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TLC Determination of Iodochlorhydroxyquin and Its Conjugate in Plasma

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Abstract ◻ A simple, specific, reliable, and sensitive method for the determination of iodochlorhydroxyquin and/or its conjugate in biological fluids is described. The method is based on a quantitative ether–acetone (1:1) extraction of plasma samples followed by TLC separation, visualization, elution, and determination at 267 nm. Iodochlorhydroxyquin released by hydrolysis of its conjugate was analyzed. Both compounds are detectable in amounts as low as 0.04 μg/ml. Application of the one-compartment open model to the data (assuming the biotransformation of the drug in conjugated form) provides a pharmacokinetic profile for the 50-mg/kg dose of iodochlorhydroxyquin in Wistar male rats.

Keyphrases ◻ Iodochlorhydroxyquin—and conjugate, TLC analysis, biological fluids ◻ TLC—analysis, iodochlorhydroxyquin and conjugate, biological fluids ◻ Antiamebic agents—iodochlorhydroxyquin, TLC analysis, biological fluids

Iodochlorhydroxyquin (I) has been widely used for over 30 years to treat various intestinal and vaginal infections. In view of debatable evidence of its neurotoxicity (1, 2), it is surprising that a method for the determination of I and/or its conjugate (II) in blood is not available. This paper describes a fast and simple TLC procedure for the detection of I and II in biological fluids and its use in pharmacokinetic studies.

EXPERIMENTAL

Reagents and Materials—Compound I was used as received[1]. All solvents were analytical reagent grade[2], and the developing solvent [1-butanol-acetone-diethylamine-water (30:20:4:30)] was prepared fresh daily. Silica gel plates, 250 μm thick, were activated at 105° for 1 hr[3].

Instrumentation—A 250-nm UV lamp[4], grating spectrophotometer[5], and 1-cm quartz microcells[6] were used.

Standard Preparation—Compound I was dissolved in acetone or 3 *N* HCl. Aliquots equivalent to 1, 2, 4, and 6 μg were placed in glass-stoppered centrifuge tubes and evaporated to dryness under a gentle stream of nitrogen. Plasma, 1 ml, from untreated rats was added to each tube and mixed well. These standards and the appropriate blanks were handled in the same manner as described for the plasma specimens.

Sample Preparation—One volume (1–5 ml) of plasma from treated animals and five volumes of 0.1 *N* NaOH were introduced into a 100-ml separator. Compound I was extracted with two 10-volume portions of ether–acetone (1:1 v/v).

Free I—The ether–acetone extracts were pooled, washed with five volumes of 0.1 *N* NaOH and five volumes of water, dried with sodium sulfate, and flash evaporated to about 5 ml. This final amount as well as the ether–acetone washes was transferred to a glass-stoppered centrifuge tube and evaporated to dryness under a gentle stream of nitrogen. The standards and the appropriate blanks were handled in a similar manner.

Compound II—The water phase was heated with one volume of 8 *N* HCl in a boiling water bath for 40 min, and three volumes of 3 *N* NaOH were added to the mixture. After the mixture was cooled at room temperature, I was released by hydrolysis and treated as described under *Free I*.

TLC—Each sample was taken up in 0.1 ml of acetone. Aliquots of 0.09 ml were spotted 2 cm from the edge of the chromatographic plate. Aliquots equivalent to 1.1, 2.2, 4.4, and 6.6 μg of I standard acetone solution were also spotted on the plate.

The chromatograms were developed in an ascending system of 1-butanol-acetone-diethylamine-water (30:20:4:30) in a saturated atmosphere. After 90 min, the solvent front was marked and the plates were dried at room temperature. Spots of I, visualized by exposure to UV light, were scraped into glass-stoppered centrifuge tubes, eluted with 0.6 ml of 3 *N* HCl, and shaken every 15 min for 45 min. A similar spotless area (blank) was scraped off and subjected to an identical procedure.

The test tubes were then centrifuged at 4000 rpm for 10 min, and the relative absorbance of the supernates was measured at 267 nm against a blank.

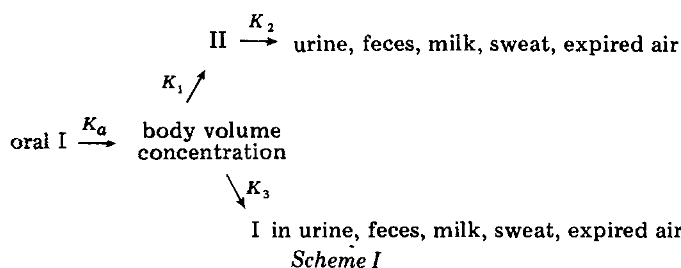
Calculations—Calibration curves were prepared by plotting absorbance at 267 nm against a known concentration of I in either plasma or 3 *N* HCl. Values for the unknown concentration of I in plasma specimens were calculated from the slope of the standard curve.

Drug Administration to Rats—Male albino Wistar rats, 200–250 g, were divided into eight groups of three animals each. Compound I was suspended in an aqueous vehicle consisting of 0.9% sodium chloride, 0.4% polysorbate 80, 0.5% carboxymethylcellulose sodium, and 0.9% benzyl alcohol and administered by gavage at a dose of 50 mg/kg in a constant volume of 5 ml/kg. Blood samples were obtained by cardiac puncture by means of a heparinized syringe at time intervals ranging from 30 min up to 24 hr after drug administration. Plasma samples were obtained by centrifugation at 2000 rpm for approximately 10 min.

Application to Pharmacokinetic Analysis—Experimental data were fitted by the method of least squares (3) applied to each exponential segment of the blood level–time curve. The extent of biotransformation of I to II was measured by employing a one-compartment open model with extravascular drug administration. The blood concentration–time curve of I after oral administration of 50 mg/kg in male rats is represented by the following biexponential equation:

$$\mathrm{I} = I_0 \frac{K_a}{(K_a - K)}(e^{-Kt} - e^{-K_a t}) \qquad \text{(Eq. 1)}$$

[1] Gianni, Milan, Italy.
[2] Merck.
[3] Merck 60 F_{254}.
[4] Mineral Light UVS 11.
[5] Beckman DBG.
[6] Hellma 108/2 QS.



Scheme I

while that of the conjugate II is represented by the following triexponential equation:

$$II = I_0 K_a K_1 \left\{ \left[\frac{e^{-Kt}}{(K_a - K)(K_2 - K_a)} - \frac{e^{-K_a t}}{(K_a - K)(K_2 - K_a)} \right] + \frac{e^{-K_2 t}}{(K_2 - K_a)(K_2 - K)} \right\} \quad \text{(Eq. 2)}$$

where I_0 is the intercept of the back-extrapolated monoexponential overall elimination line or monoexponential absorption line with the ordinate; t is the time intervals following I administration; and K_a, K, K_1, and K_2 represent rate constants of I absorption, I overall elimination, II formation, and II elimination, respectively, calculated from the appropriate relationship (4).

The one-compartment body model is illustrated in Scheme I, where K_a, K, K_1, and K_2 are as defined and K_3 is the rate constant of I excretion in urine, feces, milk, sweat, and expired air. It follows that $K = K_1 + K_3$.

The goodness of fit of the one-compartment model was tested by the paired t test between the observed mean data at each time interval and the calculated value.

RESULTS

Evaluation of Assay Method—Known amounts of I were added to plasma or acetone. The percentage recovery of I from plasma compared to that from acetone directly spotted on the plate provides an accuracy index of 96.3% (Table I). This method is adequately specific, since it is unaffected by the presence of endogenous substances.

Preliminary chromatograms of I added to plasma indicate that a recovery of 100 ± 2.2% can be obtained following an elution time of 45 min. Compound I has an R_f value of 0.58. Due to a sufficiently high molar absorptivity ($E^{1\%}_{1\,cm}$ in 3 N HCl at 267 nm is 852), I can be determined in amounts as low as 0.04–0.2 µg/ml.

Ten determinations to assess the precision of the procedure were carried out on unconjugated portions of two plasma samples obtained at 2 and 4 hr following I administration (Table II). Statistical evaluation of the data provided the following values: relative mean standard deviation (RSD) = 4.0% and reproducibility limits = $\overline{X} \pm RSDt_{18,0.05} = \overline{X} \pm 8.4\%$ of $\overline{X}$. The time required to determine I and II concentrations in plasma varies according to number of samples, *i.e.*, about 4 hr for one and 1 day for 10 samples.

Pharmacokinetic Analysis—Experimental data shown in Fig. 1 were analyzed by using the proposed one-compartment open model; the calculated pharmacokinetic parameters are: K_a = 0.26396 hr^{-1}, K = 0.08994 hr^{-1}, K_1 = 0.07902 hr^{-1}, K_2 = 0.14238 hr^{-1}, and K_3 = 0.01092 hr^{-1}, with half-lives of 2.6, 7.7, 8.8, 4.9, and 63.6 hr, respectively.

Table I—Recovery of I Added to Rat Plasma or Acetone

Amount of I Added, µg/ml	Amount[a], µg/ml, Recovered from Plasma	Acetone
1	0.93	0.99
2	1.85	1.93
4	3.92	4.06
6	5.88	5.94
Recovery (mean ± SD), %	95.41 ± 3.11	99.12 ± 3.74

[a] Mean of three replications.

Table II—Assay Precision Evaluation[a]

Sample	2 hr	4 hr
Mean	0.58	1.30
SD	0.02	0.06
RSD	0.34	4.61

[a] See text for discussion.

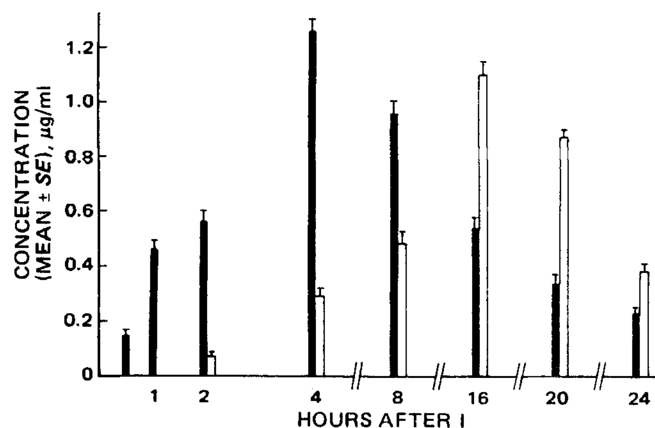


Figure 1—*Concentration of I in plasma of male rats at different time intervals after oral administration of 50 mg/kg. Key:* ■, *I; and* □, *II.*

The exponential functions representing the blood level–time curve of I and II are:

$$I = 3.11739\,(e^{-0.08994t} - e^{-0.26396t}) \quad \text{(Eq. 3)}$$

$$II = 4.63386\,e^{-0.08994t} + 1.99868\,e^{-0.26369t} - 6.63250\,e^{-0.142377t} \quad \text{(Eq. 4)}$$

The test for goodness of fit (t_{15}) = 0.63.

DISCUSSION

Because of insufficient sensitivity, previous methods (5–7) were limited to the determination of I and/or II in urine. The reported method is rapid, reliable, and sensitive enough for the determination of I and/or II in biological fluids of both experimental animals and humans. The usual therapeutic dosage of I ranges from 500 to 750 mg three times daily (8). Since intestinal absorption of labeled I amounts to 43.6% of the administered dose as calculated from urinary excretion during the 10–18-day observation period (9), plasma levels of I and/or II are well within the ranges of the sensitivity of this method.

The present method has two definite advantages: (a) its specificity, because I can be determined in amounts as low as those detectable by the radiochemical method (10); and (b) its usefulness, since I and II can be determined separately. This model provides a pharmacokinetic profile for this oral dose of I in rats; the K_a and K values indicate fairly rapid absorption and elimination rates.

The very small difference between K and K_1 suggests that biotransformation by the liver plays a major role in the process of irreversible drug elimination. Excretion of unaltered I in the urine, feces, expired air, milk, and sweat is insignificant.

Results of the goodness of fit test clearly indicate that the model is appropriate. It could be usefully employed clinically for: (a) estimation of drug absorption following oral administration or topical application and, therefore, indication of potential systemic toxicity (11); and (b) study of absorption and/or metabolic disposition of I as influenced by the presence of other substances (12).

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Phytochemical Investigation of *Adenium obesum* Forskal (Apocynaceae): Isolation and Identification of Cytotoxic Agents

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Abstract □ An ethanol extract of *Adenium obesum* exhibited cytotoxic activity against the human epidermoid carcinoma of the nasopharynx test system. Constituents in the active extract were identified as the cardenolides somalin, hongheloside A, 16-acetylstrospeside, and honghelin and the flavonol 3,3′-bis(*O*-methyl)quercetin. Their identities were proven by IR, UV, proton magnetic resonance, and mass spectrometry; elemental analyses; preparation of derivatives; and melting-point determinations. An inactive triterpene, dihydroifflaionic acid, and an inactive flavonol, 3-*O*-methylkaempferol, also were isolated and identified.

Keyphrases □ *Adenium obesum*—ethanol extract of aboveground parts, various cardenolides and flavonol isolated and identified, evaluated for cytotoxic activity □ Cardenolides, various—isolated from ethanol extract of aboveground parts of *Adenium obesum*, evaluated for cytotoxic activity □ Flavonols—3,3′-bis(*O*-methyl)quercetin isolated from ethanol extract of aboveground parts of *Adenium obesum*, evaluated for cytotoxic activity □ Cytotoxic activity—evaluated in various cardenolides and flavonol isolated from ethanol extract of aboveground parts of *Adenium obesum*

In the continuing search for plants having antitumor properties, an ethanol extract of the stems, leaves, and flowers of *Adenium obesum* Forskal (Apocynaceae)[1] exhibited cytotoxic activity against the human epidermoid carcinoma of the nasopharynx (KB) test system[2].

DISCUSSION

The chloroform fraction, obtained from an ethanol extract of the ground stems, leaves, and flowers of *A. obesum* by partitioning between chloroform and water, was systematically fractionated by solvent extractions. The highly active ether-soluble fraction, consisting of seven major constituents, was subjected to multiple column chromatography, preparative TLC, and fractional crystallizations.

The seven compounds were identified as the triterpene dihydroifflaionic acid, the flavonols 3,3′-bis(*O*-methyl)quercetin and 3-*O*-methylkaempferol, and the cardenolides somalin, hongheloside A, 16-acetylstrospeside, and honghelin by means of IR, UV, proton magnetic resonance (PMR), and mass spectrometry; elemental analyses; preparation of derivatives; and melting-point determinations. In addition, the structures of the flavonols were substantiated by microanalytical UV spectroscopy. Somalin and hongheloside A, previously isolated from other *Adenium* species (1–5), were hydrolyzed to their respective genins and carbohydrate moieties.

Somalin, honghelin, hongheloside A, 16-acetylstrospeside, and 3,3′-bis(*O*-methyl)quercetin exhibited cytotoxic activities at levels < 0.01, 0.02, 0.035, 0.035, and 3.08 μg/ml, respectively. Dihydroifflaionic acid and 3-*O*-methylkaempferol were inactive. Activity in the KB test system is defined as $ED_{50} \leq 20$ μg/ml (6).

EXPERIMENTAL[3]

Isolation Procedure—The stems, leaves, and flowers (15 kg) of *A. obesum* were ground to a powder and extracted exhaustively in a Lloyd-type extractor with petroleum ether and then with 95% ethanol. The air-dried ethanol extract (1.2 kg) was partitioned between chloroform (10 liters) and water (10 liters). The air-dried chloroform fraction (468 g) was stirred mechanically with petroleum ether three times, the residue (324 g) was stirred mechanically with acetone three times, and the air-dried filtrate was stirred mechanically with ether three times.

The ether-soluble fraction (100 g) was subjected to a silica gel G (1.5 kg) column (7 × 81 cm) eluted with petroleum ether–ether–chloroform (1:3:1). Fractions were collected with an automatic fraction collector (25 ml/tube) and combined according to their TLC profiles. The first fractions (A) contained dihydroifflaionic acid. The next set of fractions (B) consisted of a mixture of two flavonols. The third set (C) contained somalin. The fourth set (D) consisted mainly of hongheloside A.

The fifth fraction (E, 50 g) was rechromatographed on a silica gel 60 (1.2 kg) column (6 × 70 cm) eluted with chloroform–methanol–water (188:12:1). The first fractions consisted of mainly 16-acetylstrospeside, the middle fractions contained an overlap mixture, and the latter fractions contained honghelin.

Isolation of Dihydroifflaionic Acid—The air-dried residue of Fraction A was treated with petroleum ether. The resultant precipitate was filtered, washed with petroleum ether, dissolved in chloroform–petroleum ether (1:1), and stored at 5° overnight. The resultant powder was filtered and subsequently crystallized from methanol to yield stout white crystals of dihydroifflaionic acid, mp 259–260° [lit. (7) mp 260°]; mass spectrum: *m/e* 456 (M^+) and 248 (base); IR[4]: ν_{max} 3400–3500, 1700, 1450, 1390, 1280, 1020, and 990 cm^{-1}; PMR[4]: δ 4.50 (m, 1H), 3.42 (broad

[1] Identification was confirmed by Dr. Robert E. Perdue, Chief, Medicinal Plant Resources Laboratory, U.S. Department of Agriculture, Beltsville, Md., where a reference specimen (PR 26176) is maintained. The plant was collected in the Kilifi District of Kenya by R. W. Spjut and P. D. Ensor in November 1972.
[2] Data on the cytotoxic activity were provided through the courtesy of the Divison of Cancer Treatment, National Cancer Institute, National Institutes of Health, Bethesda, Md.

[3] Carbon and hydrogen analyses were performed by Chemalytics, Inc., Tempe, Ariz. PMR, IR, UV, and mass spectral data were determined using a Varian T-60 spectrophotometer, a Beckman IR-33, a Beckman DB-G spectrophotometer, and a Hitachi Perkin-Elmer double-focusing spectrometer (model RMU-6E), respectively. Melting points were determined on a Kofler hot-stage apparatus and are uncorrected.
[4] These data were not reported previously.