U. S.A.). Heliotrine was purchased from Chemasea Manufacturing (Peakhurst, Australia).

Chromatographic analyses were performed on a microprocessor-controlled Hewlett-Packard 5840A gas chromatograph equipped with a ^{63}Ni electron-capture detector. Glass columns (4 ft. × 2 mm I.D.) were packed with 3% OV-101 on Supelcoport (80–100 mesh) and conditioned overnight (295°, gas flow-rate 30 ml/min) prior to analysis. Injector, oven and detector temperatures were 250, 185 and 345°, respectively. Carrier gas (5% methane–argon; Air Products, Allentown, Pa., U.S.A.) flow-rate was 30 ml/min.

Indicine N-oxide analysis

Heparinized plasma or urine (1 ml) was placed in a 12-ml screw-capped cen-

trifuge tube*, diluted with 1 ml water and 10 μg heliotrine N-oxide (1 μg/μl in MeOH) added as the internal standard. The pH was adjusted to 10 with 1 *M* NaOH (3 drops to plasma, 6 to urine), chloroform added (6 ml) and the samples shaken for 20 min on a mechanical shaker. The samples were centrifuged at 1000 *g* for 10 min and the chloroform layer removed and saved for analysis of indicine. The chloroform extraction was repeated and the chloroform combined with the first chloroform extract and reserved for indicine analysis.

Zinc dust (0.35 g) was added to the extracted plasma or urine, followed by either 6 *M* acetic acid (2 ml) for plasma or 4 *M* ammonium chloride (2 ml) for urine. After thorough mixing, samples were centrifuged at low speed for 2–3 min to control frothing. Following vigorous mixing, samples were shaken at a 45° angle for 30 min on a mechanical shaker, centrifuged at 1000 *g* for 5 min and 2 ml of the aqueous layer transferred to a 12-ml centrifuge tube containing either 1 ml ice-cold 5 *M* NaOH (plasma) or 0.5 ml phosphate buffer and 0.5 ml 5 NaOH (urine). Chloroform (6 ml) was added, the tubes shaken for 25 min and centrifuged at 1000 *g* for 10 min. The chloroform layer was transferred to another tube containing a small amount of anhydrous sodium sulfate, the tube briefly vortexed, 4.5 ml transferred to a 12-ml conical centrifuge tube and the solvent evaporated under a stream of nitrogen. Samples were stored under nitrogen until derivatization.

Indicine analysis

Heliotrine (10 μg, 1 μg/1 μl in MeOH) was added as an internal standard to the combined chloroform extracts containing free indicine. After drying with anhydrous sodium sulfate, the extracts were evaporated to dryness.

GC analysis

PFPA (0.1 ml) was added, and the samples heated at 75° for 15 min. The PFPA was removed under nitrogen and 0.2 ml ethyl acetate added and removed under nitrogen to remove all traces of PFPA. The samples were dissolved in 0.1–1 ml ethyl acetate and 1 μl injected for analysis. Concentrations were determined by peak area ratios of drug to internal standard compared to plasma and urine standard curves.

RESULTS AND DISCUSSION

Initial studies revealed that although indicine could be determined directly by GC analysis, underivatized indicine N-oxide decomposed to yield several chromatographic peaks, one of which was determined to be indicine by mass spectrometric analysis[17]. In addition, it was found that detection by flame-ionization or nitrogen–phosphorous flame-ionization did not provide the required sensitivity. Derivatization of the N-oxide with fluorinated acylating agents was not possible due to polymerization of the pyrrole product after treatment with anhydride, a well-described reaction of pyrrolizidine alkaloids[18]. We then turned to derivatization of indicine with PFPA to form an electron-capture sensitive species. Reaction with PFPA at 75° was found to be complete in less than 15 min without the formation of major side products,

* All glassware was silanized prior to use.

and picogram amounts of the derivative could be measured by GC analysis with an electron-capture detector.

Reduction of pyrrolizidine N-oxides by sulfuric acid and zinc dust has been reported in the chemical literature[19]. We were able to reduce indicine N-oxide in plasma by this method but encountered significant losses at low drug concentrations. Replacement of sulfuric acid with acetic acid gave much better results with complete reduction of indicine N-oxide and heliotrine N-oxide. Because of possible acid hydrolysis of conjugates of N-oxide or metabolites in the urine, 2 *M* ammonoum chloride was used instead of acid for the reduction in urine. There was a slight loss of sensitivity using ammonium chloride, but this was not a problem since urine contained relatively large amounts of indicine N-oxide. The acid reduction conditions were employed for plasma analysis, as there were no conjugates which could be hydrolyzed under reduction conditions.

Extraction of indicine formed either by *in vivo* reduction of indicine N-oxide or after chemical reduction was optimal with chloroform. Two extractions prior to reduction gave 80% recovery of indicine added to plasma and 97% recovery of heliotrine. Indicine N-oxide and heliotrine N-oxide are not extracted under these conditions and remaining unextracted indicine represents an insignificant quantity compared to the indicine produced after reduction at dosages of indicine N-oxide employed in our animal and human studies. Only one extraction of indicine after reduction was necessary using an internal standard as sensitivity was not a limiting factor in the analysis. While pre-extraction to remove plasma or urine indicine is an extra step, the use of the same solvent for extractions of reduced indicine N-oxide results in unusually clean chromatograms. A plasma blank and patient sample which was found to contain 2.6 μg/ml indicine N-oxide (both containing 10 μg heliotrine N-oxide internal standard) were analyzed following the reduction procedure giving the chromatograms shown in Fig. 2A and B. Chromatograms of the free indicine present in the

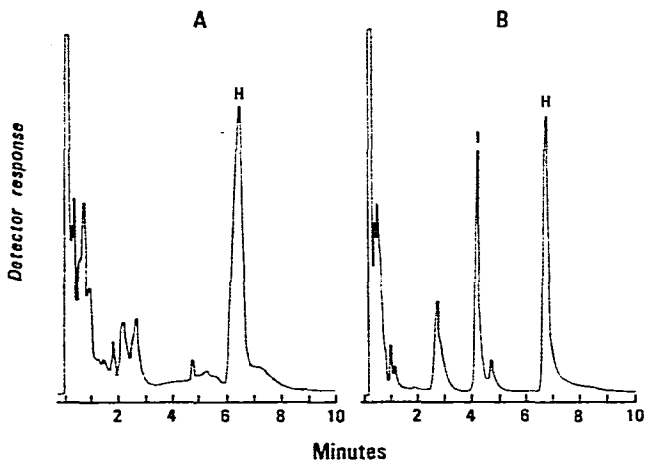


Fig. 2. Chromatograms of (A) blank plasma sample and (B) patient plasma sample found to contain 2.6 μg/ml indicine N-oxide (both samples contained 10 μg heliotrine N-oxide added as internal standard). Samples were pre-extracted with chloroform, reduced and analyzed as described in Materials and methods. (I = indicine N-oxide and H = heliotrine N-oxide).

initial chloroform extracts of urine are very clean; those of plasma contain extraneous peaks. However none of the substances found in plasma interfere with the determinations of indicine or the internal standard heliotrine.

Indicine N-oxide standard curves in plasma (Fig. 3) were linear over the range of concentrations found under biological conditions. Reliable quantitative results were obtained at 100 ng/ml concentrations. Similar results were obtained as illustrated by the standard curve in urine, with slightly reduced sensitivity. Standard curves for the analysis of free indicine in plasma and urine (employing heliotrine as internal standard) were also linear.

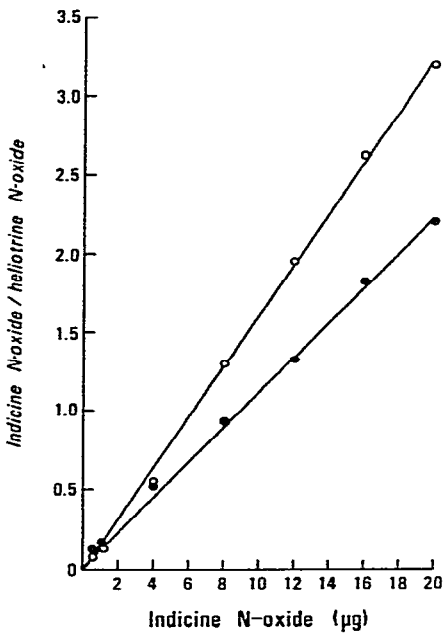


Fig. 3. Standard curves for indicine N-oxide in (○) plasma and (●) urine with 10 μg heliotrine N-oxide as internal standard.

The assay has been used to detect indicine N-oxide and indicine in the plasma and urine of rabbits administered a single intravenous dose of indicine N-oxide (25 mg/kg). The plasma concentrations time curve is biphasic (Fig. 4) and there is an appreciable difference in the disposition of the compound between the two rabbits. No free indicine could be detected in the plasma. The cumulative urinary excretion of indicine N-oxide and indicine is shown in Fig. 5. The indicine N-oxide was rapidly excreted mostly within 2 to 3 h of administration. In 24 h the total urinary excretion of indicine N-oxide was 97% in rabbit A and 53% in rabbit B. Free indicine was present in the urine at concentrations of approximately 50 μg/ml and was formed continuously over the 5 h of observation. In 24 h rabbit A had excreted 1091 μg of free indicine and rabbit B 561 μg free indicine.

The assay has also been used to measure the levels of indicine N-oxide in the

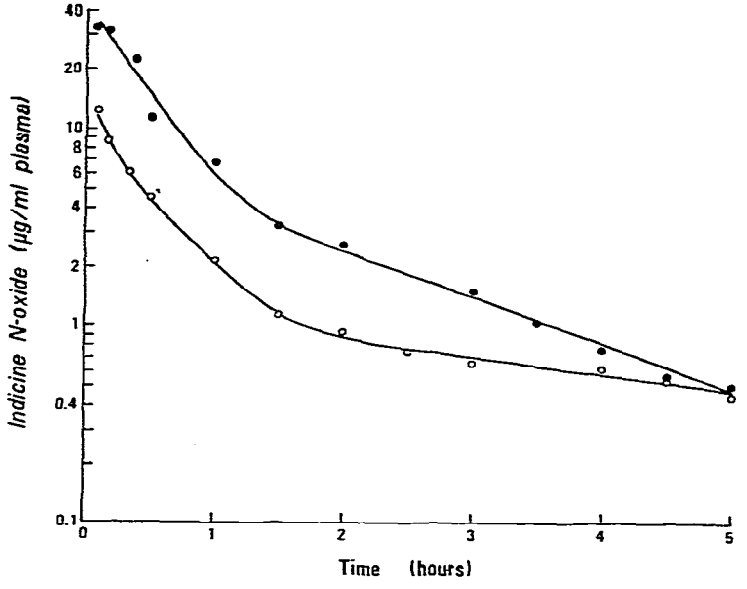


Fig. 4. Plasma levels of indicine N-oxide in the rabbit. Indicine N-oxide was administered by rapid i.v. injection to two male rabbits at a dose of 25 mg/kg. Both rabbits weighed 2.6 kg. ○, Rabbit A; ●, rabbit B. No free indicine could be detected in the plasma.

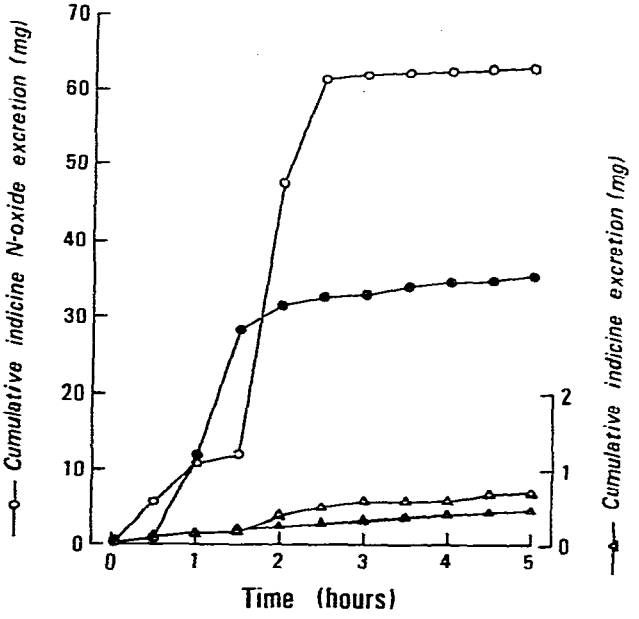


Fig. 5. Cumulative excretion of free indicine N-oxide and indicine in rabbit urine. Indicine N-oxide was administered by rapid i.v. injection to two male rabbits. ○,△, Rabbit A; ●,▲, rabbit B at a dose of 25 mg/kg (65 mg total). The cumulative urinary excretion of free indicine N-oxide is shown by ○,● and the cumulative urinary excretion of free indicine by △,▲.

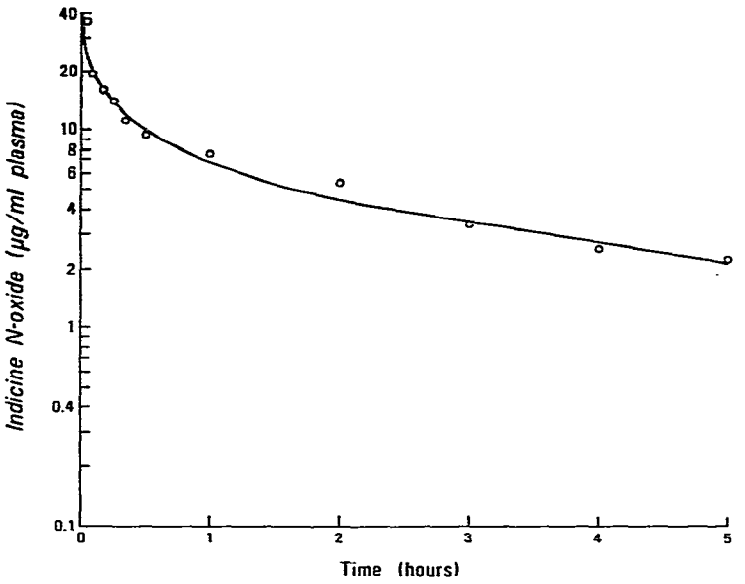


Fig. 6. Plasma levels of indicine N-oxide in man. Indicine N-oxide was administered by i.v. injection to a 64.4-kg female patient at a dose of 450 mg/m^2 (12 mg/kg).

plasma of one patient who received the compound (450 mg/m^2). and the plasma concentration time curve is shown in Fig. 6. The curve is also biphasic in nature.

The assays described here have provided a method for the determinations of indicine N-oxide and indicine in plasma and urine. The method is sensitive and specific and has been successfully applied to both animal and human studies at dosages being used in clinical trials of indicine N-oxide. In both rabbit and man, indicine N-oxide appears to have a rapid distribution phase and a markedly slower elimination phase. The reduction product, indicine, was not observed in plasma. However, significant quantities of indicine were isolated (free and conjugated) in the urine, suggesting that reduction to the parent alkaloid may play a role in the metabolism of the N-oxide.

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