Spectrofluorometric Determination of Substituted Dihydroisoquinolines and Tertiary Tetrahydroisoquinolines as Their Isoquinolinium Derivatives

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Abstract □ A spectrofluorometric method was developed for the determination of a substituted dihydro- and a tertiary tetrahydroisoquinoline in biological fluids based on a chemical and/or photochemical oxidation reaction to produce the isoquinolinium derivatives which fluoresce strongly in acidic media. This principle was used for the development of assays of these compounds in blood and urine with sensitivity limits in the submicrogram range. TLC was used to establish the specificity of the assays for the intact drugs and their biotransformation products. The method was applied to the determination of blood levels and the urinary excretion of these compounds administered in the dog and in man. Both compounds were found to be rapidly absorbed and extensively metabolized by both species.

Keyphrases □ 3,4-Dihydro-1-isoquinoline acetamide—determination of blood levels and urinary excretion in dogs and man, spectrofluorometric assay developed □ 8-Methoxy-2-methyl-1,2,3,4-tetrahydroisoquinoline—determination of blood levels and urinary excretion in dogs and man, spectrofluorometric assay developed □ Isoquinoline derivatives—spectrofluorometric assay in blood and urine □ Blood levels—3,4-dihydro-1-isoquinoline acetamide and 8-methoxy-2-methyl-1,2,3,4-tetrahydroisoquinoline, dogs, man □ Excretion, urinary—3,4-dihydro-1-isoquinoline acetamide and 8-methoxy-2-methyl-1,2,3,4-tetrahydroisoquinoline, dogs, man □ Spectrophotofluorometry—analysis, dihydro- and tetrahydroisoquinoline derivatives in blood and urine

The chemistry of the isoquinoline alkaloids has been extensively investigated (1-3) and has led to the synthesis of a large number of chemical derivatives of widely different pharmacological properties (4) such as analgesic (5), antiviral (6-9), and antihypertensive (10, 11) activities. This report describes the spectrofluorometric determination of two new members of this class of compounds, one showing antiviral activity and the other antihypertensive activity. 3,4-Dihydro-1-isoquinoline acetamide hydrochloride (I-HCl)[1] was synthesized by Creighton *et al.* (12) (Scheme I and Table I) and was shown to have antiviral activity (13–15). 8-Methoxy-2-methyl-1,2,3,4-tetrahydroisoquinoline hydrochloride (II-HCl) was synthesized by Schenker *et al.* (16) (Tables I and II) and was clinically evaluated as a hypotensive agent (17).

Tertiary tetrahydroisoquinolines can be readily oxidized to their 3,4-dihydroisoquinoline derivatives, which exhibit UV absorption and fluorescence in acidic media (18). This oxidative reaction was first described by Knabe (18) for the oxidation of tertiary amines using mercuric acetate in ethylenediaminetetraacetic acid. He also found it to be a useful general reaction applicable to a variety of tertiary tetrahydroisoquinolines. Knabe's method was modified by Schwartz and Rieder (19), who omitted ethylenediaminetetraacetic acid and used mercuric acetate in acetic acid and 0.1 *N* H_2SO_4. This modification, which was reported to give more quantitative and reproducible yields of the oxidation products, was used for the determination of emetine and dehydroemetine in biological fluids (19) and for the determination of methopholine and its metabolites in man and in the rabbit (20).

The chemical oxidation step usually introduces only one double bond in the 1,2-position of the tetrahydroisoquinoline ring to form the 3,4-dihydroisoquinoline derivatives. This report describes a photochemical oxidation which introduces a second double bond in the 3,4-position of the dihydroisoquinoline to form the fully aromatized isoquinolinium derivatives (21, 22) (Table II), which are highly fluorescent in acidic media. This photochemical oxidation was used to develop sensitive assays for the quantitation of I and II in blood and urine.

EXPERIMENTAL

Determination of 3,4-Dihydro-1-isoquinoline Acetamide Hydrochloride (I-HCl) and 1-Isoquinoline Acetamide (I-A) in Blood and Urine

Reference Standards—I-HCl, mol. wt. 224.69, m.p. 195–197° dec.

Stock Solution—Weigh out 11.940 mg. of I-HCl equivalent to 10.000 mg. of free base, transfer into a 100-ml. volumetric actinic flask, and dissolve in 100 ml. of methanol. This stock solution contains 100-mcg. equivalents of free base/ml.

Working Solution—Dilute 1.0 ml. of the stock solution to 10 ml. with methanol. This solution contains 10.0-mcg. equivalents of free base/ml., suitable aliquots of which are used as internal standards added to blood for the determination of percent recovery.

Standard Solution of I-A—1-Isoquinoline acetamide, mol. wt. 186.21, m.p. 204.5–205° dec.

Stock and Working Solutions—Weigh out 10.000 mg. of I-A and follow the procedure exactly as described for I-HCl.

External Standard of I—The determination of percent recovery from blood of added internal standards requires the use of a standard curve of I in 30% trichloroacetic acid exposed to UV light under similar conditions as the internal standard.

Weigh out 11.940 mg. of I-HCl equivalent to 10.000 mg. of free base, transfer into a 100-ml. volumetric actinic flask, and dis-

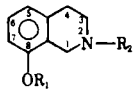
[1] The Roman numerals refer to the free base of the compound; the salts of a given compound are indicated by the appropriate abbreviations.

Table I—Chemical Names and Physical Properties of Compounds Referred to in Scheme I and Table II

Compound	Chemical Name	Molecular Weight	Melting Point	Reference Synthesis
I-HCl	3,4-Dihydro-1-isoquinolineacetamide hydrochloride	224.69	195–197° dec.	12
I-A	1-Isoquinolineacetamide	186.21 (B)[a]	204–205° dec.	—[b]
II-HCl	8-Methoxy-2-methyl-1,2,3,4-tetrahydroisoquinoline hydrochloride	213.70	215–216°	16, 21
III-HCl	8-Methoxy-1,2,3,4-tetrahydroisoquinoline hydrochloride	199.68	261–262.5°	16, 21
IV-HCl	2-Methyl-1,2,3,4-tetrahydro-8-isoquinolinol hydrochloride	199.71 163.21 (B)	243–244° 173–174.5° (B)	16 21
V-HCl	1,2,3,4-Tetrahydro-8-isoquinolinol hydrochloride	185.68 149.18 (B)	271–272° 181–181.5° (B)	16 21
II-A-HI	8-Methoxy-2-methyl-3,4-dihydroisoquinolinium iodide	303.15	182–182.5° dec.	21
III-A-HCl	8-Methoxy-3,4-dihydroisoquinoline hydrochloride	197.70	175–175.5°	21
IV-A	2-Methyl-3,4-dihydro-8-isoquinolinol	162.18	—	N.S.[c]
V-A	3,4-Dihydro-8-isoquinolinol	147.18	156–157°	[b]
II-B-HI	8-Methoxy-2-methylisoquinolinium iodide	301.13	231–232°	16, 21
III-B-HCl	8-Methoxyisoquinoline hydrochloride	195.65 159.15 (B)	170–170.5° dec. 50.5–51.5° (B)	16, 21 16, 21
IV-B-HI	8-Hydroxy-2-methylisoquinolinium iodide	287.11	220–221° dec.	16, 21
V-B-HCl	8-Isoquinolinol hydrochloride	181.65 145.15 (B)	240–242° 210–211° (B)	22 22

[a] (B) = free base of compound. [b] F. Schenker, Department of Chemical Research, Hoffmann-La Roche Inc., Nutley, N. J., unpublished data, 1967. [c] N.S. = not synthesized.

Table II—Postulated Chemical Reactions of II and Its Desmethyl Analogs

R Group R_1	R Group R_2	Parent Compound (tetrahydroisoquinoline)	chemical oxidation → Intermediate (3,4-dihydro derivative)	photochemical oxidation → Fluorophore (isoquinolinium derivative)	Wavelength (nm.) of Activation	Wavelength (nm.) of Emission
			mercuric acetate-acetic acid; 0.1 *N* H_2SO_4 at 100° for 30 min.	irradiated for 15 min.; Pyro-Lux R-57 lamp		
$-CH_3$	$-CH_3$	II	II-A	II-B	370	458
$-CH_3$	$-H$	III	III-A	III-B	370	458
$-H$	$-CH_3$	IV	IV-A	IV-B	425	525
$-H$	$-H$	V	V-A	V-B	425	525

solve in a minimal amount of water. Add 0.6 ml. of 0.1 *N* NaOH to liberate the free base[2], which is insoluble in aqueous media, resulting in a turbid solution. Add 25 ml. of 30% trichloroacetic acid solution to dissolve the free base. Make to volume with 30% trichloroacetic acid solution. This stock solution of I contains 10 mg. free base/100 ml. or 100 mcg./ml.

Make serial dilutions of this stock solution in 30% trichloroacetic acid to give standard solutions containing 0.125, 0.25, 0.50, 1.0, and 2.0 mcg. of free base/ml. to be used in making an external standard curve.

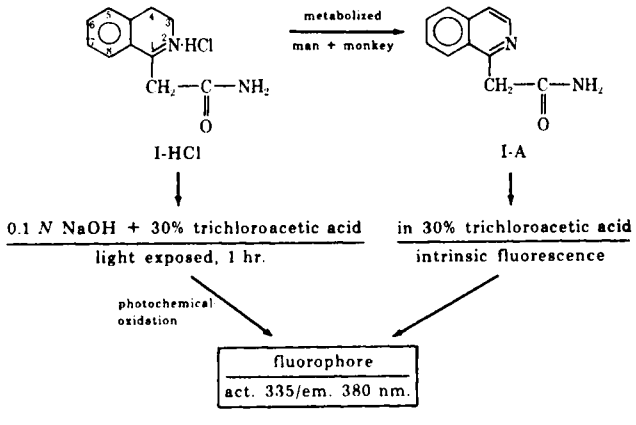
External Standards of I-A—Dissolve 10.000 mg. of compound in 100 ml. of 30% trichloroacetic acid. Make serial dilutions of this solution in 30% trichloroacetic acid to give standard solutions containing 0.125, 0.25, 0.50, 1.0, and 2.0 mcg./ml. Use these solutions to prepare an external standard curve of I-A (described later).

Reagents—The following were used: ether[3] (peroxide free), 1.0 *M* pH 9.0 borate–potassium carbonate–potassium chloride buffer solution (23), 30% trichloroacetic acid in distilled water, 5 *N* NaOH in distilled water, 1 *N* HCl in distilled water[4], and 0.1% bromthymol blue indicator in 50% alcohol.

Procedure for Blood and Urine—To 1.0 ml. of blood in a 50-ml. centrifuge tube, add 5 ml. of pH 9.0 buffer and 15 ml. of ether. Stopper the tube and shake for 15 min. on a reciprocating shaker. Along with the samples, run a 1-ml. specimen of control blood (taken prior to medication) and duplicate 1-ml. specimens of control blood containing 1.0 mcg. of both I-HCl and I-A (0.1 ml. of each working solution) added as internal standards.

To 1.0 ml. of urine, add 4 ml. of 2 *N* NaOH and 15 ml. of ether and extract as described for blood. Along with the samples, run 1.0 ml. of control urine and duplicate 1.0-ml. specimens of control urine containing 1.0 mcg. of both I-HCl and I-A added as internal standards.

Centrifuge all samples for 10 min. at 2000 r.p.m., preferably at 0–4° in a refrigerated centrifuge; then transfer the ether layer



Scheme I—Postulated chemical reactions of I and its biotransformation to I-A

[2] This procedure is necessary because only the free base (I) dissolved in 30% trichloroacetic acid gives an optimal, photochemically produced yield of I-A. When I-HCl was dissolved directly in 30% trichloroacetic acid and irradiated, it gave only 25% of the fluorescence yield as that produced by I.

[3] Mallinckrodt anhydrous analytical reagent grade diethylether from a freshly opened can may be used for up to 4 days after opening.

[4] Acculate.

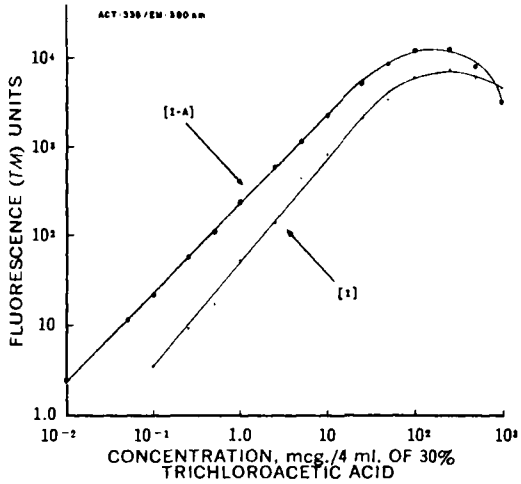


Figure 1—*Linear dynamic range of fluorescence* versus *concentration of I and I-A in 30% trichloroacetic acid.*

quantitatively into another 50-ml. centrifuge tube and evaporate to dryness. Repeat the extraction of each specimen with another 15-ml. portion of ether, combine the ether extracts, and evaporate to dryness.

Each residue is then dissolved in 100 μl. of methanol, which is transferred quantitatively onto a 20 × 20-cm. silica gel G/F_{254} thin-layer chromatoplate[6]. The chromatogram is developed in ethyl acetate–methanol–concentrated ammonium hydroxide (95:4:2), ascending for 12 cm. The intact drug (I, R_f 0.6) and the metabolite (I-A, R_f 0.3) are detected by their intrinsic blue-green fluorescence under shortwave UV light and are identified by reference to the R_f values of authentic standards run alongside the sample extracts. The areas on the plate corresponding to I and I-A are scraped off immediately after development[6] and transferred into separate 15-ml. centrifuge tubes. Both I and I-A are eluted from the silica gel with 4 ml. of 30% trichloroacetic acid by slurring vigorously on a high-speed mixer[7] for 60 sec. After centrifugation of the silica gel, the clear supernates are transferred into a fresh set of 15-ml. centrifuge tubes. At this point in the assay, a series of external standards of I and I-A in 30% trichloroacetic acid are prepared by transferring 4-ml. aliquots of each standard solution into separate 15-ml. centrifuge tubes. The external standards and biological specimens containing I *only* are then stoppered and exposed for 60 min. to high intensity light from a lamp[8] contained in an aluminum foil-lined box. The samples are placed in a single row 30.48 cm. (12 in.) away from the light source to standardize the photochemical formation of the fluorophore prior to fluorometric analysis. The intrinsic fluorescence of I-A in the external and internal standards and of the metabolite in the biological specimen is measured directly *without* prior light exposure.

Fluorometric Analysis—Read the fluorescence (TM)[9] of all the solutions in a 1-cm. path cell at 380 nm., activating at 335 nm. and using 4 ml. of irradiated 30% trichloroacetic acid as the reagent blank.

Plot external standard curves for I and I-A of the respective fluorescence units corrected for blank *versus* concentration of standard (micrograms per milliliter) (Fig. 1), from which the recovery of the internal standards of I-HCl and I-A added to blood or urine can be determined.

The fluorescence readings of the biological samples are corrected for control blood or urine blank readings, while that of the external standards of I and I-A are corrected for reagent blank (30% trichloroacetic acid) readings. The concentration of each compound in the unknown is determined by direct comparison to the fluorescence reading of the respective internal standard. The percent recovery of the added internal standards of I-HCl and I-A may be determined by direct comparison to the fluorescence reading of the respective external standards.

Calculations—*Determination of I and I-A in Blood and Urine*—

$$\frac{TM_{380} \text{ of unknown}}{TM_{380} \text{ of internal standard}} \times \frac{\text{concentration of internal standard (1.0 mcg. I or I-A)}}{\text{ml. sample assayed}} = \text{mcg. I and I-A/ml. blood or urine} \quad \text{(Eq. 1)}$$

Determination of Percent Recovery of I and I-A—

$$\frac{[TM/\text{mcg./ml.}] \text{ internal standard}}{[TM/\text{mcg./ml.}] \text{ external standard}} \times 100 = \text{percent recovery} \quad \text{(Eq. 2)}$$

The percent recovery of internal standards should be determined routinely as a check on analytical precision and reproducibility.

Determination of 8-Methoxy-2-methyl-1,2,3,4-tetrahydroisoquinoline Hydrochloride (II-HCl) and Its Metabolites in Blood and Urine

Reference Standards—II-HCl, mol. wt. 213.70, m.p. 213–214°.

Stock Solution—Weigh out 12.10 mg. of II-HCl equivalent to 10.00 mg. of free base, transfer into a 10-ml. volumetric actinic flask, and make to volume in absolute ethanol. This stock solution contains 1 mg. of II/ml.

Working Solution—Make two serial 1:10 dilutions of the stock solution with ethanol to yield a working solution containing 10 mcg./ml. Suitable aliquots of this solution are used as internal standards added to blood for the determination of percent recovery.

External Standard of II—The determination of percent recovery from blood of added internal standards requires the use of a standard curve of II in 0.1 N H_2SO_4, oxidized in mercuric acetate–acetic acid and exposed to light under the same conditions as the internal standards.

Weigh out 12.100 mg. of II-HCl equivalent to 10.000 mg. of free base, transfer into a 10-ml. volumetric actinic flask, and dissolve in a minimal amount of water (1–1.5 ml.). Add 0.6 ml. of 0.1 N NaOH to liberate the free base, which is insoluble in aqueous media, resulting in a turbid solution. Add 5 ml. of 0.1 N H_2SO_4 solution to dissolve the free base, and make to volume with 0.1 N H_2SO_4 solution. This stock solution contains 1.0-mg. equivalent of II/ml. Make serial dilutions of this solution with 0.1 N H_2SO_4 to give standard solutions containing 0.125-, 0.25-, 0.50-, 1.0-, and 2.0-mcg. equivalents of II/ml. to be used in making a standard curve (Fig 2.).

Reagents—The following were used: ether[3] (peroxide free), 0.1 N H_2SO_4 in distilled water, and 1.0 and 0.1 N NaOH solutions in distilled water. For mercuric acetate reagent (pH 6.0), dissolve 32.4 g. of sodium acetate (anhydrous), 1.0 g. mercuric acetate, and 3 ml. of glacial acetic acid/100 ml. of distilled water. This solution must be made fresh every 2–3 days due to precipitation and crystallization of the salt. For 1.0 M phosphate buffer (pH 5.5), dissolve 138.01 g. of $NaH_2PO_4 \cdot H_2O$ in distilled water and make to 1 l. Titrate to pH 5.5 with 1.0 N NaOH, and mix well by inversion.

An enzyme preparation[10] containing 100,000 units of glucuronidase and 50,000 units of sulfatase/ml. is available commercially.

Procedure—*Extraction of Blood and Urine*—To 1.0 ml. of blood or 4.0 ml. of urine in a 50-ml. centrifuge tube, add 4 ml. of 1.0 N NaOH solution and 15 ml. of ether. Stopper the tubes and shake for 15 min. on a reciprocating shaker. Along with the samples, run a 1-ml. specimen of control blood or 4 ml. of control urine (taken prior to medication) and duplicate 1-ml. specimens of control blood

[6] Brinkmann F_{254}.

[6] Compounds I and I-A are unstable to atmospheric oxidation and light exposure, especially on a thin-layer chromatoplate. Both compounds undergo extensive oxidation on exposure to the atmosphere and also when irradiated on the TLC plate by a Pyro-Lux R-57 lamp. Therefore, immediately following TLC separation of the biological extracts, the silica gel areas corresponding to I and I-A must be scraped off, eluted, and processed for fluorometric analysis as described.

[7] Vortex Super-Mix.

[8] Pyro-Lux R-57, Luxor Corp., N. Y.

[9] (TM) = transmittance (T) × meter multiplier factor (M). All fluorescence measurements were made in a Farrand spectrofluorometer equipped with a xenon arc energy source and an RCA IP-28 photomultiplier. The 10-nm. slit arrangement was used in all four positions. The monochromators were corrected for the fundamental mercury lines, but the fluorescence measurements were not corrected for energy and other instrumental artifacts. The instrument sensitivity was adjusted for maximum energy for each day, using a Pyrex reference rod and a standard solution of the compound to be analyzed.

[10] Glusulase, Endo Laboratories, Inc., Garden City, N. Y.

or 4-ml. specimens of control urine containing 0.50 mcg. II (50 μl. of the working solution evaporated to dryness under nitrogen).

Centrifuge all samples for 10 min. at 2000 r.p.m., preferably in a refrigerated centrifuge at 0–5°, and transfer the ether phase quantitatively into another 50-ml. centrifuge tube. Repeat the extraction with a second 10-ml. portion of ether, centrifuge, and combine the ether extracts. The whole blood specimen is discarded at this point, but the urine specimen is saved for further analysis of the conjugated metabolites.

Assay in Blood—The combined ether extracts of blood are back-extracted with 2 ml. of 0.1 *N* H_2SO_4 by shaking for 15 min. Centrifuge at 2000 r.p.m. at room temperature for 5 min., and aspirate off the supernatant ether layer.

The sulfuric acid extract is backwashed with two 10-ml. portions of ether by shaking for 5 min. each, centrifuging, and aspirating off the ether layer. To the ether-washed sulfuric acid extract, add 2 ml. of mercuric acetate reagent and place in a boiling water bath. Allow 5 min. for equilibration to expel all residual ether from the acid.

At this stage in the assay, pipet 2-ml. aliquots of each standard solution of the external standards of II into 15-ml. centrifuge tubes, add 2 ml. of the mercuric acetate reagent into each tube, and process these samples through the rest of the procedure along with the biological extracts. Stopper the tubes tightly (seal with a drop of distilled water), and allow the chemical oxidation step to proceed for 30 min. in a boiling water bath (100°). Then cool the samples to room temperature, wipe the tubes dry prior to irradiation, and expose all samples including the external standards of II directly to high intensity light from a lamp[8] contained in an aluminum foil-lined box. The samples must be arranged in a single row in a suitable rack and placed 30.48 cm. (12 in.) from the light source. Expose for 15 min. to complete the photochemical oxidation reaction for optimal fluorophore formation. The fluorescence of each sample is read in a 1-cm. path quartz cell at 458 nm., activating at 370 nm., within 1 hr. after light exposure. The concentrations in the unknowns are calculated by direct comparison to the corrected fluorescence of the internal standards:

$$\frac{TM_{458} \text{ of unknown}}{TM_{458} \text{ of internal standard}} \times \frac{\text{concentration of internal standard (0.5 mcg.)}}{\text{ml. sample assayed}} = \text{mcg. II/ml. blood} \qquad \text{(Eq. 3)}$$

Assay in Urine—The initial ether extract of the urine contains unconjugated metabolites in addition to the parent compound. To assay for conjugated metabolites, the ether-extracted urine specimen is transferred into a 50-ml. erlenmeyer flask, titrated potentiometrically to pH 5.5 with 2 *N* HCl, and buffered with 5 ml. of 1.0 *M* phosphate buffer (pH 5.5). The enzyme preparation is added to a final concentration of 1% (v/v), and the flask is stoppered loosely with cotton and incubated at 37° for 2 hr. in a Dubnoff incubation shaker with mild reciprocation. After incubation, the samples are cooled to room temperature, transferred into 50-ml. centrifuge tubes, and extracted at pH 5.5 with 2 × 15-ml. portions of ether to remove the deconjugated weakly acidic phenolic metabolites. The urine specimen is then made strongly alkaline (pH 14) with sufficient 10 *N* NaOH and is reextracted with 2 × 15-ml. portions of ether to remove the deconjugated basic metabolites.

The three separate ether extracts of urine are evaporated to dryness, and the residues are dissolved in 100 μl. of methanol and transferred quantitatively onto separate 20 × 20-cm. silica gel G/F thin-layer chromatoplates[5]. The TLC plates are developed in a vapor-saturated chamber containing ethyl acetate–methanol–ammonium hydroxide (90:10:1) until the solvent front has ascended 12–15 cm. The plates are air dried after development, sprayed with 0.1 *N* H_2SO_4 followed by the mercuric acetate–acetic acid reagent, and then heated for 10 min. in an oven at 100°. The parent drug (II) is located as a highly fluorescent (blue-green) spot (R_f 0.4–0.5) by reference to the R_f of an authentic standard of 1.0 mcg. of II run alongside the sample extracts. All drug-related components fluoresce with characteristically different colors when viewed under longwave UV light, especially the polar metabolites seen at the origin and slightly above it.

The major urinary metabolites, the *N*-desmethyl analog (III, R_f 0.10), the *O*-desmethyl analog (IV, R_f 0.20), and the tetrahydro-8-isoquinolinol analog (V, R_f 0.08) can be readily identified by the very distinctive colors of the fluorophores formed on the TLC plate after the initial oxidative spray treatment and by comparison with the R_f values of authentic standards run alongside the sample extracts.

Scrape the silica gel from the fluorescent areas on the plate corresponding to II into 15-ml. conical centrifuge tubes, add 3 ml. of 0.1 *N* H_2SO_4, and slurry on a supermixer[7] for 1 min. to dissolve the compound in the acid phase. Wash the acid phase with 5 ml. of ether by slurring on the supermixer for 1 min., centrifuge, and then remove the ether layer by aspiration. If the acid phase does not appear clear (water white) when viewed against the light, repeat the ether wash. Transfer a 2-ml. aliquot of the ether-washed sulfuric acid solution into another 15-ml. centrifuge tube, taking care not to disturb the silica gel at the bottom of the 15-ml. tube. Proceed as described for blood to complete the chemical and photochemical oxidation process for fluorophore formation and quantitate fluorometrically as described for blood.

Determination of Percent Recovery of II from Blood and Urine—The recovery of internal standards of II may be determined by comparison of the corrected fluorescence of the internal standard against that of an external standard as described earlier for I.

RESULTS AND DISCUSSION

Tertiary tetrahydroisoquinolines are basic compounds and are extracted into polar solvents such as ether or ethyl acetate at a pH greater than 9.0. Compounds I-HCl and II-HCl were quantitatively extracted from blood or urine into ether at pH 9.0 or above as their respective free bases and back-extracted into dilute acid (0.1 *N* H_2SO_4 or HCl). This property was used as an effective "cleanup" step prior to quantitation. Both compounds have sufficient UV absorbance in dilute acids for quantitation with sensitivity in the microgram range. However, much higher sensitivity is achieved by fluorometric determination of these compounds as their isoquinolinium derivatives.

Compound I-HCl—Studies on the *in vitro* metabolism of I-HCl by Schwartz and Taylor[11], using the 9000 × *g* microsomal fraction of liver from phenobarbital-pretreated rats, indicated a moderate conversion of I to a fluorescent metabolite. This metabolite was isolated and characterized by mass and UV spectrometry as having a structure compatible with 1-isoquinoline acetamide (I-A). An authentic reference sample was synthesized by Schenker[12] and was used to confirm the identity of the metabolite by TLC migration (R_f) values. Further metabolic studies on orally administered I in the monkey showed that the major component in the blood was not the intact drug but was a metabolite (I-A) whose fluorescent properties were similar to that of photochemically oxidized I dissolved in 30% trichloroacetic acid. Authentic I-A was also used as a reference standard in the studies on elucidating the chemical nature of the fluorophore of I.

Characterization of Fluorophore of I—The characterization of the chemical nature of the photochemically produced fluorophore of I was undertaken using UV absorption spectra, activation–emission spectra, TLC, and differential pulse polarographic techniques. It was noted initially that I-HCl dissolved in 0.1 *N* HCl or in 30% trichloroacetic acid exhibited weak intrinsic fluorescence at 380 nm., activating at 335 nm. The fluorescence yield was greatly increased by irradiating I-HCl in the high intensity light from a lamp[8]. The fluorescence yield after irradiation in 30% trichloroacetic acid was about 20 times greater than that produced in 0.1 *N* HCl. The free base I dissolved directly in 30% trichloroacetic acid after irradiation produced 2.7 times the fluorescence yield of I-HCl dissolved in the same medium. This indicated that I is more amenable to photochemical oxidation than is I-HCl.

It was also noted that I-A in 0.1 *N* HCl or 30% trichloroacetic acid exhibited strong intrinsic fluorescence at 380 nm., activating at 335 nm., and that the fluorescence yield in 30% trichloroacetic acid was about four times greater than that in 0.1 *N* HCl. These observations indicated that 30% trichloroacetic acid was the medium of choice for effecting the optimal photochemical conversion of I to its fluorophore and also for measuring the optimal fluorescence yield. The fact that the activation–emission spectral characteristics

[11] M. A. Schwartz and M. K. Taylor, Hoffmann-La Roche, Inc., Nutley, N. J., unpublished data on file, 1967.

[12] F. E. Schenker, Department of Chemical Research, Hoffmann-La Roche Inc., Nutley, N. J., unpublished data, 1967.

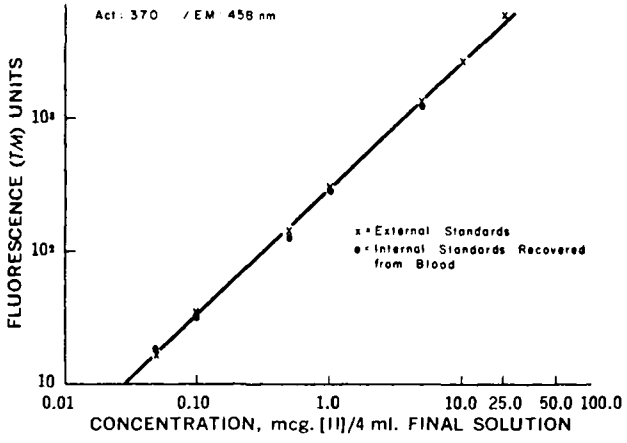


Figure 2—*Linear dynamic range of fluorescence* versus *concentration of external standards and blood-recovered internal standards of II after chemical and photochemical oxidation to their isoquinolinium derivatives.*

of I-A and the photochemically produced fluorophore of I in 30% trichloroacetic acid were identical indicated that I was oxidized (dehydrogenated) to produce I-A (Scheme I). The ratio of the fluorescence yields for equivalent (microgram) amounts of the two compounds (Fig. 1) indicated a conversion efficiency of about 20–25% at low concentrations of I (0.1–1.0 mcg./4 ml.), which increased to about 40–45% at higher concentrations (5–15 mcg./4 ml.). Although the photochemical conversion of I to I-A is not quantitative, it is sufficiently reproducible and linear to be analytically usable.

Further characterization of the fluorophore I-A was effected by a comparison of the UV absorption spectra of I, I-A, and the fluorophore produced by the photochemical oxidation of I. The UV absorption spectra of the two compounds in 0.1 *N* HCl (Fig. 3) show a single absorption maximum for I at 282 nm. (A/mcg./ml. = 0.052), whereas I-A has two absorption maxima, an intense one at 228 nm. (A/mcg./ml. = 0.260) and a weak one at 335 nm. which also corresponds to the wavelength of activation for fluorescence measurements. The reaction products of photochemically oxidized I were extracted into ether (after making the medium strongly alkaline with sodium hydroxide) and back-extracted into 0.1 *N* HCl, and its UV spectrum was determined. The UV spectrum indicated that the major peak of I at 282 nm. had decreased significantly, and absorption maxima at 228 and 335 nm. similar to those of I-A were observed. The reaction products were also analyzed by two-dimensional TLC (Fig. 4), which indicated that the major reaction product had the same R_f as I-A and also exhibited strong intrinsic fluorescence under longwave UV light when the plate was sprayed with 30% trichloroacetic acid. In addition to some unconverted parent compound, a second minor component migrating lower than I-A was also seen. This component showed weak intrinsic fluorescence in 30% trichloroacetic acid and was also present in

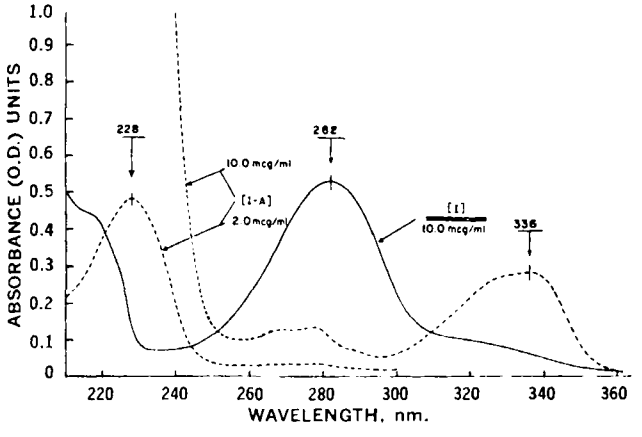


Figure 3—*UV absorption spectra of I and I-A in 0.1* N *HCl.*

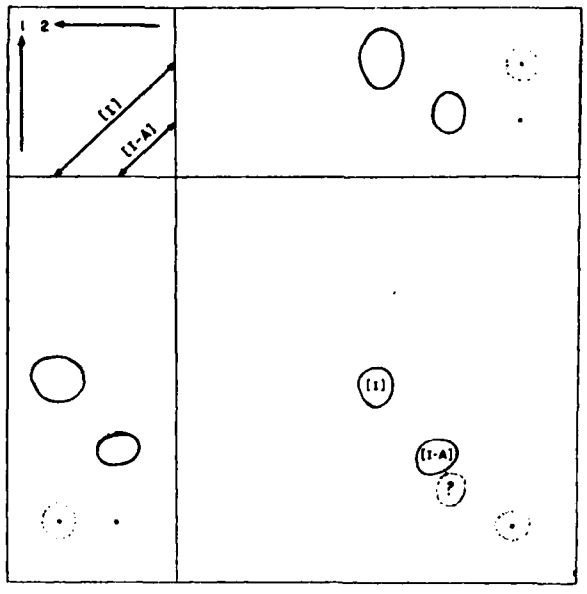


Figure 4—*Two-dimensional TLC of the photochemical oxidation products of I in 30% trichloroacetic acid. Key: 1, ethyl acetate–methanol–ammonium hydroxide (95:4:2); and 2, ethyl acetate–chloroform–ethanol (40:40:20).*

trace amounts when I-A dissolved in 30% trichloroacetic acid was irradiated for over 60 min. It appears to be a breakdown product of I-A produced by prolonged irradiation.

Electrochemical studies on the conversion of I to I-A, using differential pulse polarographic analysis (24), revealed that I-HCl gave a distinct peak at −0.700 v. *versus* standard calomel electrode (SCE) in 1.0 *N* HCl; this was attributed to the reduction of the azomethine (>C=N–) bond (25-27) in the isoquinoline ring. The peak also showed a characteristic pH shift to more negative potential with decreasing acidity from −0.700 v. *versus* SCE in 1.0 *N* HCl to −0.750 v. *versus* SCE in 0.1 *N* HCl and −0.990 v. *versus* SCE in pH 7.0 (1.0 *M* KCl/0.1 *M* PO_4) buffer solution, respectively (Fig. 5*a*). The isoquinolinium derivative (I-A), on the other hand, gave no reduction waves in any of the above media. Similar loss of electrochemical activity was reported for quinoline and isoquinoline in aqueous supporting electrolytes when compared with either nonaqueous or mixed electrolytes (28, 29) and was attributed to the adsorption of the electroinactive substance on the electrode surface. However, I-A gives a well-defined polarographic peak at −1.90 v. *versus* SCE in 95% dimethylformamide–5% water, which is about three times the height as that obtained with an equivalent concentration of I-HCl [E_p (peak potential) = −2.19 v. *versus* SCE) in the same supporting electrolyte. The E_p value for I-A is in close agreement with that reported for isoquinoline (−1.84 v. *versus* SCE) in the same electrolyte (30). Analogous electrochemical activity was noted with the products of the photochemical oxidation of I in 30% trichloroacetic acid.

These experiments demonstrated that I irradiated in 30% trichloroacetic acid, was photochemically oxidized (dehydrogenated) to 1-isoquinoline acetamide (I-A), the major fluorophore produced in this reaction.

Recovery of I and I-A—The direct assay procedure for I gave an overall recovery of 86 ± 5.0% (Table III). Since the TLC separa-

Table III—Overall Recovery Data on I-HCl, I-A, and II-HCl from Blood and Urine

Compound	Blood		Urine	
	Direct Analysis	With TLC	Direct Analysis	With TLC
	Overall Percent Recovery			
I-HCl	86 ± 5.0	63 ± 4.0	≃61	45 ± 6.0
I-A	≃57	41 ± 3.0	≃55	37 ± 5.0
II-HCl	89 ± 5.0	≃60	≃55	33 ± 5.0

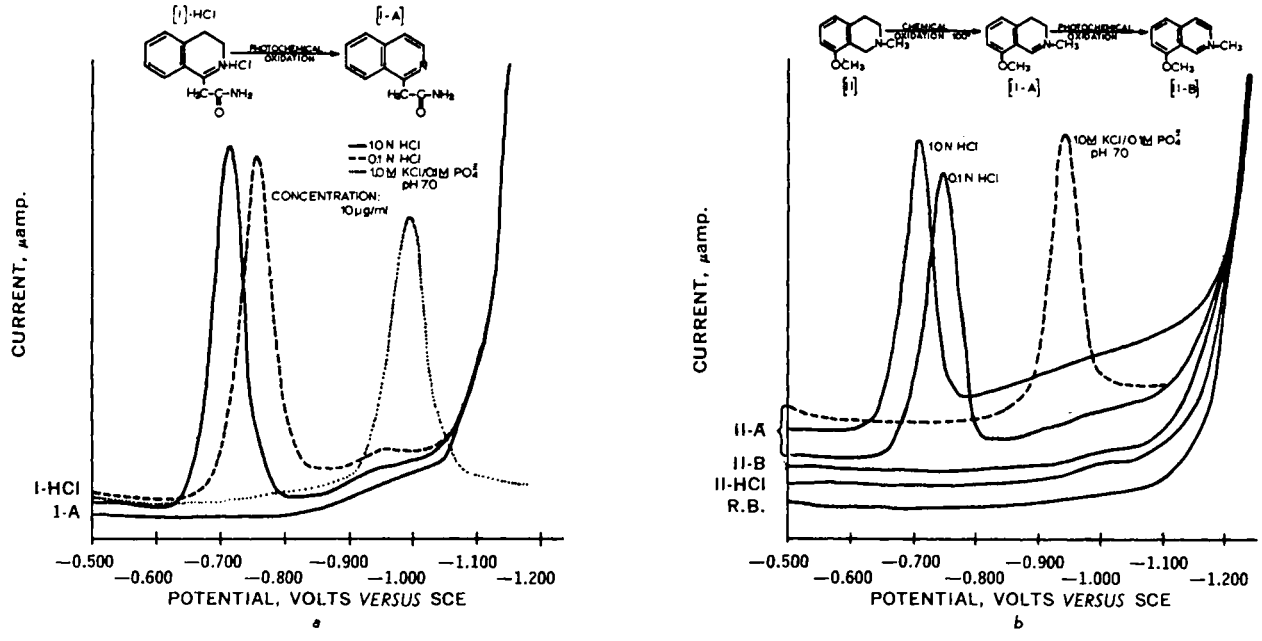


Figure 5—(a) *Differential pulse polarograms of I-HCl and I-A.* (b) *Differential pulse polarograms of II-HCl and the products of chemical oxidation (II-A) and the combined chemical and photochemical oxidations (II-B).*

tion step is essential to ensure the specificity of the assay for the intact drug (I) and its metabolite (I-A) in both blood and urine, the overall recovery of both compounds was determined after inclusion of the TLC separation step. The overall recovery from blood of I was 63 ± 4.0%, while that of I-A was 41 ± 3.0%. The overall recoveries of I and I-A from urine were 45 ± 6.0 and 37 ± 5.0%, respectively. The sensitivity limits of the assay were of the order of 0.25–0.50 mcg. I and 0.25 mcg. I-A/ml. of blood and 0.75–1.00 mcg. I and 0.50 mcg. I-A/ml. of urine, respectively.

Compound II-HCl—Compound II-HCl is a weak base (pKa = 8.2), and it is quantitatively extracted as its free base into ether from alkalinized blood or urine and is back-extracted into 0.1 *N* H_2SO_4 or 0.1 *N* HCl. The compound exhibits UV maxima at 272 and 278 nm. of about equal intensity (*A*/mcg./ml. = 0.077). Following chemical oxidation in the mercuric acetate–acetic acid–sulfuric acid mixture, a significant hypsochromic shift of the UV maxima was observed together with a significant hyperchromic effect. The UV absorption maxima shifted to 264 and 267 nm., indicative of aromatization, and the absorbance at either wavelength (*A*/mcg./ml. = 0.184) also increased twofold over that seen at 272 and 278 nm. Irradiation for 15 min. with UV light[8] produced no further shifts in the UV maxima. The absorbance, however, showed a further increase to 3.5 times that of the parent compound.

The reaction products of the chemical oxidation step and the combined chemical–photochemical oxidation process were also examined by differential pulse polarography in the same aqueous supporting electrolytes as were used for I-HCl. The polarograms shown in Fig. 5*b* indicate that the parent compound (II-HCl) has no intrinsic electrochemical activity. The product of chemical oxidation, II-A, showed peaks typical of a 3,4-dihydroisoquinoline and were almost superimposable on those shown by I-HCl (Fig. 5*a*). However, the isoquinolinium derivative (II-B) formed by the combined chemical and photochemical oxidation reaction showed no activity as did I-A. Attempts to study these reactions in 95% dimethylformamide–5% water were unsuccessful due to trace amounts of coextracted impurities from the reaction medium which interfered with the peaks in the potential region of from −1.80 to −2.00 v. *versus* SCE.

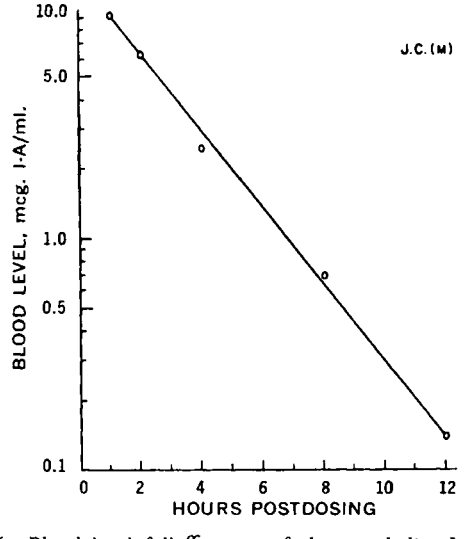


Figure 6—*Blood level falloff curve of the metabolite I-A in man following a single, 1000-mg. oral dose of I-HCl.*

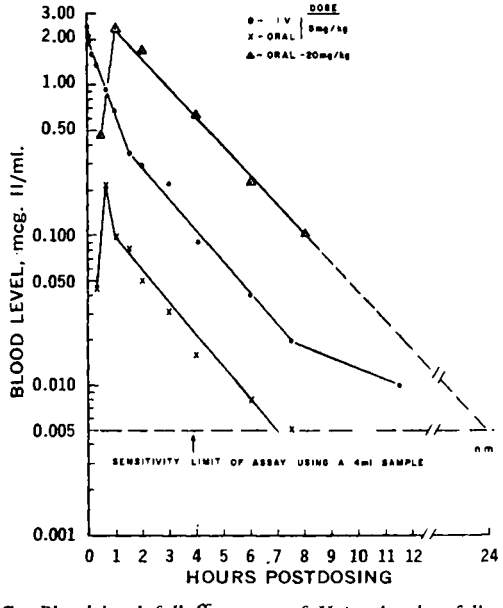


Figure 7—*Blood level falloff curves of II in the dog following intravenous and oral administration.*

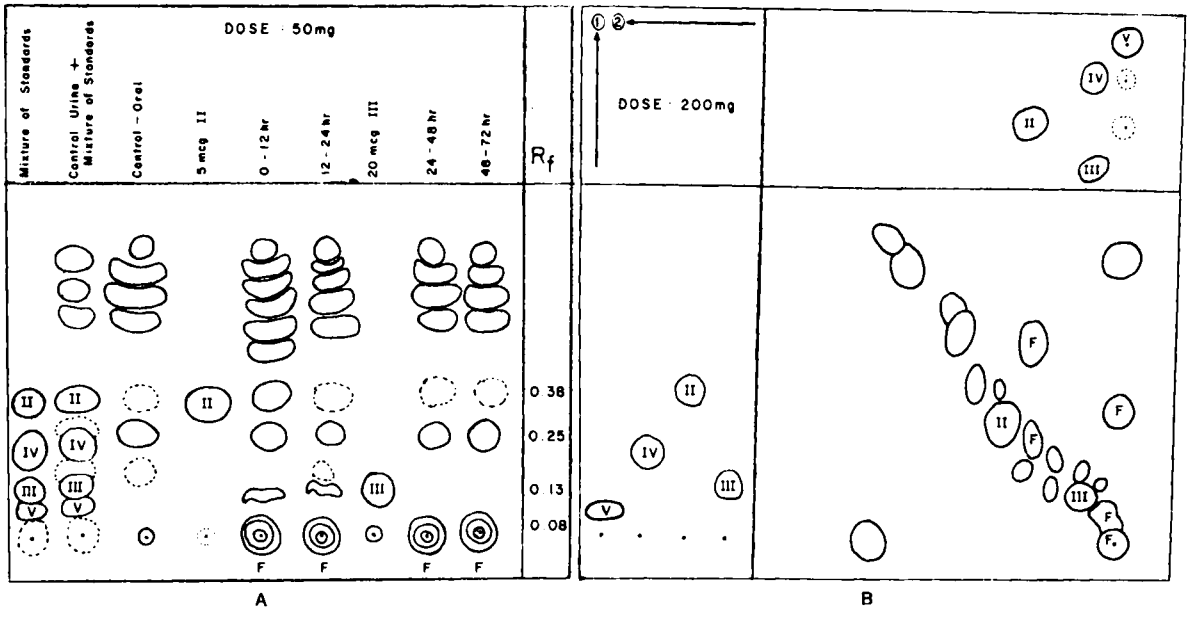


Figure 8 *Thin-layer chromatograms of the direct ether extracts of urine (made strongly alkaline, pH 14) from a dog following oral administration of II. F = fluorescent metabolites. Key: A, developed in one dimension; solvent system of ethyl acetate–methanol–ammonium hydroxide (90:10:1); B, developed in two dimensions; solvent system of (1) ethyl acetate–methanol ammonium hydroxide (90:10:1) or (2) benzene–methanol–glacial acetic acid (90:10:1).*

Polarographic and spectrophotometric studies on the analogous chemical reduction products by partial hydrogenation of isoquinoline to 3,4-dihydroisoquinoline and of 3,4-dihydropapaverine to papaverine also were reported (31) and showed similar stepwise changes in electrochemical activity.

Further characterization of the reaction products of II following either chemical oxidation or the combined chemical–photochemical oxidation reaction was obtained using TLC. The reaction products were extracted at alkaline pH into ether, analyzed by one-dimensional TLC in ethyl acetate–methanol ammonium hydroxide (90:10:1), and examined under longwave UV light. Under both sets of conditions, two fluorescent spots were present, one at the origin and the other close to the solvent front (R_f 0.90), in addition to some residual II (R_f 0.50). The fluorescence of the two spots was of about equal intensity following only chemical oxidation, whereas the fluorescence of the lower spot increased several fold in intensity following the combined oxidation procedure. The two fluorescent spots were eluted into 0.1 *N* H_2SO_4 and scanned for their spectral characteristics. The two compounds had identical UV absorption and activation–emission spectra, indicating that these reaction products are a mixture of fluorophores whose "total" fluorescence in solution is apparently reproducible and analytically acceptable.

Similar reactions were observed with the other tetrahydroisoquinoline analogs (III, IV, and V) shown in Table II. However, the fluorescence yield decreased markedly with the removal of the methyl group in either or both positions of II. Thus, the fully aromatized isoquinoline derivatives of II showed the highest fluorescence (at 370/458 nm.), the fluorescence yield being over 25 times greater than that of the *N*-desmethyl analog (III) at the same wavelengths. Of the two phenolic analogs, the isoquinoline derivative of the *O*-desmethyl analog (IV) exhibited about 2.5 times more fluorescence (at 425/525 nm.) than III (at 370/458 nm.), while the *N*- and *O*-desmethyl analog (V) had the weakest fluorescence (at 425/525 nm.) of them all. Thus, the fluorophores produced by equiv-

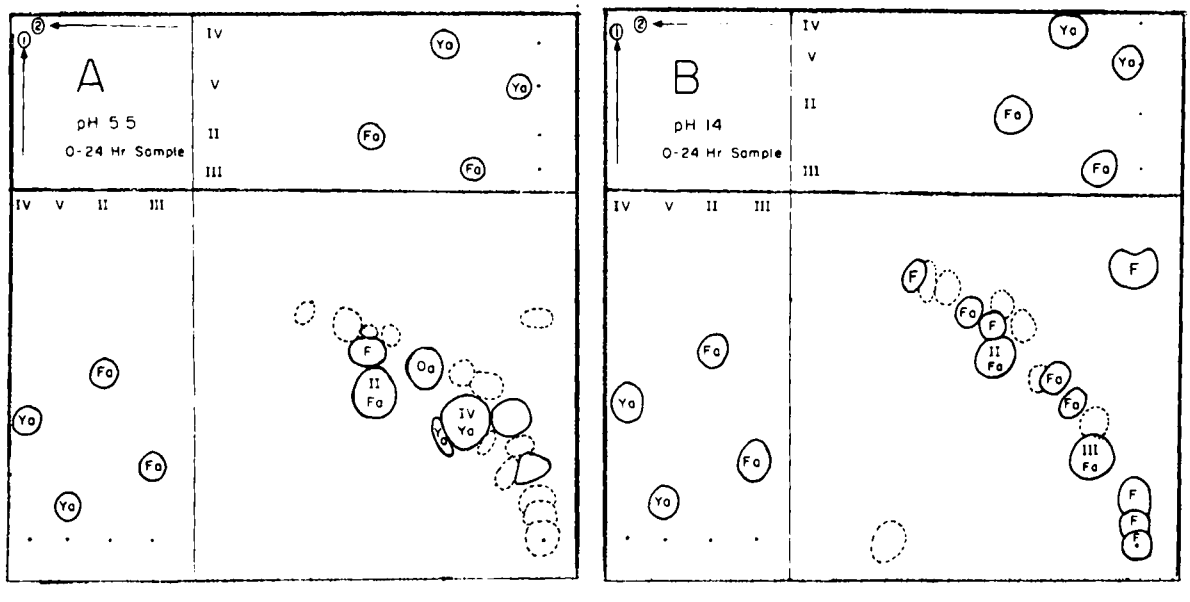


Figure 9 *Two-dimensional thin-layer chromatograms of ether extracts of dog urine after enzyme incubation following oral administration of a 20-mg./kg. dose of II. Key: solvent system: 1, ethyl acetate–methanol–ammonium hydroxide (90:10:1); and 2, benzene–methanol–glacial acetic acid (90:10:1). F = fluorescent before and after oxidation, Fa = fluorescent only after oxidation, Ya = yellow colored only after oxidation, and Oa = orange colored only after oxidation. A = acidic pH 5.5 extract, and B = basic pH 14 extract.*

alent (microgram) amounts of the respective tetrahydroisoquinolines (read at their respective wavelengths of maximum activation–emission) arranged in decreasing order of fluorescence emission are II > IV > III > V. These studies showed that in the overall oxidation process the first step (chemical oxidation) introduces a double bond in the 1,2-position of the tetrahydroisoquinoline ring while the second step (photochemical oxidation) introduces a second double bond in the 3,4-position to produce the fully aromatized isoquinolinium derivative (Table II). These derivatives exhibit high UV absorption and fluorescence in acidic media due to the increase in the aromaticity in the molecule.

Recovery of II from Blood and Urine—Recovery experiments with II from blood and urine indicated that the overall recovery from blood using the direct assay procedure was 89 ± 5.0% (Table III) while that from urine, which included the TLC separation step, was significantly lower (33 ± 5.0%).

The sensitivity limits of the assay for blood and urine are of the order of 0.05–0.10 mcg. II/ml. and can be extended to 0.005 mcg./ml. of blood using a 4-ml. specimen. The linear range of fluorescence *versus* concentration of oxidized II as external standards and as internal standards recovered from blood is of the order of 0.05–25.0 mcg. II/4 ml. of final solution (Fig. 2).

Application of Methods to Biological Specimens—*Compound I-HCl: Blood Levels in Man following a Single 1000-mg. Oral Dose*—A pilot blood level study was performed with one volunteer who was administered a single dose of 1000 mg. of I-HCl orally. Whole blood, oxalated, was drawn at 0, 1, 2, 4, 8, 12, 24, 48, 72, and 96 hr. after administration and was analyzed. No measurable levels of the intact drug (I) were seen at any time point. Measurable levels of a metabolite I-A were seen; however, the peak blood level of 9.0 mcg./ml. at 1 hr. declined rapidly within 12 hr., with an apparent half-life of about 2 hr. After 12 hr. the metabolite levels were no longer measurable (Fig. 6). The data indicate rapid absorption of I-HCl and rapid biotransformation to I-A.

Specificity of Assay for I and Characterization of Blood Metabolite I-A in Man and Monkey—Blood ether extracts from man and monkey following oral administration of I-HCl were analyzed by one-dimensional TLC in ethyl acetate–methanol–concentrated ammonium hydroxide (95:4:2). The chromatoplate did not show the presence of any intact drug (I, R_f 0.60). The major component was a metabolite seen in both man and monkey blood whose R_f 0.28 was identical with that of authentic I-A chromatographed alongside the sample extracts. This TLC step, which is also used in the assay, ensures its specificity for I and I-A. The metabolite and compound I-A, when cochromatographed, ran as one spot having the same R_f as the reference standard, indicating that the two compounds were identical. Thus, I-A was identified as the major ether-extractable metabolite of I-HCl in the blood of man and in the monkey. On-going studies[13] on the metabolism of I-HCl in man and in the dog also indicate extensive metabolism, resulting in several polar metabolites present in blood and urine which can be analyzed by selective solvent extraction and TLC separation

Compound II-HCl—Blood levels in the dog were determined in one animal following the administration of a 5-mg./kg. (total 50-mg.) dose of II-HCl by intravenous and oral routes and in another animal following a single oral 20-mg./kg. (total 200-mg.) dose. The blood level falloff curves are shown in Fig. 7. Following the 5-mg./kg. i.v. dose, a biphasic falloff pattern in the blood levels of II was seen, with the α-component (distribution phase) showing a rapid apparent half-life of 40 min., while the β-component (elimination phase) showed an apparent half-life of 1.4 hr.

Following oral administration of the 5-mg./kg. (50-mg.) dose, the blood levels of II appeared to reach a peak level (0.22 mcg./ml.) at 40 min. followed by a progressive decline, with an apparent half-life of 1.5 hr. between 1.0 and 7.5 hr. The blood level falloff data determined following a single 20-mg./kg. (200-mg.) oral dose indicated a peak blood II level of 2.33 mcg./ml. 1 hr. after dosing, which declined to nonmeasurable levels in 24 hr., again with an apparent half-life of 1.5 hr.

The recovery of the intact drug into ether from dog urine made alkaline (pH 14) was very low. After the 5-mg./kg. i.v. dose, only 1.5% of the drug was recovered in the 0–24-hr. period; following the 5- and 20-mg./kg. oral doses, only 0.21 and 3.9% of the respective administered doses were recovered in the same excretion period. Again, TLC of the direct ether extracts of urine (Figs. 8A and 8B) indicated the presence of the intact drug, the *N*-desmethyl analog (III), and several highly fluorescent, incompletely resolved compounds near the origin.

The 0–24-hr. urine sample from the dog dosed with 20 mg./kg. following enzyme incubation at pH 5.5 was extracted at pH 5.5 and 14 and analyzed by two-dimensional TLC. The chromatogram of the pH 5.5 ether extract (Fig. 9A) indicated the presence of the parent drug (II), the phenolic analog (IV), and three unidentified acidic metabolites. The pH 14 ether extract (Fig. 9B) indicated the presence of II, the *N*-desmethyl analog (III), and at least eight other discernible basic metabolites which were strongly fluorescent either intrinsically or after oxidation with mercuric acetate–acetic acid. The basic metabolites showed a typical blue-green isoquinoline-like fluorescence similar to that of oxidized II, whereas the acidic phenolic metabolites IV and V showed characteristic yellow- or orange-colored fluorescence after oxidation.

The highly fluorescent metabolites seen at the origin of the TLC plate were those present in the most significant amounts, as indicated by the intrinsic fluorescence obtained upon their elution from silica gel into 4 ml. of 0.1 *N* H_2SO_4 and measurement of the fluorescence at the same wavelengths as for oxidized II. Based on the assumption that the specific intrinsic fluorescence of these metabolites is approximately the same as that of oxidized II, the total amount of the fluorescent metabolites recovered from the chromatoplate represents about 37% of the dose. The intact drug (II) recovered from both acidic and basic extracts accounted for about 8% of the administered dose. These fluorescent metabolites appear to be chemically similar to the isoquinoline derivatives of II (oxidized) but have yet to be characterized. The percent of the dose recovered as the acidic phenolic metabolites also appears to be considerable. It is conservatively estimated that more than 50% of a given dose can be accounted for in the urine over a 24-hr. excretion period, indicating rapid and extensive biotransformation of the drug.

Blood levels of II were determined in man (17) during the clinical evaluation of the drug, when doses ranging from 50 to 300 mg. were administered orally. Peak blood II levels were seen 1–2 hr. after dosing, followed by a rapid decline in blood levels over the next 10 hr. The urinary excretion data indicated extensive biotransformation of the drug with less than 1% of the orally administered dose recovered in the 0–24-hr. period (17).

Specificity of the Assay—The blood level assay is apparently specific for the intact drug (II) after single doses in the dog, since only the intact drug was seen in the blood ether extracts analyzed by TLC. Chronic administration to dogs at high doses (20 mg./kg.), however, showed the presence of trace amounts of III and another unidentified fluorescent metabolite[14]. This would necessitate the use of TLC to establish the specificity of the assay in blood for II and its metabolites. In the direct assay in blood, small amounts of III would not seriously interfere with the assay values for II because of the 25-fold difference in the fluorescence intensities of the isoquinolinium derivatives of II over III. However, the highly fluorescent isoquinoline-like metabolites seen in urine, if present in blood following chronic administration, would have to be separated by TLC prior to the determination of II.

The urine assay requires the use of the TLC separation step to resolve the parent drug (II) from the many metabolites present, especially the highly fluorescent isoquinoline-like metabolites which have similar fluorescence properties as that of oxidized II.

CONCLUSION

The chemical oxidation of tertiary tetrahydroisoquinolines is a useful general reaction for converting these compounds to their 3,4-dihydroisoquinoline derivatives (18–20). These derivatives can be further oxidized by photochemical oxidation by exposure to UV radiation in acidic media to yield the fully aromatized isoquinolinium derivatives, which are highly fluorescent and amenable to sensitive quantitation in the nanogram range. Both principles have been effectively used in developing assays for the spectrofluorometric quantitation of these types of compounds in biological fluids. Photochemical methods are useful on the microscale for

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derivatization reactions and are also used extensively in organic synthesis (32), especially in the preparation of isoquinoline alkaloids (33).

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Serum Protein Binding of Erythromycin, Lincomycin, and Clindamycin

R. C. GORDON, C. REGAMEY, and W. M. M. KIRBY▲

Abstract □ Serum protein binding determinations for erythromycin, lincomycin, and clindamycin utilizing ultrafiltration of serum containing antibiotic in a concentration of 5 mcg./ml. and tube dilution techniques revealed a high degree of binding: erythromycin base, 73%; erythromycin propionate, 93%; lincomycin, 72%; and clindamycin, 94%.

Keyphrases □ Erythromycin and erythromycin propionate—serum protein binding □ Lincomycin—serum protein binding □ Clindamycin—serum protein binding □ Antibiotics—serum protein binding of erythromycin, lincomycin, and clindamycin □ Serum protein binding—erythromycin, lincomycin, and clindamycin

Serum protein binding of antibiotics is of potential clinical significance since bound antibiotic has been found to have no antibacterial activity and it is probable that the level of free antibiotic in the tissues is no greater than the peak level in the blood (1). The extent of protein binding of erythromycin, lincomycin, and