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ANALYSIS OF IODOCHLORHYDROXYQUIN IN BIOLOGICAL MATERIALS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A reversed-phase high-performance liquid chromatographic system using a mobile phase of 0.05 *M* phosphoric acid—methanol (30:70) was developed for determination of iodochlorhydroxyquin (clioquinol, I) in biological material. I was extracted from samples with diethyl ether. Conjugates of I were hydrolyzed to free I and extracted by the same method. The ether phases were evaporated to dryness, reconstituted in the mobile phase and chromatographed using a microparticulate C18 column, a pre-column and a UV detector set at 256 nm. Quantitation of I in the range of 0.20—2.0 μg/ml of urine, 0.50—2.0 μg/g of liver, and 0.25—2.0 μg/g of feces was obtained with coefficients of variation of 0.02, 0.05, and 0.06, respectively. The detection limit of I was 0.2 μg. Extensive absorption of I upon topical application to dogs was also demonstrated.

INTRODUCTION

Iodochlorhydroxyquin (clioquinol, I) has been widely used in many countries as an antidiarrheal, antimycotic and antibacterial agent. Subacute myelo-opticoneuropathy (SMON) is the major toxicological manifestation associated with high doses of I [1—4], and an iron chelate of I has been isolated from the urine and feces of patients with SMON.

Several analytical methods have been developed to study the distribution and excretion of I. Electron-capture gas—liquid chromatography (GLC), which requires expensive instrumentation and time-consuming derivatization, has been used [5—10]. Chen et al. [10] developed a solvent extraction method for separating I and its glucuronide and sulfate conjugates in biological fluid prior to GLC. A spectrophotometric method was reported for quantitating I and its conjugates in the urine [11, 12]. This procedure is not sufficiently sensitive for the determination of the drug in other biological materials. Chen et al.

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[13] reported a method for the determination of I conjugates by high-performance liquid chromatography (HPLC). This method could not be applied to tissue or plasma because of the presence of constituents absorbing at 254 nm and free I could not be measured. Tsuji et al. [14] reported a method whereby I was chelated with aluminum and measured fluorometrically. In our laboratory a procedure for analysis of I in plasma was developed [15]. In addition, methods for analysis of I and hydrocortisone in creams and ointments [16, 17] have been developed using simple isocratic HPLC, based upon our initial procedure for the analysis of I in plasma [15]. Hayakawa et al. [18] have recently published an HPLC procedure for the analysis of I and its conjugates in biological materials.

This report describes a new HPLC method for the measurement of I in urine, feces, and liver. The procedure combines sensitivity and simplicity not attainable by previously described methods.

EXPERIMENTAL

Apparatus

The liquid chromatograph consisted of a reciprocating pump (Milton Roy Model 396-31), a stainless-steel tube (1 m × 6.35 mm O.D. and 4.76 mm I.D.) as a pulsation damper, a 34.5 MPa (5000 p.s.i.) pressure gauge (Laboratory Data Control), a fixed volume sample injector (Rheodyne) with a 20-μl loop, and a variable-wavelength UV detector (Spectro-Monitor III, Laboratory Data Control). A multivoltage 25.40-cm stripchart recorder (Beckman Instruments) was connected to the UV detector. A microparticulate reversed-phase chromatographic column (250 × 2.6 mm) packed with ODS-HC-SIL-X-I (particle size 10 μm, serial No. 1303, Perkin-Elmer) and a 40 X 5 mm guard column containing RP-18-MPLC (Rheodyne) were connected to the HPLC system.

Materials

Iodochlorhydroxyquin (clioquinol, I) was obtained from CIBA Pharmaceuticals (Summit, NJ, U.S.A.). Diethyl ether anhydrous analytical reagent, anhydrous sodium sulfate, benzene, sodium hydroxide, hydrochloric acid, sodium fluoride, and theophylline were purchased from Mallinckrodt (St. Louis, MO, U.S.A.). Methanol distilled in glass, residue free, and acetone were obtained from Burdick and Jackson Labs. (Muskegon, MI, U.S.A.). β-Glucuronidase (1,000,000 units per 2.25 g) and alumina were purchased from Sigma (St. Louis, MO, U.S.A.). Sodium acetate, acetic acid and diphenylcarbazone were obtained from Fisher Scientific (Fair Lawn, NJ, U.S.A.). Pyridine was obtained from J.T. Baker (Phillipsburg, NJ, U.S.A.). 4,7-Dichloroquinoline, 5,7-dichloro-8-hydroxyquinoline, 10-chloro-9-anthracene—methanol, 9-hydroxy-4-methoxy-acridine and α-naphthoflavone were purchased from Aldrich (Milwaukee, WI, U.S.A.). 4-Chloro-3-methylphenol, 5-nitroso-8-quinolinol, α-bromo-*p*-nitrotoluene, 4,4-dimethylbenzophenone, benzanilide, 1-naphthylamine, 2,7-naphthalenediol, and dibenzofurane were all purchased from Eastman Organic Chemicals (Rochester, NY, U.S.A.). Xanthin-9-ol was obtained from Matheson Coleman and Bell (Norwood, OH, U.S.A.). Trihexyphenidyl was a gift from Lederle Laboratories (Pearl River, NY, U.S.A.).

HPLC conditions

The UV detector was set at 256 nm with recorder chart speed at 0.254 mm/min. Methanol—0.05 *M* phosphoric acid (70:30) was used as mobile phase at a flow-rate of 1 ml/min. The mobile phase was filtered using a 0.2-μm filter, degassed under vacuum and maintained at 40° C during chromatography. 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.

Sample preparation

Urine. Aliquots of 1—5 ml of urine were transferred to 15-ml screw-capped and PTFE-lined centrifuge tubes. Known amounts of I working standard solution were used to spike urine samples which were then extracted three times with 1—5 ml of diethyl ether by vortexing for 10 sec, and the phases were separated by centrifuging at 3000 *g* for 10 min at 15° C. The ether extracts were dried over anhydrous sodium sulfate and evaporated to dryness at 40° C under a stream of nitrogen. The residues were redissolved in the mobile phase and 20 μl of each was injected onto the column. When a determination of the total I in urine was made, conjugates (glucuronides and sulfates) were hydrolyzed with β-glucuronidase (final concentration 200 units/ml) at pH 5 using 1.0 *M* acetate buffer, incubating at 37° C for 2 h. Complete hydrolysis of sulfate conjugates was ensured by adjusting the solution to 1 *N* using 6 *N* hydrochloric acid and incubating at 40° C for an additional 2 h [10]. The hydrolysates were neutralized with 3 *N* sodium hydroxide prior to extraction three times with 5 ml diethyl ether as described above. After evaporation of the ether phases the residues were dissolved in 0.5 ml benzene, adsorbed on to an alumina column and washed successively with 2 ml each of benzene—pyridine (7:1), acetone, 0.1 *N* acetic acid in methanol and methanol. The alumina was transferred to another 15-ml screw-capped glass tube with 1 ml of a saturated aqueous solution of sodium fluoride and extracted twice with 5 ml diethyl ether. The ether phases were evaporated to dryness, the residues were dissolved in the moble phase, and 20-μl aliquots were injected onto the column.

Tissue. To 1-g samples of liver in 25-ml glass Potter-Elvehjem homogenizer tubes 2 ml of the mobile phase and a known quantity of the standard solution of I in the mobile phase were added and homogenized. The homogenates were transferred to 15-ml screw-capped and PTFE-lined centrifuge tubes, the homogenizer tubes were each washed with 1 ml of mobile phase and the washings were transferred to the centrifuge tubes. The homogenates were extracted three times with 5 ml diethyl ether by vortexing for 1 min and centrifuging at 3000 *g* for 10 min. The ether phases were collected and evaporated to dryness. The residues were dissolved in 0.50 ml benzene, adsorbed onto an alumina column and processed as described above for the urine.

Feces. To 1-g samples of feces in 25-ml glass Potter-Elvehjem homogenizer tubes 5 ml of mobile phase were added. Each was spiked with working standard solution of I in methanol and homogenized. The homogenized mixtures were transferred to 15-ml screw-capped and PTFE-lined centrifuge tubes and subjected to hydrolysis and the washing procedures described above for the urine extraction.

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 two weeks without deterioration. Working solutions were prepared fresh daily. Dilutions were made using methanol—0.05 *M* phosphoric acid (70:30) to prepare the working standards, which were used to spike samples and to calculate recovery from samples after extraction.

RESULTS AND DISCUSSIONS

The UV absorbance spectrum of a standard solution of iodochlorhydroxyquin (I) in methanol—0.05 *M* phosphoric acid (70:30) was determined, and exhibited maxima at 256 nm (absorptivity, $\delta = 0.15$) and 204 nm ($\delta = 0.11$). For optimum sensitivity the UV detector was set at 256 nm for subsequent HPLC analysis [15, 16].

Representative chromatograms of canine urine samples in the presence and absence of I using a column packed with ODS-HC-SIL-X-1 (10 μm) are given in Fig. 1. Fig. 1A represents an analysis of an extract of a blank (control) urine. No peaks which might interfere with I are present. Fig. 1B is a chromatogram of a urine sample to which I had been added. I is represented by peak 1 and has a retention time of approximately 7.5 min, while peak 4 is the solvent front with a retention time of 2.0 min. Peaks 2 and 3 are unknown substances extracted from urine with retention times of approximately 5.0 and 2.5 min, respectively. The capacity factor k' for I was 2.75.

Fig. 1C is a chromatogram of an organic extract of urine from a dog which had been treated topically with 5 g of a cream containing 3% I. The cream had been applied to the shaved back over a 200 cm^2 area. Since I is readily conjugated with glucuronic acid and sulfate [3], urine samples were hydrolyzed and washed as described under Experimental and the extracts chromatographed. The total I in the urine sample was 1.8 μg/ml. The washing procedure during sample preparation was necessary to remove interfering substances.

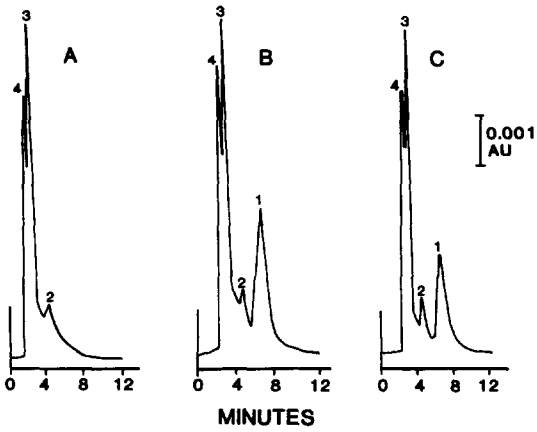


Fig. 1. Chromatograms of (A) blank canine urine; (B) urine containing 2.5 μg/ml iodochlorhydroxyquin (I); (C) urine from a dog treated topically with I following hydrolysis and alumina column clean-up procedure. The detector was set at 0.02 aufs and conditions are as described under Experimental. Peaks: 1 = I; 2, 3 = unknowns; 4 = solvent front.

The chromatograms of canine liver extracts in the absence and presence of I are shown in Fig. 2A and B, respectively. In Fig. 2B the tissue was spiked with 1.5 μg I per g. I is represented by peak 1, and peak 2 is the solvent front. As can be seen, no substances which interfered with the elution of I were extracted from the liver. Fig. 2C is a chromatogram of an organic extract of liver from a dog which had been treated with I topically. The liver contained 0.9 μg I per g of tissue.

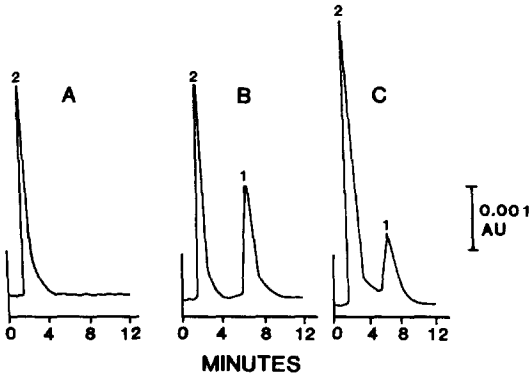
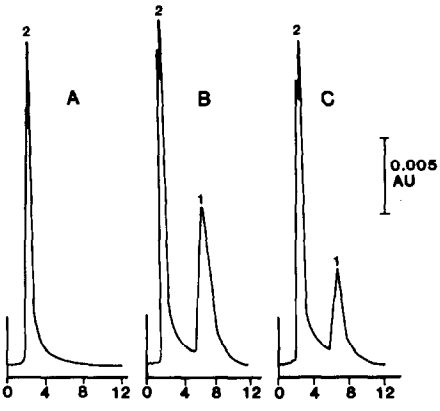


Fig. 2. Chromatograms of (A) control dog liver; (B) 1.0 g canine liver to which had been added 1.50 μg I; (C) liver from a dog treated topically with I. The detector was set at 0.02 aufs and chromatographic conditions used are described under Exprimental. Peaks: 1 = I; 2 = solvent front.

Hayakawa et al. [18] recently reported a HPLC system for the measurement of I and its conjugates in biological fluids as bile, plasma and urine. We previously reported a sensitive method for determining I in plasma [15]. Hayakawa et al. [18] also presented a chromatogram of an extract of 0.2 g of kidney containing 2 μmole (610 μg) of I. We were able to measure less than 1 μg I per g liver (Fig. 2), suggesting that our extraction procedure is more efficient. The limits of detection for I reported by Hayakawa et al. [18] and the procedure described herein were 0.3 and 0.2 μg, respectively.

Phoon and Stubley [17] developed a HPLC method for the determination of I in ointments. The system they reported is a modification of the procedure which we developed for plasma [15]. We have also modified this method for measuring I in ointments and creams [16].

Representative chromatograms of extracted feces samples from dogs in the absence and presence of I are presented in Fig. 3A and B, respectively. The feces samples employed in Fig. 3B was spiked with 20.0 μg I per g. Peak 1 represents I with a retention time of approximately 7.5 min, and peak 2 is the solvent front. As with the urine and liver samples, no peaks interfered with I. Most of the I excreted in the feces has been reported to exist as the glucuronide and sulfate conjugates [3]. Therefore, feces samples were hydrolyzed, extracted and washed as described in Experimental; a representative chromatogram is presented in Fig. 3C. The sample was obtained from a dog which had been treated topically with I, and contained approximately 11.2 μg per g. The primary route of excretion of I appears to be the feces following formation of conjugates which are concentrated in the bile [3].



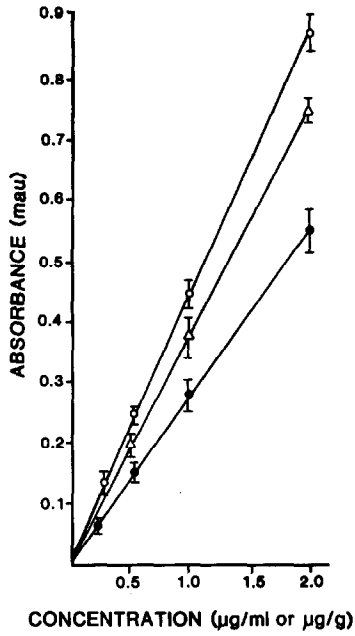


Fig. 3. Chromatograms of (A) control dog feces; (B) feces containing 20.0 µg I per g; (C) feces from a dog treated topically with I. The detector was set at 0.05 aufs, and chromatographic conditions are described under Experimental. Peaks: 1 = I; 2 = solvent front.

Fig. 4. Standard curves of iodochlorhydroxyquin (I) from urine (•——•), liver (△——△), and feces (○——○) spiked with I.

The amounts of I extracted from urine samples spiked with 0.20, 0.50, 1.0, and 2.0 µg/ml, feces samples spiked with 0.25, 0.50, 1.0, and 2.0 µg/g, and liver samples spiked with 0.50, 1.0, 2.0, and 4.0 µg/g are presented in Fig. 4. The correlation coefficient r for the standard curves for I from extracted urine, feces, and liver was 0.99 in each case. Concentrations as high as 60 µg/g of I in feces and 40 µg/ml in urine were also shown to give linear results.

The percent recovery of I following extraction of urine, feces, and liver was determined and is presented in Table I. Each value represents the mean of three determinations with the standard deviation. An average recovery of 96.85% of I was obtained by direct extraction of urine spiked with I without subjecting the samples to the hydrolysis and washing procedure which were ncessary to release conjugated I and remove interfering substances occurring as a result of the hydrolysis [1]. Approximately 28% of the I was recovered from urine following hydrolysis and the sample clean-up procedure on alumina columns.

Liver samples were spiked with I. The samples were not hydrolyzed but were subjected to the washing procedure to remove interfering substances. As such, the recovery of I was approximately 36% (Table I). The recovery of I from feces samples which had been spiked with I, hydrolyzed and washed was approximately 41% (Table I). The results indicate that the greatest loss of I occurs during the alumina column clean-up procedure which was necessary to remove substances that intefered with HPLC of I. The washing procedure was

TABLE I

RECOVERIES OF IODOCHLORHYDROXYQUIN FROM BIOLOGICAL MATERIALS

Biological sample	Added I (μg/ml or μg/g)	Percent recovery	Average percent recovery
Urine*	0.20	97.3 ± 2.1	96.8
	0.50	96.4 ± 2.3	
Urine	0.50	30.5 ± 2.7	27.8
	1.0	25.2 ± 0.4	
Liver	0.50	45.4 ± 2.9	36.0
	2.0	26.7 ± 2.0	
Feces	0.50	52.0 ± 3.1	41.0
	2.0	30.0 ± 1.5	

*Direct extraction of sample without subjection to hydrolysis and washing process.

previously employed by Tamura et al. [1] for sample preparation for the gas chromatography of I. Although the hydrolysis and washing procedures that were used reduced the percent recovery of I, extremely good reproducibility was obtained in each case.

An attempt was made to find an internal standard for the assay of I in biological tissues and fluids, and the chromatographic characteristics of the compounds presented in Table II were determined. Only α-naphthoflavone had a suitable retention time (13.5 min). However, α-naphthoflavone was sufficient as an internal standard only when samples were extracted and chromato-

TABLE II

HPLC RETENTION TIMES OF VARIOUS COMPOUNDS EXAMINED AS POSSIBLE INTERNAL STANDARDS

Substance	Retention time (min)
Iodochlorhydroxyquin	7.5
4,7-Dichloroquinoline	6.0
5,7-Dichloro-8-hydroxyquinoline	6.2
10-Chloro-9-anthracene—methanol	7.5
9-Hydroxy-4-methoxyacridine	3.0
Trihexyphenidyl hydrochloride	5.4
Diphenylcarbazone	2.6
4-Chloro-3-methylphenol	3.0
α-Bromo-*p*-nitrotoluene	3.5
1-Naphthylamine	3.0
5-Nitroso-8-quinolinol	2.5
Tribenzylamine	5.7
Xanthin-9-ol	5.5
Dibenzofurane	8.0
4,4-Dimethylbenzophenone	6.0
Theophylline	2.3
Benzanilide	3.0
2,7-Naphthalenediol	2.5
α-Naphthoflavone	13.5

graphed directly without subjection to the alumina column washing and clean-up procedure. Due to the solubility of the α-naphthoflavone in the washing solvents, the α-naphthoflavone did not adhere to the alumina and was lost. Therefore, this compound can only be used as a standard when added before injection of an extract onto the column. Hayakawa et al. [18] have used 5,7-dichloro-8-hydroxyquinoline as an internal standard without an alumina column washing and clean-up procedure.

The present method provides a rapid, sensitive, and reproducible procedure for the determination of I in different biological materials and will be useful for toxicity and metabolism studies. I has been previously shown to be well absorbed upon oral administration and is associated with neuromuscular toxicity [14]. The present study indicates that I is extensively absorbed upon topical application to dogs.

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