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Stability-Indicating Colorimetric Assay for Indicine *N*-Oxide Using TLC

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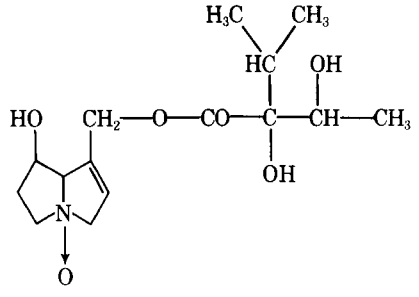
Abstract □ Aliquots of aqueous solutions in which indicine *N*-oxide may be degraded were mixed with 0.5 *M* formic acid (1:3) to adjust the pH to ~2–4 to quench the reaction and to ensure adequate TLC resolution. Silica-coated aluminum sheets were used to isolate indicine *N*-oxide by cutting the appropriate region from the chromatogram. By a modification of a known procedure, the silica gel then was treated with an acetic anhydride–diglyme mixture, and the mixture was heated to convert the drug to a pyrrole, which was then coupled with 4-dimethylaminobenzaldehyde to produce a color. The absorbance of the resulting solution was determined at 566 nm, and the apparent molar absorptivity, ϵ, based on the final indicine *N*-oxide concentration was 6.13×10^4. The recovery was ~92%, and the assays were readily reproducible with a coefficient of variation of 4.4%.

Keyphrases □ Indicine *N*-oxide—stability-indicating colorimetric assay using TLC □ Alkaloids—indicine *N*-oxide, stability-indicating colorimetric assay using TLC □ Antineoplastic agents, potential—indicine *N*-oxide, stability-indicating colorimetric assay using TLC

Indicine *N*-oxide[1] (I), an unsaturated pyrrolizidine alkaloid found in *Heliotropium indicum* Linn (Boraginaceae) (1), is undergoing clinical testing as an anticancer agent (2). While stability data have not been reported, alkaline ester hydrolysis is predicted from degradation studies of related alkaloids.

BACKGROUND

Approximate half-lives for the decomposition of 12 pyrrolizidines were estimated in 0.5 *N* aqueous or hydroalcoholic sodium hydroxide at room temperature (3). The relatively facile hydrolysis of esters of trachelanthic and viridifloric acids was attributed to their potential for β-hydroxyl participation, presumably *via* hydrogen bonding (3). This potential for intramolecular catalysis is present in I, which also is a trachelanthic acid ester (of retronecine *N*-oxide). The products obtained from the hydrolysis of indicine in 2 *N* NaOH at 100° for 2 hr were shown to be retronecine and a diastereoisomer of trachelanthic acid (4). It is not known whether the presence of the *N*-oxide in I gives rise to additional degradation pathways. Kugelman *et al.* (1) found that the properties of I extracted from *H. indicum* Linn (Boraginaceae) did not agree with those of synthesized I. Although these differences were ascribed to solvation problems, they also might reflect chemical instability.



I

Two assays for I in biological samples have been reported (2, 5). An electron-capture GLC assay after formation of the pentafluoropropionic anhydride derivative of indicine was applied to the analysis of mixtures of indicine and I in plasma and urine (2). Prior to analysis, indicine was extracted selectively with chloroform; the I remaining in the raffinate then was reduced to indicine. A GLC–mass spectrometric method using selective-ion monitoring recently achieved nanogram sensitivity through formation of the trimethylsilyl derivative of I (5). Although the method is selective for I, the equipment required is sophisticated and expensive.

This study was undertaken to develop a simple, specific assay for I in the presence of its degradation products. A colorimetric assay for unsaturated pyrrolizidine alkaloids using modified Ehrlich reagent (6) was adapted to assay I under aqueous conditions, in which it was shown to be unstable. TLC on aluminum sheets was employed to isolate I from buffers and reaction products. After the appropriate region was cut and scraped, the silica gel mixture was treated to convert the pyrrolizidine structure to a pyrrole. The pyrrole then was coupled with 4-dimethylaminobenzaldehyde to produce a color which was measured spectrophotometrically.

EXPERIMENTAL

Materials and Chemicals—The TLC aluminum sheets were precoated with 0.2 mm of silica gel 60 F-254[2]. Ether[3], absolute ethanol[4], ammonium hydroxide solution[5], acetic anhydride[6], and acetone[3] were analytical reagent grade. The diglyme[7] was kept free of peroxides (6). Modified Ehrlich reagent was prepared by dissolving 2% (w/v) 4-dimethylaminobenzaldehyde[8] in an ethanolic solution containing 14% (w/v) boron trifluoride, which was incorporated as its etherate complex (6, 7).

pH Adjustment—The assay results, obtained from absorbance values, were consistent provided that the sample to be spotted had a pH of 2–4.5. At pH < 2, erratic absorbance values were obtained. The pH was maintained at <4.5 since I degraded in alkali but not in acid. One part of 0.5 *M* formic acid was effective in controlling the pH when it was mixed with three parts of simulated reaction solutions of pH 2–13. Indicine *N*-oxide solutions treated in this manner were stable for several weeks under refrigeration.

Optimum Conditions—The amount of acetic anhydride used in the conversion of I to the pyrrole was varied from 0.1 to 0.4 ml. The best results were obtained with 0.2 ml. The optimum heating time for this step was 3.5 min. An optimum heating period of 4.5 min was observed for the color-producing step using modified Ehrlich reagent.

Assay of I in Aqueous Solutions or Reaction Mixtures—An aliquot of ~0.01 *M* I solution or reaction mixture at pH 2–13 was mixed with

[1] NSC-132319.

[2] EM Laboratories, Elmsford, N.Y.
[3] Mallinckrodt, St. Louis, Mo.
[4] U.S. Industrial Chemicals Co., New York, N.Y.
[5] Allied Chemical Co., Morristown, N.J.
[6] Drake Brothers, Menomonee Falls, Wis.
[7] Aldrich Chemical Co., Milwaukee, Wis.
[8] J. T. Baker Chemical Co., Phillipsburg, N.J.

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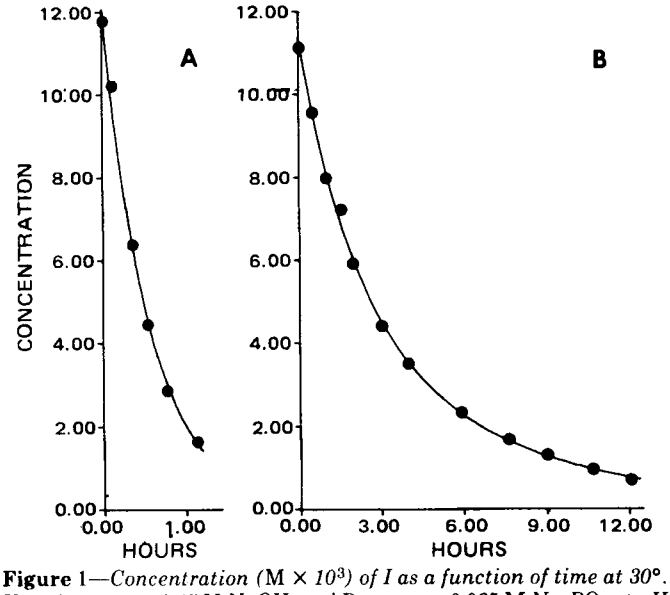

Figure 1—*Concentration (M × 10³) of I as a function of time at 30°. Key: A, aqueous 0.15 N NaOH; and B, aqueous 0.065 M Na_3PO_4 at pH 12.18.*

one-third of its volume of 0.5 *M* formic acid to adjust the pH to 2–4. An 8-μl sample was spotted on a TLC sheet, and the spot was air dried. Eight microliters of a reference solution consisting of I (~8×10^{-3} *M*) also was spotted on the same sheet. The sheet was developed (12 cm) with ether–ethanol–ammonium hydroxide solution–water (5:4:1:1) and air dried.

The entire channel containing the reference spot was cut out, sprayed with sulfuric acid–ether (1:4), and heated in an oven to locate I, which appeared at $R_f \sim 0.42$. The area in the sample channel corresponding to the I reference spot was scraped into a glass-stoppered test tube (12 × 1.5 cm). One milliliter of diglyme, followed by 0.2 ml of acetic anhydride, was added; the mixture was agitated thoroughly and heated in a boiling water bath for 3.5 min, during which it was agitated thoroughly at the end of 1 and 2 min. The tube was cooled to room temperature using a water bath (10–15°), and 1 ml of modified Ehrlich reagent was added.

The mixture then was agitated thoroughly and heated in a water bath at 57.5° for 4.5 min, during which it was agitated at the end of 100 and 200 sec. The tube was cooled, and 2 ml of acetone was added with thorough agitation followed by centrifugation at 3400 rpm for 1.5 min. The clear supernate was swirled carefully around the sides of the tube to wash down any silica gel particles. The tube was centrifuged a second time for 3.5 min, and the absorbance of the clear supernate was determined immediately at 566 nm against a similarly prepared blank.

The concentration of I in the initial solution may be calculated from:

$$[\mathrm{I}] = \frac{(A_{566})(R)}{\epsilon} \qquad \text{(Eq. 1)}$$

where A_{566} is the measured absorbance at 566 nm (λ_{max}), ϵ is the apparent molar absorptivity in the final solution (6.13×10^4), and R is the ratio of the final volume of the assayed solution to the sample volume spotted on the TLC sheet.

Beer's Law Plots—Aliquots of 2, 4, 6, 8, and 10 μl of a solution of I (8×10^{-3} *M*) in 0.125 *M* formic acid were chromatographed and assayed as described. A Beer's law plot was constructed using the absorbance data and the I concentration in the final assay solution.

Indicine *N*-oxide (8×10^{-3} *M*) was dissolved in a 3:1 mixture of phosphate buffer (pH 11.93, [Na_3PO_4] = 0.0328 *M*) and 0.5 *M* formic acid. Aliquots were chromatographed and used to prepare a Beer's law plot as described.

Percent Recovery—Aliquots of 2, 4, 6, 8, and 10 μl of an aqueous solution of I (8×10^{-3} *M*) were placed in glass-stoppered test tubes (12 × 1.5 cm) and dried under a dry nitrogen stream since water interferes with the assay (6). The residues were assayed without TLC separation. A Beer's law plot of absorbance at 566 nm *versus* the I concentration in the final assay solution was constructed.

RESULTS AND DISCUSSION

Beer's Law Plots—The apparent molar absorptivity, ϵ, from the Beer's law plot prepared by chromatographic assays of solutions of I in 0.125 *M* formic acid was 6.13×10^4 ($r^2 = 0.9996$). The molar absorptivity from the Beer's law plot prepared by assaying the I solution in the phosphate buffer–formic acid mixture was 6.37×10^4 ($r^2 = 0.9996$), which is within 4% of that obtained for I in 0.125 *M* formic acid alone. The Beer's law plots passed through the origin, and all experimental points were on the regression lines.

Assay reproducibility was assessed by estimating ϵ 18 times, each using two known concentrations of I in solutions simulating a variety of reaction conditions ($\bar{\epsilon} = 6.18 \times 10^4$, $s = 0.27 \times 10^4$).

TLC Assay Recovery of I—The percent recovery of I following chromatographic separation was evaluated relative to solutions that were not chromatographed by comparing their Beer's law plots. The ϵ value without chromatographic separation was 6.69×10^4 ($r^2 = 0.9990$). Therefore, the percent recovery of I in the TLC assay was 91.6% as calculated from:

$$\%\ \text{recovery} = \frac{(100)(6.13 \times 10^4)}{(6.69 \times 10^4)} \qquad \text{(Eq. 2)}$$

Application to Kinetics of I Degradation—Preliminary studies indicated that I was unstable in alkali. The assay successfully measured the decreasing concentration of I as a function of time under a wide variety of aqueous alkaline conditions. Figure 1 shows two examples that illustrate the stability-indicating efficacy of this assay. Detailed studies on the kinetics and mechanisms of I degradation are in progress.

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