Metabolism of Mono- and Dichlorohydroxyquinolines-Cl^{36} in the Rat and Calf

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A balance study with chlorine-36 labeled mixture of 5-chloro-8-hydroxyquinoline and 5,7-dichloro-8-hydroxyquinoline showed the following distribution of Cl^{36} in the urine and feces of the rat and calf: rat, urine 39.3% and feces 60.4%; calf, urine 80.5% and feces 10.5%. Chlorine-36 tissue distribution is given for the calf fed 3 weeks with chloroquinolines at 40 ppm level. Chlorine-36 accumulation and depletion have been studied in rat tissues. Tissue balance was established within 6 days, and 95% depletion occurred in 3 days. Free or conjugated unchanged chloroquinolines were excreted in urine, and unchanged chloroquinolines were present in the tissues. There was 12% molecule dechlorination.

Nutriquinol (CHQ) is a mixture of 65% 5,7-dichloro-8-hydroxyquinoline, 31% 5-chloro-8-hydroxyquinoline, and 4% 7-chloro-8-hydroxyquinoline (Figure 1), used as a feed additive for improving growth of calves. Although these quinolines have been used for a long time in the treatment of human amoebic and bacillary dysenteries (de Alencar and Sampaio, 1963; Heseltine and Campbell, 1960; Heseltine and Freeman, 1959), their metabolism has never been studied. However, the metabolism of related halogenated derivatives 5-iodo-7-chloro-8-hydroxyquinoline (ICOQ) and 5,7-dibromo-8-hydroxyquinoline (DBHQ) has been investigated. 38 to 50% ICOQ was found in the urine of rabbits receiving oral doses of 20 mg/kg/day for 7 days (Haskins and Luttermoser, 1953). A man receiving a single dose of 0.1 to 1 mg of ICOQ excretes 43% in his urine as glucuronide and sulfate conjugates within 10 to 18 days (Liewendahl and Lamberg, 1967). This confirms other experiments (Ritter and Jermann, 1966). The proportions of free and conjugated ICOQ in urine, feces, and plasma of rats receiving a 1-mg oral dose of I^{125}-labeled drug have been determined (Liewendahl, 1968). The same experiment also showed iodine-125 tissue distribution in rats 8 hr, 24 hr, and 48 hr after a 1-mg oral administration of labeled ICOQ; deiodination of the molecule was weak, being about 6%. 58% of the DBHQ administered was found in human urine as glucuronide conjugate after 24 hr; debromination occurred for 1.3% (Rodriguez and Close, 1968).

A preliminary balance study using colorimetric determination of chlorohydroxyquinolines enabled the recovery in the urine and feces of only 60% of the orally administered dose. It was necessary to use a labeled molecule for establishing this balance, and for studying incorporation, distribution, and depletion in the tissues. 5-Chloro- and 5,7-dichloro-8-hydroxyquinolines labeled with chlorine-36 were synthesized. This labeling seemed appropriate, as there was little dehalogenation of the ICOQ and DBHQ.

SYNTHESIS OF Cl^{36}-LABELED CHQ

Synthesis was carried out by controlled chlorination of 8-hydroxyquinoline with Cl^{36}–sulfuryl chloride (New England Nuclear). It gave a mixture of 5,7-dichloro- and 5-chloro-8-hydroxyquinoline having radiochemical purity higher than 99% as determined by radiochromatography. The ponderal composition of the mixture was determined by spectrophotometry (read at 254 nm) after the two substances had been separated by thin-layer chromatography (tlc). It contained 79% dichlorinated derivative and 21% monochlorinated derivative. The radioactive composition determined by radiochromatography was 90 and 10%, respectively. The approximate specific activity of the mixture was 0.102 mCi/m*M*.

ADMINISTRATION OF THE DRUG

Rats. For the balance study, three male Wistar rats, of the same age and weight (250 g), were put in metabolism cages and given feed and water *ad libitum*. In a first experiment, a single dose of 3.75 mg (1.85 μCi) of Cl^{36}–CHQ (representing 250 ppm of the diet) dissolved in 1 ml of peanut oil was administered to the rats by stomach intubation. In a second experiment, the labeled drug was blended at the same level in the feed. Urine and feces were collected separately and stored at −25 °C.

The experimental procedure used for determining long-term accumulation involved giving Cl^{36}–CHQ at 80 ppm level in the diet (1.2 mg or 0.6 μCi per day) to six male Wistar rats having the same age and weight (150 g). The animals were sacrificed after 1, 3, 6, 9, 12, and 15 days, and the tissues analyzed for radioactivity.

For the depletion study, six male Wistar rats of the same age and weight (150 g) were fed for 15 days with 100 ppm of unlabeled CHQ, then with 100 ppm of labeled CHQ (1.5 mg or 0.75 μCi per day) for 8 days. The animals were then sacrificed every 12 hr, and chlorine-36 tissue levels determined.

Calf. A balance study was carried out on a 2-month-old French Frisian calf put in a metabolism cage and fed with 10 l. of milk a day. 30 μCi of labeled CHQ in capsule form (about 60 mg), representing 50 ppm of the calf's diet, were intubated into the stomach. Urine and feces were collected separately for 15 days, homogenized, and samples stored at −25 °C.

The CHQ residues in the tissues were studied. The same calf, still fed with 10 l. of milk per day, received 40 ppm of unlabeled CHQ (about 50 mg) for 15 days, then for the next 8 days received 25 mg of nonradioactive CHQ in the morning and 25 mg of labeled CHQ (12 μCi) in the evening, both in capsule form. The calf was slaughtered on the morning of the ninth day, and the tissues were excised, ground in liquid nitrogen, and lyophilized.

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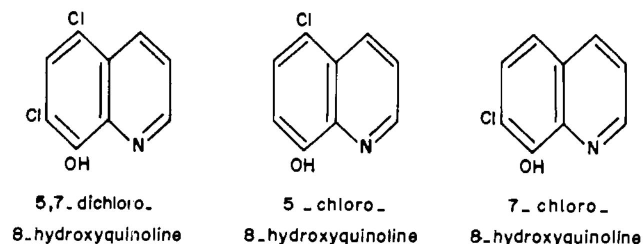

Figure 1. CHQ components

RADIOACTIVITY ASSAYS

The assays were performed by liquid scintillation on a Nuclear-Chicago 6860 Counter. The samples were introduced in aqueous solution into a liquid scintillator containing Triton-X 100, which gives stable gel between 0° and −5°C (Patterson and Green, 1965). Quenching corrections were made by external standardization method. The settings were as follows: window 0.5–9.9 V, attenuation F_0 and E_0 for Cl^{36}, and external standard, respectively. The counting time was calculated according to the radioactivity samples to give a standard deviation of less than 5%. Counting efficiency varied between 50% for the kidney and spleen samples and 92% for the urine and fat.

Urine Assay. Radioactivity determinations were performed on 1 and 2 ml of each sample.

Feces Assay. Feces were dissolved at 70°C in 1 *M* NaOH, and 1- and 2-ml samples counted. In order to avoid chemiluminescence phenomenon in alkali medium, the counting vials were left 24 hr in the counter before being measured.

Tissue Assay. For saturation and depletion experiments in rats, fresh tissues were finely ground in water until homogeneous; 1-ml and 2-ml samples were counted. For the calf tissue residue assays, a sample of ground, lyophilized tissues was dissolved in 1 *M* NaOH and counted using normal conditions. The bile was collected with a syringe in the gall bladder immediately after sacrifice, diluted with water, and counted. Hydrogen peroxide was used to improve counting efficiency of the highly colored samples (kidney, spleen, bile).

IDENTIFICATION OF Cl^{36}-LABELED METABOLITES

Thin-Layer Chromatography (tlc) of 8-Hydroxyquinoline and Chlorinated Derivatives. 5 × 20 tlc plates, coated with a 0.25-mm layer of silica gel H (Merck) containing EDTA to chelate heavy metal traces (1 g of EDTA for 30 g of silica), were activated 30 min at 105°C. The development solvent used was methanol–butanol–ethyl acetate–ammonium hydroxide (3:2:11:2), and the quinolines were detected by spraying a copper acetate solution in methanol. By increasing R_f, the separation order was 5,7-chlorohydroxyquinoline, 5-chlorohydroxyquinoline, and 8-hydroxyquinoline. The radioactive spots were located by a Packard 7201 radiochromatogram scanner.

Determination of the Elimination Compounds. During the long-term calf experiment a representative 8-day excretion sample was taken. Free hydroxyquinolines were extracted from neutralized urine by diethyl ether, and the conjugated forms were hydrolyzed from extracted urine in three processes. 6 *N* hydrochloric acid, refluxed for 1 hr, liberated all the bound forms. At pH 4.9 and 37°C for 24 hr, the action of a crude solution from Helix pomatia (type H.2 Sigma), essentially containing β-glucuronidase and sulfatase, liberated glucuronic acid and sulfuric acid-bound compounds. At pH 6.2 (acetic 0.2 *M* buffer) and 37°C for 24 hr, β-glucuronidase (type B-10, bovine liver, Sigma) liberated glucuronic acid-bound compounds.

Identification in Calf Urine. Part of the same urine sample was hydrolyzed in 6 *N* hydrochloric acid medium. After ammonia neutralization, the hydrolyzate was extracted four times with 250 ml of diethyl ether. The extracts were pooled, dried on sodium sulfate, and then evaporated under vacuum. The residue was dissolved in 1 ml of chloroform and chromatographed on a preparatory scale (1 mm-thick layers) using the tlc described. Radioactive bands were located by autoradiography (Kodirex film, Kodak). The silica was scraped and eluted by chloroform. The ultraviolet spectra were recorded directly on the chloroform eluate with a Bausch & Lomb spectronic 505 spectrophotometer. Infrared spectra were obtained with a double beam infrared spectrophotometer (Perkin-Elmer model 457) using KBr disks containing 50 to 100 μg of the compound coming from the chloroform evaporation and recrystallized twice in isooctane.

Identification in Calf Kidneys. The kidneys were chosen because they were the most radioactive and the sensitivity threshold of the radiochromatographic method described could be reached. Radioactive metabolites were extracted from several grams of lyophilized tissue by a 200-ml. mixture of acetone–1 *N* hydrochloric acid (3:1). The acetone was evaporated under vacuum after filtration, the aqueous solution neutralized by concentrated ammonia, and then extracted four times by diethyl ether. The extracts were dried on sodium sulfate, and then evaporated to dryness under vacuum. The residue was dissolved in 0.1 ml of chloroform, then chromatographed according to the process described. Radioactivity was located with a radiochromatogram scanner.

Evaluation of CHQ Dechlorination. Figure 2 shows the plan used to isolate and identify chlorine-36-labeled chloride ions in a calf urine sample. Chromatography of Cl^{36}-ions was done using Grassini and Ossicini's method (1968) on Whatman paper No. 1 employing isopropyl alcohol–1.5 *M* ammonia (1:1) development solvent. Cl^{36}-Ions were revealed by silver nitrate; then radioactivity was located by the radiochromatogram scanner. Counting was done after silver chloride precipitation, redissolution of the precipitate in thiosulfate, and incorporation into the scintillation mixture described above.

RESULTS AND DISCUSSION

A preliminary remark should be made about the radioactive product used which has a slightly different composition than that of Nutriquinol as a result of perfected microsynthesis. The metabolism of each component of the labeled product could not be studied separately because of the large quantities of the product required for the calf experiments. In view of the fact that this study was to determine the range in amount of tissue residue rather than to make a strict evaluation of it, we hypothesized that the two chlorinated derivatives had a rather similar metabolism. Thus, we gave overall consideration to the mixture of the two radioactive substances and expressed radioactive residues in tissues in per million parts of Nutriquinol. Moreover, some results show that this approximation was correct.

Tables I and II show the results of balance studies in the rat and calf. Urinary and fecal withdrawal was very rapid, with more than 90% of the radioactivity being eliminated within 48 hr in the rat. These results are similar to those obtained in experiments carried out with ICOQ and DBHQ (Liewendahl, 1968; Ritter and Jermann, 1966; Rodriguez and Close, 1968). Urinary elimination is predominant in the calf

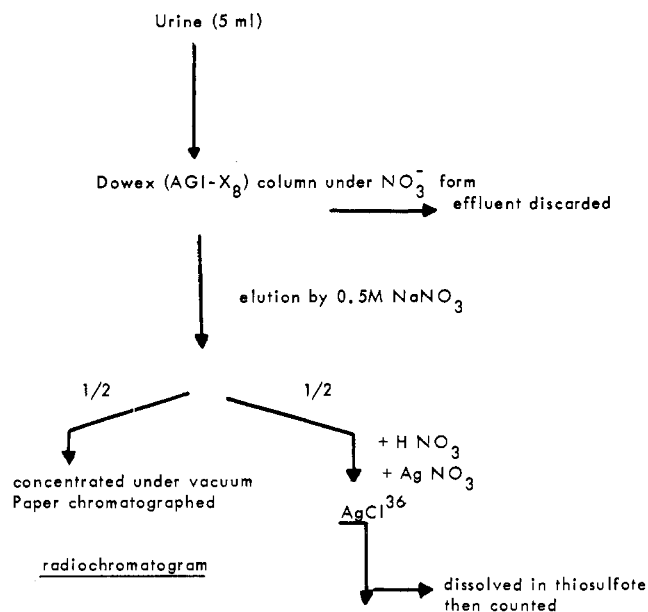

Figure 2. Scheme used for the identification and evaluation of urine chloride ^{36}Cl content

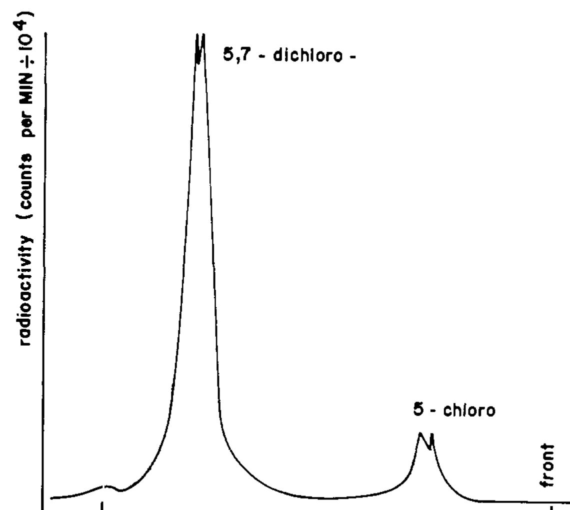

Figure 3. Radiochromatogram of labeled metabolites isolated from calf urine

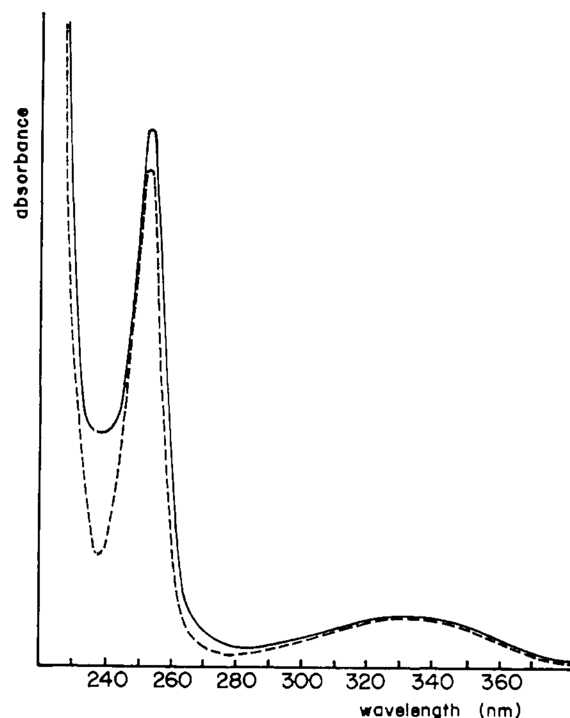

Figure 4. Ultraviolet absorption spectrum of main metabolite isolated from calf urine. Solid line, unknown; broken line, 5,7-chloro-8-hydroxyquinoline reference standard

Table I. Rat, Radioactivity Percentage Recovered

Dose administered	CHQ fed 4.93 μCi		CHQ intubated 4.35 μCi	
Days	Urine	Feces	Urine	Feces
1	19.3	33.5	31.8	0.3
2	4.6	32.9	5.5	53.0
3	1.2	2.0	1.0	6.4
4	0.4	0.4	0.3	0.6
5	0.2	Traces	0.3	0.1
6	0.2		0.2	Traces
7	0.1		0.1	
8	Traces		0.1	
	26.0	68.8	39.3	60.4
Total	94.8		99.7	

Table II. Calf, Radioactivity Percentage Recovered

Dose administered	CHQ intubated, 28.19 μCi	
Days	Urine	Feces
1	4.6	0.2
2	40.8	5.3
3	11.9	2.7
4	11.9	1.1
5	4.7	1.0
6	2.5	0.2
7	0.8	Traces
8	1	Traces
9	0.9	Traces
10	0.7	Traces
11	0.4	Traces
12	0.2	Traces
13	0.1	Traces
14	Traces	
	80.5	10.5
Total	91	

and fecal elimination is predominant in the rat; these species differences already are being observed by Haskins and Luttermoser (1953) with ICOQ.

Nutriquinol is eliminated both in free and conjugated forms, and undergoes limited dechlorination. Radioactive distribution in calf urine is the following: 54.3% free, 31.7% conjugated, and 12.1% in the form of chlorides. The three types of hydrolysis applied to calf urine, from which the free radioactive fraction has been extracted, cause liberation of the same amount of radioactivity (about 30%). This suggests that conjugated metabolites may be in the form of glucuronides. The radiochromatogram (Figure 3), and ultraviolet (Figure 4) and infrared (Figure 5) spectra show the identity between urinary metabolites (free or conjugated) and 5,7-dichloro- and 5-chloro-8-hydroxyquinolines of the original product. The respective percentages of the two chlorinated derivatives revealed by radiochromatography and expressed in radioactivity are 16 and 84%, while the product administered had a composition of 10 and 90%. The lower elimination of 5,7-dichloro derivative may correspond either to its preferential storage or to a partial or total dechlorination.

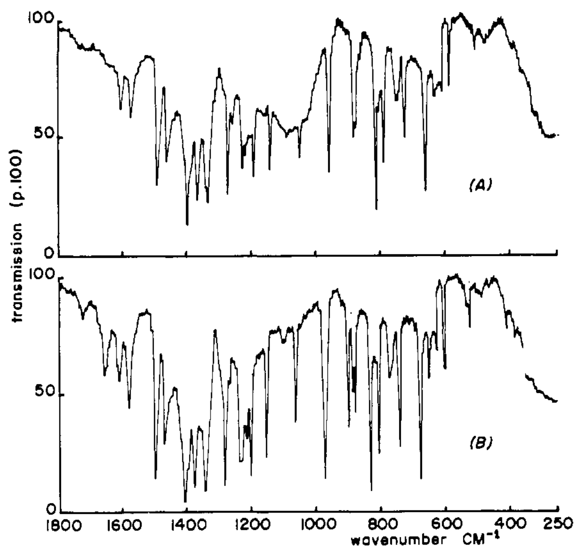

Figure 5. Infrared spectrum of standard 5,7-dichloro-8-hydroxyquinoline (A) and main urinary metabolite (B)

Table III. Residues in Tissues of Calf Fed 3 Weeks with 40 ppm of Cl^{36}–CHQ

Tissues	ppm[a]
Liver	0.12
Kidney	1.00
Spleen	0.23
Heart	0.07
Thymus	0.09
Muscle	0.025
Fat	0.12
Serum	0.4 μg/ml
Bile	0.8 μg/ml

[a] Parts per million calculated as CHQ equivalents.

The fact that part of the radioactivity (12.1%) is in the form of chlorides in the urine, and that 8-hydroxyquinoline was not determined, would lead us to suppose a partial dechlorination. Only 5% of the radioactivity in calf and rat feces was extracted. This corresponds to free metabolites, it not being possible to extract more than 5% by 6 *N* hydrochloric acid hydrolysis or 70% methanol reflux. Given the chelating properties of 8-hydroxyquinolines and the low solubility of resulting chelates (iron in particular), it may be hypothesized that most of the radioactivity eliminated in the feces may be a chelated form.

Calf tissue, serum, and bile residue levels are given in Table III. This shows that after long-term administration at a 40 ppm level, the quantities of radioactivity are very low, most being found in the liver and kidneys, where metabolism and elimination occur. Radioactivity level in the bile indicates considerable elimination by this route. Residues of 5-chloro- and 5,7-dichlorohydroxyquinoline were found in the kidneys in proportions similar to those of the administered product.

Table IV. Incorporation of Cl^{36} in Tissues of Rats Fed 2 Weeks with 80 ppm of Cl^{36}–CHQ, ppm[a]

Tissues	Days on medication: 1	3	6	9	12	15
Kidney	0.82	1.34	1.59	1.35	1.91	1.69
Liver	0.50		1.11	1.17	1.31	1.34
Muscle	0.25	0.39	0.49	0.37	0.64	0.51
Fat	0.20	0.24	0.35	0.35	0.50	0.45

[a] Parts per million calculated as CHQ equivalents.

Table V. Depletion of Cl^{36} from Tissues of Rats Fed 2 Weeks with 100 ppm of Cl^{36}–CHQ, ppm[a]

Tissues	Hours after withdrawal of medication: 12	24	36	48	60	72
Kidney	0.82	0.36	0.02	0.02	0.06	0.09
Liver	0.31	0.16	0.03	0.02	0.03	0.03
Muscle	0.22	0.20	0.16	0.14	0.12	0.13
Fat	0.10	0.05	0.06	0.04	0.03	0.02

[a] Parts per million calculated as CHQ equivalents.

Table IV shows that Cl^{36} balance in most rat tissues is established within 6 to 9 days, and that no accumulation occurs. After administering 80 ppm of the drug, residue levels are 2 ppm in the kidneys and about 0.5 ppm in the muscles and fat.

The depletion of radioactivity following withdrawal of drug is shown in Table V. Chlorine-36 is rapidly eliminated from liver and kidneys, the residue levels reaching that of the other tissues after 36 hr.

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