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Effect of Clioquinol on Metals in Rabbit

KAZUICHI HAYAKAWA,*,[a] TOSHIHIKO OGAI,[a] YOSHIAKI IRIKURA,[a]
MOTOICHI MIYAZAKI[a] and ZENZO TAMURA[b]

Faculty of Pharmaceutical Sciences, Kanazawa University,[a] 13–1, Takara-machi, Kanazawa 920, Japan and Faculty of Pharmaceutical Sciences, University of Tokyo,[b] 7–3–1, Hongo, Tokyo 113, Japan

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Changes in the distributions and chemical forms of biological metals, copper, iron, zinc, calcium and magnesium, produced by the administration of clioquinol in rabbits were investigated. Single administration caused a decrease in plasma zinc and excretion into urine of a large amount of iron (this may have originated from iron-binding proteins such as hemoglobin). Multiple administrations increased plasma copper, which was mainly bound to ceruloplasmin. Decrease in copper in the kidney, increase in copper in the cerebrum and increase in zinc in both the kidney and liver were also observed after multiple administrations.

Keywords——clioquinol; SMON; multiple administration; biological metals distribution; biological metals chemical form

The metabolism and toxicity of clioquinol (C), the causative agent of SMON (subacute myelo-optico-neuropathy),[1] have been studied since the identification of a green pigment isolated from the green urine and green feces of SMON patients as an iron chelate of C.[2] Significant changes in concentration of several metals were found in the tissues of SMON patients[3] and experimental animals[4,5] after multiple administrations of C. Moreover, both glucuronide (CG) and sulfate (CS) of C, which are considered to be the main metabolites of C in animals, were easily hydrolyzed to chelates with many metal ions.[6] The iron chelate of C has catalytic activity in the oxidative degradation of lipids,[7] and the denaturation of neurons caused by the oxidation of lipids is considered to be a possible mechanism involved in the neuropathy in SMON patients.[8] Further, many SMON patients were administered not only C but also metal-containing drugs.[9] Thus, research into the relationship between C and metals *in vivo* seems worthwhile as an approach to understanding the pathological mechanism of SMON. This paper describes the results of such a study in rabbits.

Experimental

Chemicals——C was kindly supplied by Tanabe Seiyaku Co., Ltd., and CG and CS (sodium salt) were prepared by the methods of Matsunaga and Tamura[10] and Chen *et al.*,[11] respectively. The other reagents used in the analyses of C, CG, CS and metals were all of analytical grade. Sephadex G-150 was purchased from Pharmacia Fine Chemicals. Pellet type feed for rabbits was Oriental Yeast RC-4 diet. Other reagents were all commercially available.

Apparatus——The high performance liquid chromatography (HPLC) systems used in the analyses of C, CG and CS were the same as described in previous reports.[12,13] A Shimadzu 3BE gas chromatograph equipped with an electron capture detector was also used for analyses of C, CG and CS. Metals were determined by the use of a Hitachi 308 atomic absorption spectrophotometer. The system for gel filtration was composed of an Atto SJ-1211 mini pump, a Pharmacia K16/40 column and a Toyo SF-100P mini fraction collector. The absorbance at 280 nm was measured with a Shimadzu 100-02 spectrophotometer. The incubation was performed in a Yamato BT-21 incubater, and centrifugation was performed in Kubota KC-25 and Kokusan H-11C centrifuges.

Animals——Male Japanese white rabbits (2—3 kg in weight) were used in the experiments after acclimation for at least a week. If necessary, the duodenum was cannulated with polyethylene tubing (Igarashi No. 25) about 1 cm

below the stomach and the common bile duct was cannulated (Igarashi No. 25) about 2 cm below its exit from the liver. More tubing (Igarashi NO. 30) was used to cannulate into the bladder. These tubings were run outside the abdominal wall. The above operations were all performed under pentobarbital anesthesia (35 mg/kg, intravenously injected). After the operations, the rabbits were settled in cages for one night.

Administration——Single administration: Five hundred mg of C suspended in 3.5 ml of 0.5% sodium carboxymethyl cellulose (CMCNa) solution was orally administered to each rabbit through a stomach tube, while 3.5 ml of 0.5% CMCNa solution alone was orally administered to control rabbits. After the administration, the diluted bile obtained from a control rabbit was passed into the duodenum through the tubing at 10 ml/h. Multiple administration: Five hundred mg of C was mixed with 100 g of solid diet and 150 ml of tap water. The mixture of C and diet was given to rabbits every morning for a month. Diet without C was given to control rabbits.

Sampling——In the single administration experiment, blood, urine and bile were collected at suitable intervals. In the multiple administration experiment, blood was collected just before every administration. Six hours after the last administration in this experiment, blood, urine and bile were all collected. The tissues were also sampled after perfusion with saline.

Analyses of Metals——An aliquot of each tissue was wet-digested with HNO_3–H_2SO_4–H_2O_2 and diluted with water. Samples of plasma, urine and bile were simply diluted adequately with saline or water. Copper, iron, zinc, calcium and magnesium were determined by atomic absorption spectrophotometry (flame method). The conditions were as follows; acetylene, 3 l/min; air, 13 l/min; analytical lines Cu 3247.5 nm (lamp current 15 mA), Fe 248.3 nm (15 mA), Zn 213.9 nm (5 mA), Ca 422.7 nm (10 mA), Mg 285.2 nm (10 mA).

Analyses of C, CG and CS——C, CG and CS in plasma and tissues were determined by HPLC[12] or gas chromatography.[14] CG and CS in urine and bile were analyzed by HPLC.[13]

Gel Filtration——The conditions were as follows: Gel, Sephadex G-150; gel bed, 1.6 cm i.d. × 40 cm; eluent, 0.3 M NaCl–0.05 M Tris-HCl (pH 7.8); flow rate, 3 ml/h; fraction volume, 1 ml/tube. One ml of plasma or 1.5 ml of urine was applied to the column. The absorbances at 280 nm of the fractions were measured, then the fractions were used for the other measurements.

Measurements of Ceruloplasmin——Ceruloplasmin activity was measured by the method of Sunderman and Nomoto.[15]

Results

Metals in Plasma

A single administration of C at a dose of 200 mg/kg, caused the plasma concentration of zinc to decrease significantly. The concentration decreased to the lowest level, 30—50% of the control level, usually at 6—10 h after the administration, then it gradually increased. Administration of CMCNa solution alone did not cause a marked change in zinc. The concentrations of the other metals did not show significant differences from the control levels, although the initial levels of iron in the two groups were different (Fig. 1).

Multiple administrations of C caused the plasma concentration of copper to increase, reaching a maximum on the 14th day after the start of the administration. The administration was suspended for 3 d because of the poor physical state (loss of appetite) of the rabbits given multiple administrations of C. After the suspension, the concentration of copper was not increased further by the multiple administration of C. The other metals were hardly changed by the multiple administrations (Fig. 2).

Metals in Urine and Bile

The concentrations of copper and zinc in urine and bile were not affected by a single administration of C, but an abnormally high concentration of iron, which may have originated from iron-binding proteins such as hemoglobin judging from the gel filtration profile (data not shown), was excreted in urine. However, the urine was not green, in contrast to the urine from SMON patients. The concentrations of the three metals in urine were not changed by multiple administrations of C.

Metals in Tissues

The concentration of copper in the kidney was lower in C-administered rabbits than in control rabbits, while the concentration in the cerebrum was higher in C-administered rabbits.

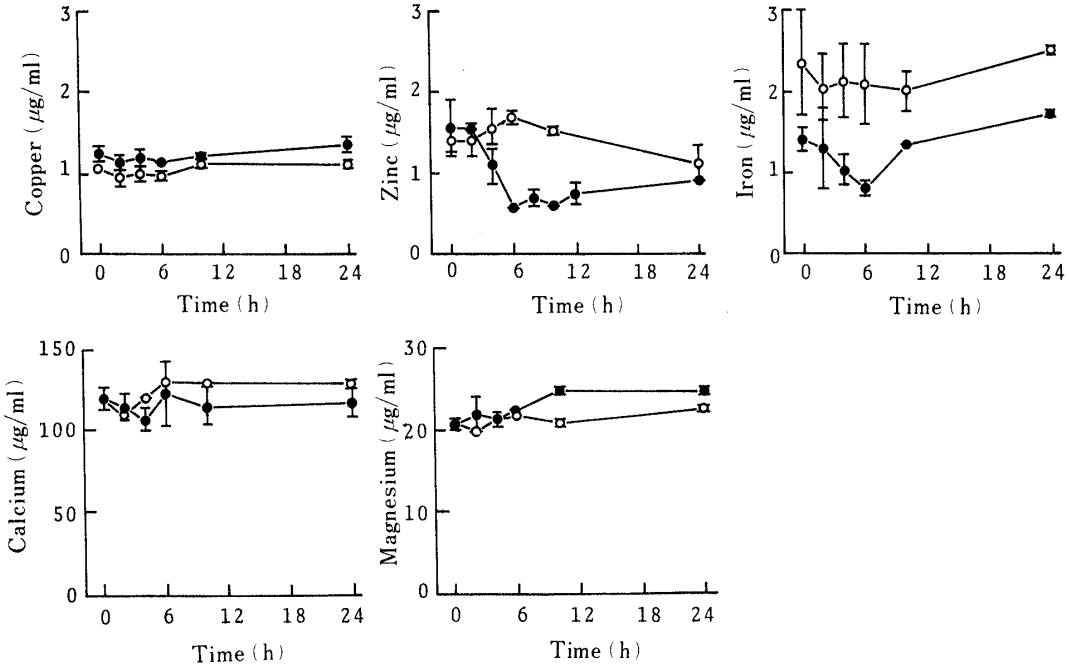

Fig. 1. Time Courses of Plasma Concentrations of Copper, Zinc, Iron, Calcium, and Magnesium after a Single Oral Administration of Clioquinol in Rabbits

○, control rabbits ($n=3$); ●, clioquinol administered to rabbits ($n=3$) at the dose of 200 mg/kg/d.
Points and vertical bars indicate the mean values and standard deviations.

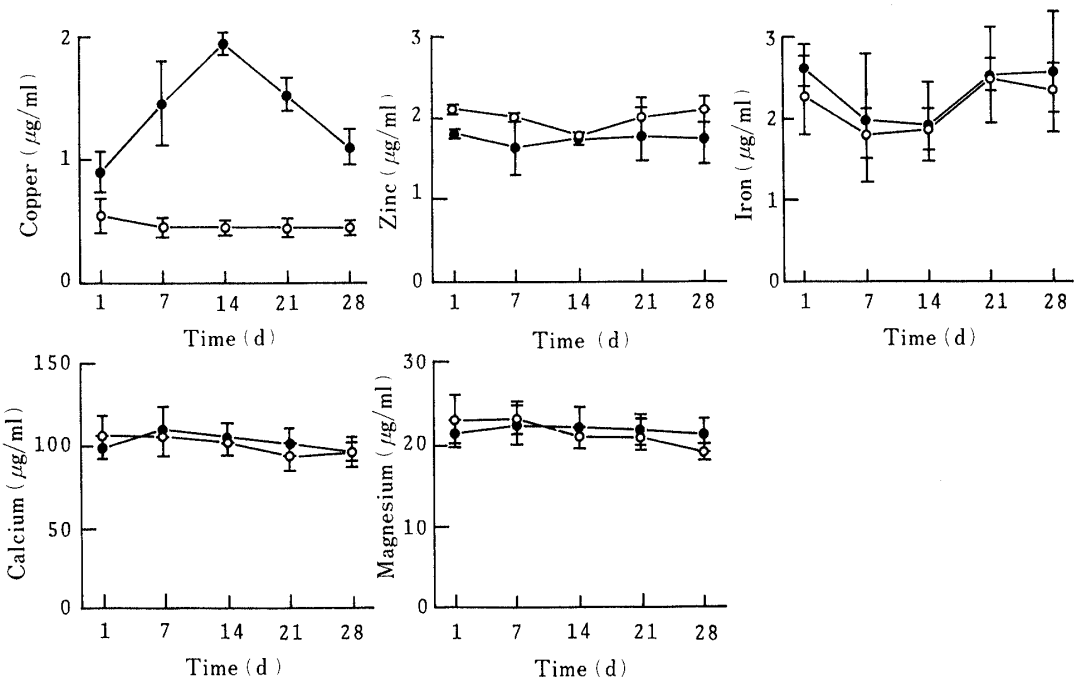

Fig. 2. Time Courses of Plasma Concentrations of Copper, Zinc, Iron, Calcium and Magnesium during Multiple Oral Administration of Clioquinol for a Month in Rabbit

○, control rabbits ($n=3$); ●, clioquinol administered to rabbits ($n=3$) at the dose of 200 mg/kg/d.
Points and vertical bars indicate the mean values and standard deviations.

TABLE I. Concentrations of Copper, Zinc and Iron in Tissues after Multiple Oral Administrations of Clioquinol for a Month in Rabbits

Tissue	Metal concentration (μg/g)					
	Control rabbits			Clioquinol-treated rabbits		
	Copper[a]	Zinc[a]	Iron[b]	Copper[a]	Zinc[a]	Iron[b]
Liver	1.9±0.2	8.0±0.7	21.9	1.9±0.3	12.7±3.8	12.0
Kidney	2.1±0.1	12.8±1.6	13.3	1.6±0.1	18.9±1.4	9.2
Cerebrum	1.5±0.1	6.2±1.1	6.6	1.9±0.3	5.9±1.1	7.2
Muscle	0.4±0.1	6.4±1.1	2.1	0.4±0.0	6.9±1.2	2.3

a) Mean value±standard deviation, $n=3$.
b) $n=1$.
Rabbits were sacrificed at 6 h after the last administration of C.

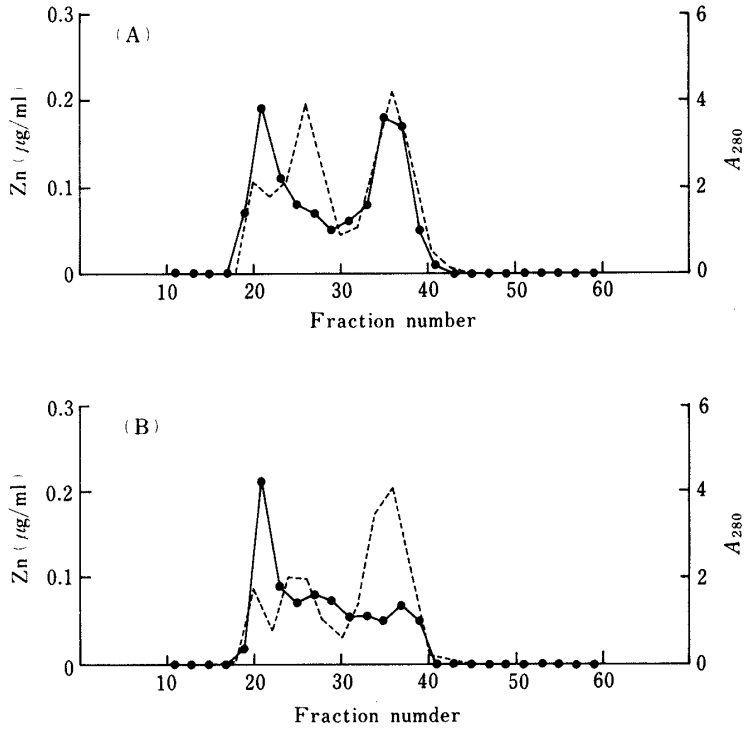

Fig. 3. Gel Filtration Elution Profiles of Zinc and A_{280} of Rabbit Plasma before and after the Administration of Clioquinol

(A), before the administration; (B), 24 h after the administration.
—●—, Zn; ----, A_{280}.

The concentration of zinc in the kidney and liver was higher in C-administered rabbits. The effects of C on the distribution of iron are not clear, since too few rabbits were available (Table I).

Discussion

Among the biological metals analyzed in plasma, only zinc was significantly affected by a single administration of C. The plasma level of zinc after administration of C at 200 mg/kg to a rabbit was the lowest at 10—24 h after the administration. The decrease of zinc in plasma of this rabbit was mainly due to decrease of albumin-bound zinc from the results of gel filtration (Fig. 3). The stability constants of C chelates of such metals as copper, iron and zinc are larger

than those of the chelates of calcium and magnesium. Albumin-bound zinc is known to be easily removable by dialysis against such chelating agents as 8-hydroxyquinoline-5-sulfonic acid,[16] whereas copper bound to ceruloplasmin and albumin, and iron bound to transferrin could not be removed by dialysis against the same reagent. The concentration of C in plasma

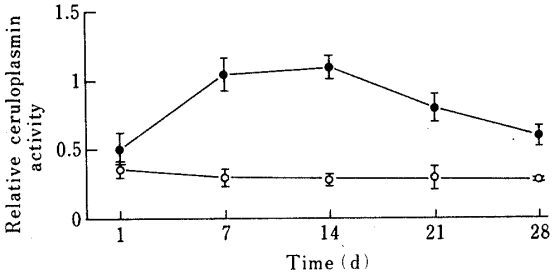

Fig. 4. Time Course of Ceruloplasmin Activity in Plasma during Multiple Oral Administrations of Clioquinol for a Month in Rabbits

○, control rabbits ($n=3$); ●, clioquinol-treated rabbits ($n=3$).
Points and vertical bars indicate the mean values and standard deviations.

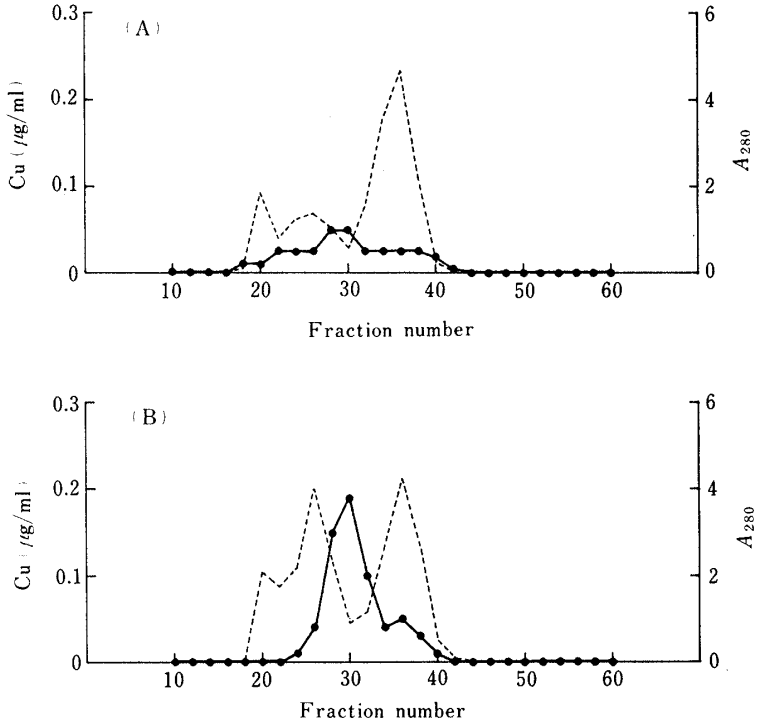

Fig. 5. Gel Filtration Elution Profiles of Plasma Copper

(A): control rabbit. (B): C-treated rabbit (C 200 mg/kg/d, 21 d).
—●—, Cu; ----, A_{280}.

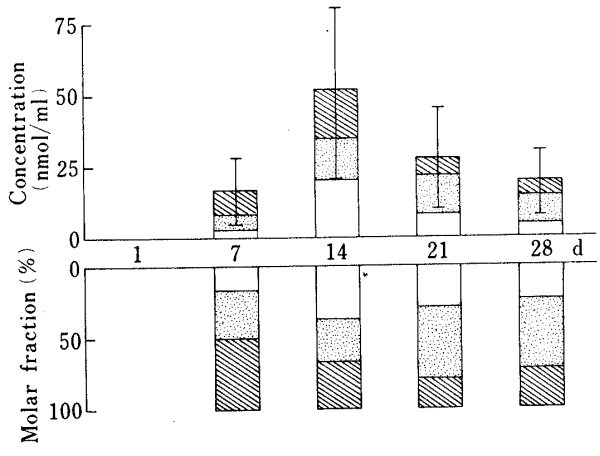

Fig. 6. Time Courses of Concentrations and Molar Fractions of C, CG and CS in Rabbit Plasma during Multiple Oral Administration of Clioquinol

Vertical bars indicate standard deviations in three rabbits.
C, ▭; CG, ▩; CS, ▥.

increased to 3×10^{-5} M—5×10^{-5} M, which is higher than that of zinc after a single administration of C, but we did not determine whether zinc could be removed from plasma albumin by C. On the other hand, diabetes was seen in rabbits given C[17] and serum zinc decreased in SMON patients when multiple administrations of C were suspended.[18] These findings suggest the easiness of change of the distribution and chemical form of zinc in the body with C. However, the maximum concentration of C in plasma would not reach 2×10^{-5} M during multiple oral administrations. This may be one of the reasons why the concentration of zinc in plasma was not changed by multiple administrations of C.

The activity of ceruloplasmin in plasma increased in parallel with copper content during multiple administrations of C (Fig. 4). The molecular weight of the main copper-binding compound was found to be 140000—160000 by gel filtration (Fig. 5). These results indicate that the increase of copper in plasma during multiple administrations of C is mainly due to ceruloplasmin-bound copper.

Both the molar fraction and concentration of C increased after the increase of ceruloplasmin activity (Fig. 6). Increased ceruloplasmin activity in plasma has been reported to be an index of such diseases as hepatitis, general infection, cancer and anemia,[19] and relationships between SMON and diseases of the liver and kidney were reported.[20-23] The changes of metals in rabbits resembled those in SMON patients[5] and rats.[7]

Moreover, the level of C in the kidney was the highest among all tissues tested after multiple administrations of C (data not shown). If the kidney was damaged by C, iron might be released from hemoglobin and be lost from the kidney to form the green iron(III) chelate of C.

Thus, C affects both the distribution and chemical form of some biological metals in rabbits. The next step is to examine the role of available metals in order to clarify the pathological mechanism of neuropathy found in SMON patients.

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