High-Performance Liquid Chromatographic Analysis of Iodochlorhydroxyquin in Plasma

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Abstract □ A simple isocratic high-performance liquid chromatographic procedure for the analysis of iodochlorhydroxyquin in human plasma is described. Protein was precipitated using perchloric acid, and the supernatant and precipitated protein fractions were extracted with ether. The ether phases were evaporated to dryness, reconstituted in mobile phase, and chromatographed. A reversed-phase microparticulate C_{18} column, a precolumn, and a UV detector at 256 nm were used. A mobile phase containing 80% methanol and 20% 0.05 *M* phosphoric acid was employed at a flow rate of 1 ml/min. Quantitation of iodochlorhydroxyquin in the 1–15-μg/ml range in human plasma was demonstrated with a coefficient of variance of 0.1–0.06. Hydrocortisone, which is used in combination with iodochlorhydroxyquin in ointments and creams, does not interfere in the assay.

Keyphrases □ Iodochlorhydroxyquin—high-performance liquid chromatographic analysis, plasma □ Anti-infectives—iodochlorhydroxyquin, high-performance liquid chromatographic analysis, plasma □ High-performance liquid chromatography—analysis, iodochlorhydroxyquin, plasma

Iodochlorhydroxyquin[1] (I) has been widely used as an antidiarrheal, antimycotic, and antibacterial agent in many countries.

Tamura *et al.* studied patients with subacute myelo-opticoneuropathy and found I chelated with iron as a green pigment in the urine and feces and also free I crystals in the urine (1). Subacute myelo-opticoneuropathy was subsequently shown to be caused by the long-term oral treatment and high dosage use of I (1–3).

BACKGROUND

Because of the toxicity associated with the oral usage of I, various analytical procedures have been developed for its determination in biological fluids. Electron-capture GLC, which requires expensive instrumentation and tedious and time-consuming derivatization, has been used (1, 4–8). Tsuji *et al.* (9) reported a method whereby I was chelated with aluminum and measured fluorometrically. A spectrophotometric method also was reported for the determination of I and its conjugates in the urine (10, 11). The spectrophotometric procedure is not sufficiently sensitive for the determination of the drug in plasma or serum. A complicated and time-consuming TLC procedure also was reported (12).

High-performance liquid chromatography (HPLC) has become an increasingly popular analytical technique in clinical and forensic laboratories. A procedure dealing with the separation and analysis of glucuronide and sulfate conjugates of I in urine using HPLC was reported previously (13) but was incapable of assaying for unconjugated I, even when present in high concentrations in the urine (13). The drug occurs predominantly in the unconjugated form in plasma (3).

A single oral dose of I of ~8 mg/kg in humans produces maximum serum levels of 3–6 μg/ml (8). Little research has been conducted on the percutaneous absorption of I. Fischer and Hartvig (14) treated four patients with widespread dermatitis of an unstated type with an ointment containing 3% I, and serum I levels were estimated by electron-capture GLC to be 0.8–1.2 μg/ml.

This report describes the analysis of therapeutic I concentrations in plasma using HPLC. The procedure combines sensitivity, specificity, and simplicity not attainable by previously described methods.

[1] 5-Chloro-7-iodo-8-quinolinol; Vioform, Ciba Pharmaceutical.

EXPERIMENTAL

Apparatus—An isocratic HPLC system assembled in this laboratory consisted of a reciprocating minipump[2]; a stainless steel tube, 6.35 mm (0.25 in.) o.d. and 4.76 mm ($^3/_{16}$ in.) i.d. × 1 m, as a pulsation damper; a 34.5-MPa (5000 psi) pressure gauge[3]; a fixed-volume sample injector[4] with a 20-μl loop; and a variable-wavelength UV detector[5]. A multivoltage 25.40-cm strip-chart recorder[6] was connected to the UV detector.

A microparticulate reversed-phase chromatographic column, 250 × 2.6 mm, packed with ODS-HC-SIL-X-I[7], and a 5 × 40-mm guard column, RP-18-MPLC[8], were connected to the HPLC system. The following reagents were used: iodochlorhydroxyquin[1]; ether anhydrous analytical reagent[9]; methanol distilled in glass, residue free[10]; concentrated perchloric acid; anhydrous sodium sulfate; and water, which was deionized, demineralized, and glass distilled in this laboratory. Iodochlorhydroxyquin (mol. wt. 305.52) decomposed at ~170°, and no impurities were detected by HPLC.

Standards—Stock solutions of I were prepared in methanol and contained 1 mg/ml and 100 μg/ml. These solutions could be maintained in the refrigerator for at least 2 weeks without deterioration. Working solutions were prepared fresh daily. Dilutions were made using 80% methanol and 20% 0.05 *M* phosphoric acid to prepare the working standards, which were used to spike plasma samples and to calculate recovery from plasma samples after extraction.

Extraction—Aliquots of 1.0-ml plasma samples were transferred to 15-ml screw-capped and polytef-lined centrifuge tubes. Human blood bank plasma was used for all studies. Aliquots of I working standard were used to spike the plasma samples. After the tubes were shaken by hand for 1 min, 100 μl of concentrated perchloric acid was added to each tube; then the tubes were vortexed for another 30 sec and centrifuged[11] at 5000 rpm for 10 min at 15°. Approximately 0.5 ml of the supernatant fractions were extracted twice with 5 ml of ether in 15-ml glass culture tubes with polytef-lined screw caps by vortexing for 10 sec, and the phases were separated by centrifuging for 10 min at 15°.

The protein precipitates were extracted once with 10 ml of ethyl ether by vortexing for 1 min and centrifuged as already described. The ether extracts were dried over anhydrous sodium sulfate[9] and evaporated to dryness at 38° under a nitrogen stream[12]. The residue from each fraction was redissolved in 500 μl of the mobile phase, and 20 μl of each was injected onto the column.

Chromatography—The parameters used throughout this investigation included a reversed-phase column and precolumn as previously described, a 20-μl injector loop, and the recorder chart speed set at 0.254 mm (0.1 in.)/min. A mobile phase containing 80% methanol and 20% 0.05 *M* phosphoric acid was used at a flow rate of 1 ml/min. The mobile phase was filtered using a 0.2-μm filter[13], degassed under vacuum, and maintained at 40° during chromatography. The UV detector was set at 256 nm. The column was flushed at the end of each day with 100% methanol. Not more than 30 min was required for column equilibration prior to use each day.

[2] Milton Roy model 396-31.
[3] Laboratory Data Control, Riviera Beach, FL 33404.
[4] Rheodyne Inc., Berkeley, CA 94710.
[5] Spectro-Monitor III, Laboratory Data Control, Riviera Beach, FL 33404.
[6] Beckman Instruments, Palo Alto, CA 94304.
[7] Serial No. 1303, Perkin-Elmer, Norwalk, CT 06858.
[8] Manufactured by Brownlee Laboratories and obtained from Rheodyne Inc., Berkeley, CA 94710.
[9] Mallinckrodt, St. Louis, MO 63147.
[10] Burdick & Jackson Laboratories, Muskegon, MI 49442.
[11] Sorvall RC2-B refrigerated centrifuge, DuPont Co., Newton, CT 06470.
[12] SC/27R sample concentrator, Brinkmann Instruments, Westbury, NY 11590.
[13] Catalog No. EGWP-04700, Millipore Corp., Bedford, Mass.

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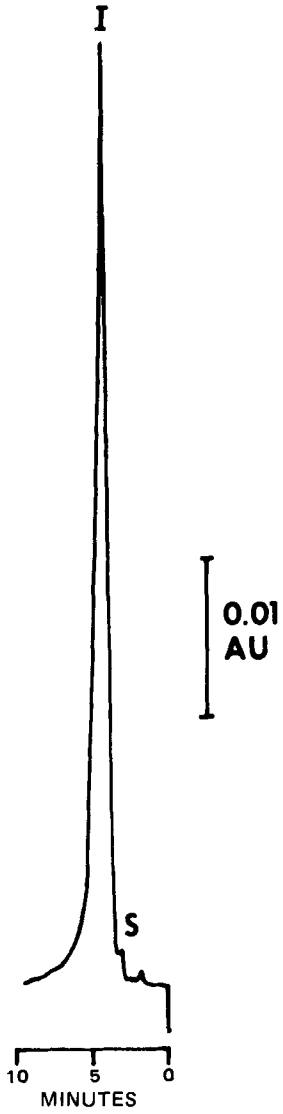

Figure 1—*Standard iodochlorhydroxyquin solution (2 μg/ml) dissolved in the mobile phase. The detector was set at 256 nm and 0.01 aufs. Other chromatographic conditions are described under* Experimental.

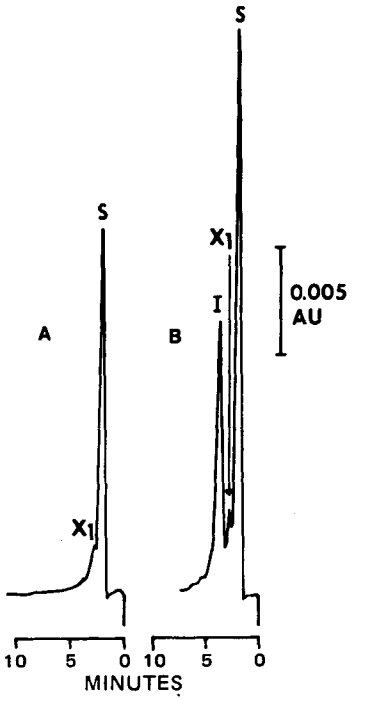

Figure 2—*The supernatant extraction from a plasma blank (A) and from plasma containing 5 μg/ml (B). The detector was set at 0.05 aufs, and other chromatographic conditions are as described in Fig. 1.*

RESULTS AND DISCUSSION

Suitable retention of I was obtained when a mobile phase containing 80% methanol and 20% 0.05 *M* phosphoric acid was used with the described reversed-phase column. A typical chromatogram of standard I in the mobile phase is presented in Fig. 1. The capacity factor (k') for I was 1.14, calculated according to the following:

$$k' = \frac{Rt_1 - Rt_0}{Rt_0} \qquad \text{(Eq. 1)}$$

where the retention time for I (Rt_1) was 3.75 min and the elution time of unretained compounds or the solvent front determined by injecting methanol onto the column (Rt_0) was 1.75 min.

Figure 2A is a chromatogram of an extracted plasma supernatant fraction without I addition. Figure 2B is a chromatogram of I extracted from a spiked plasma sample following protein precipitation using perchloric acid. Direct extraction of plasma by ether without protein precipitation resulted in the presence of unknown compounds that interfered with I upon chromatography. Preliminary studies revealed that large amounts of I were precipitated with the proteins. Therefore, both the acidified supernatant and precipitated protein fractions were extracted with ether. Extractions of I at pH values from 1 to 8 with ether, methylene dichloride, and carbon tetrachloride also were conducted. In addition, the use of ammonium sulfate as the protein precipitating agent was attempted, but the extraction efficiency of I was very poor. Ether extraction of both the supernatant and precipitated protein fractions following perchloric acid addition was most efficient and reproducible.

The representative chromatograms presented in Figs. 3A and 3B were obtained from ether-extracted protein precipitate fractions of human plasma (blank) and a plasma sample to which 5 μg of I/ml had been added, respectively. Two interfering substances were present in ether extractions of the precipitated protein fractions. The first of these compounds (X_1), which has a retention time close to I, was present in much larger concentrations in the extracts from the precipitated protein fraction (Fig. 3) as compared to the ether extracts of the supernatant fractions (Fig. 2). The second substance (X_2) was extracted from the precipitated protein fractions (Fig. 3) but not from the supernatant fractions (Fig. 2) and had a retention time of ~16 min.

Table I shows the percent recovery of I following extraction of the supernatant and precipitated protein fractions, as well as total recovery following the addition of 1–15 μg of I/ml to plasma. Each value represents the mean of three determinations with the standard deviation. The

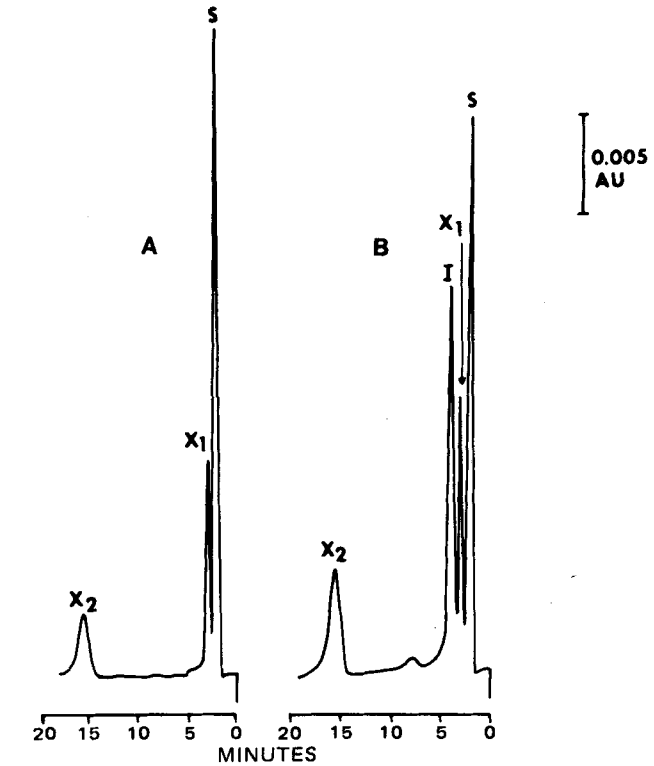

Figure 3—*Extraction of protein precipitate of a plasma blank (A) and of plasma containing 5 μg/ml (B). The detector was set at 0.05 aufs, and other chromatographic conditions are as described in Fig. 1.*

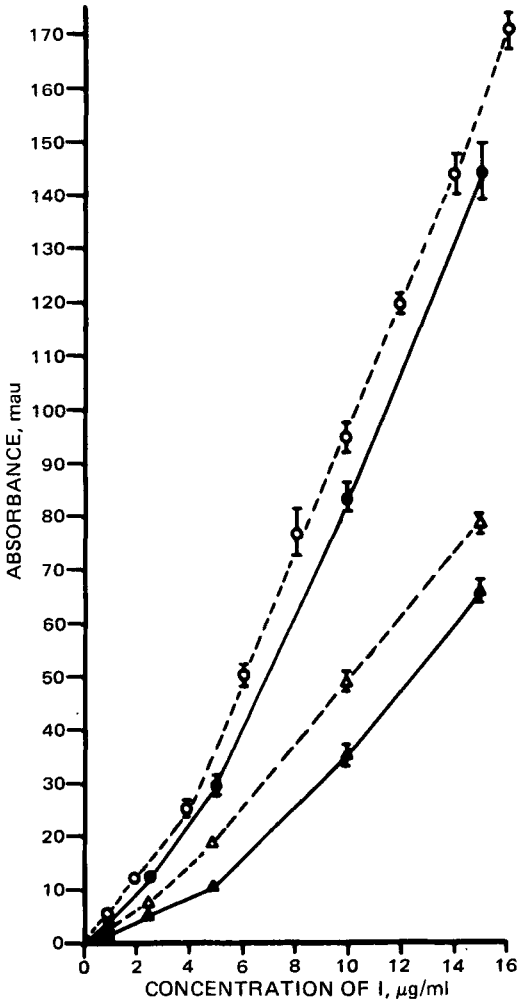

Figure 4—*Standard curve of iodochlorhydroxyquin. Key:* ▲—▲, *extracted supernatant fraction reconstituted in 500 μl of mobile phase;* △ - - △, *extracted precipitate fraction reconstituted in 500 μl of mobile phase;* ●—●, *mathematical summation of two aforementioned values; and* O - - O, *standard solutions diluted in the mobile phase. The peak height values were converted to reflect concentration in 500 μl to conform with values of extracted samples.*

coefficient of variance for each sample also is presented. From 28 to 41% of the total I added to plasma was recovered from the supernatant fraction, while 46–54% of I was recovered from the protein fraction. Total recovery of I was 77–91%.

The UV absorbance spectrum of I in the mobile phase exhibited two maxima at 256 ($\alpha = 0.15$) and 204 ($\alpha = 0.11$) nm. For optimum sensitivity, the detector was set at 256 nm. Standard solutions of I in the mobile phase were prepared at 1, 2, 4, 6, 8, 10, 12, 14, and 16 μg/ml, and 20-μl aliquots of each were injected. The standard curve for I is presented in Fig. 4. Each point on the graph represents the mean, and the bars represent the standard deviation of the mean for three injections. Some bars were omitted because the very small standard deviations of the mean could not be graphically represented.

The amounts of I extracted from the supernatant and precipitated protein fractions of plasma samples spiked with 1.0, 2.5, 5.0, 10.0, and 15.0 μg/ml of I are presented in Fig. 4. Concentrations of I higher than 15 μg/ml of plasma resulted in peak splitting for I upon HPLC. The reason for this effect is not known. The total I recovered at each concentration also is given in Fig. 4. Each point represents the mean, and the bars represent the standard deviation of the mean for three extractions. The correlation coefficients (R) for the standard curve for I, the extracted supernatant fraction, the extracted precipitated protein fraction, and

Table I—Percent Recovery of Iodochlorhydroxyquin from Extracted Plasma [a]

Plasma Concentration, μg/ml	From Supernatant Fraction	From Precipitated Protein Fraction	Total
1	30.0 ± 3.0 (0.17)	54.0 ± 3.1 (0.09)	84.0 ± 6.1 (0.12)
2.5	30.8 ± 2.2 (0.11)	45.9 ± 2.1 (0.07)	76.7 ± 4.0 (0.08)
5	28.0 ± 1.5 (0.09)	48.9 ± 2.2 (0.07)	76.9 ± 2.6 (0.06)
10	35.8 ± 3.2 (0.17)	50.4 ± 2.0 (0.07)	86.2 ± 3.4 (0.07)
15	41.3 ± 2.3 (0.08)	49.5 ± 1.3 (0.04)	90.8 ± 3.0 (0.06)

[a] Each value is the mean with the standard deviation for three determinations. The values in parentheses are the coefficients of variance.

the total extracted I were 0.995, 0.978, 0.995, and 0.992, respectively, where:

$$R = \frac{m\sigma_x}{\sigma_y} \qquad \text{(Eq. 2)}$$

The slope is:

$$m = \frac{\sum xy - \frac{\sum x \sum y}{N}}{\sum x^2 - \frac{(\sum x)^2}{N}} \qquad \text{(Eq. 3)}$$

where σ is the standard deviation, x is the concentration of I, y is the peak height, and N is the number of samples. The y intercept is:

$$b = \frac{(\sum y - m)\sum x}{N} \qquad \text{(Eq. 4)}$$

The primary current application of I is as a topical antibacterial agent, and hydrocortisone is the drug most commonly found in creams and ointments in conjunction with I. Under the experimental conditions described, hydrocortisone elutes with the solvent front and is therefore easily resolved from I. The primary urinary metabolites of I are the glucuronide and sulfate conjugates; with the analytical system employed, these two compounds do not chromatograph.

The HPLC procedure described here provides a rapid and reproducible method for determining plasma I concentrations. Previously described methods are insufficiently sensitive to detect I in small plasma samples (10–12). Chromatography of I by direct ether extract was not possible due to interfering compounds. The most accurate and rapid assay for I was achieved by extracting the plasma supernatant fraction following protein precipitation with perchloric acid. Only a small amount of an unknown compound was extracted as compared to the extraction of two unknown compounds from the precipitated protein fraction.

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