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# Relationship between Neurological Symptoms and Concentrations of Clioquinol in Serum and Nervous Systems of Beagle Dogs

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Beagle dogs were orally administered with a fixed dose of clioquinol, 300 mg/kg/day, in gelatin capsules every day. Other beagle dogs were orally administered with powder clioquinol, 300 mg/kg/day after increasing dose, in milk twice a day. All beagle dogs administered with clioquinol developed weakness of the hind-limbs and hip swaying in several ten days. Individual differences and daily variations in serum levels of clioquinol, its sulfate and its glucuronide were large. Clioquinol was detected at higher concentrations not only in central nervous system but also in peripheral nervous system, and was retained for a long time.

**Keywords**——clioquinol (chinoform); SMON; beagle dogs; neurological symptoms; concentrations in serum; concentrations in nervous system

Since the end of 1950's Japanese people noticed an occurrence of myelitis-like illness preceded by abdominal symptoms. This disease was designated as subacute myelo-optico-neuropathy (SMON) of unknown etiology. Severe outbreaks of SMON had occurred in Japan since 1967.[2]

In 1970, Yoshioka and Tamura[3] isolated green pigment from the green urine and green feces of SMON patients, and identified the pigment as the iron (III) chelate of 5-chloro-8-hydroxy-7-iodoquinoline (clioquinol, chinoform). These findings, reported at the general meeting of the SMON Research Commission, led to epidemiological surveys by Tsubaki[4] and others.[5]

The results indicated the existence of correlation between SMON and clioquinol. By the official ban of the sale of clioquinol, number of occurrence of SMON patient rapidly decreased, and since 1974, no case has been reported. This result strongly nominated clioquinol for a main etiological agent.

Many animal experiments on neurological toxicity of clioquinol were carried out and symptoms like SMON were recognized in case of dogs,[6–9] monkeys,[6,9] cats,[6] rabbits,[10,11] fowls,[12] and quails.[13] The most representative work has been done by Tateishi and co-

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2) I. Sobue, K. Ando, M. Iida, T. Takayanagi, Y. Yamamura and Y. Matsuoka, *Neurology*, **21**, 168 (1971).
3) M. Yoshioka and Z. Tamura, *Igaku no Ayumi*, **74**, 320 (1970).
4) T. Tsubaki, Y. Honma and M. Hoshi, *Report of the SMON Research Commission*, No. 2, 143 (1971).
5) Y. Yoshitake and A. Igata, *Igaku no Ayumi*, **74**, 598 (1970).
6) J. Tateishi, S. Kuroda, A. Saito and S. Otsuki, *Psychiatrica et Neurologia Jap.*, **74**, 739 (1972).
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9) Y. Egashira, *Annual Report of SMON Research Commission*, **1973**, 53.
10) A. Igata and Y. Toyokura, *Igaku no Ayumi*, **75**, 309 (1970).
11) T. Takeuchi, *Annual Report of SMON Research Commission*, **1972**, 110.
12) Y. Ikeda, M. Tobe, Y. Suzuki, K. Kobayashi, S. Suzuki and Y. Kawasaki, *Report of the SMON Research Commission*, No. 3, 190 (1971).
13) S. Otaki and Y. Egashira, *Report of the SMON Research Commission*, No. 9, 38 (1972).

workers.[8] By the oral administration with continuous increasing daily dose of clioquinol, beagle dogs showed paralysis of the hind-limbs, and also showed pathological changes in the spinal cord and the optic tracts, which were quite similar to those in SMON patients. Hess, *et al.* reported that beagle dogs did not show any symptom after the fixed dose administration of clioquinol, in spite of high dose and long duration.[14] Another report supporting Tateishi's finding appeared recently.[15]

Chen, *et al.* demonstrated that the large species difference among man, monkeys, and mongrel dogs was caused by the difference in easiness of increasing serum level of clioquinol.[16] But the serum concentrations in beagle dogs were not examined.

There remained the discrepancy between pathological feature and distribution pattern of $^{14}$C-clioquinol. In SMON patients, the spinal cord as well as the peripheral nerves were degenerated, while the incorporation of the radio-labelled clioquinol into the former of dogs was much smaller than into the latter.[17] It seemed to be probable that this discrepancy was caused by the difference in transferring speed of clioquinol owing to the existence and absence of the blood-brain barrier, and that the concentrations of clioquinol in both of the nervous systems would be similar after the continuous administration of clioquinol.

Therefore, we administered beagle dogs with clioquinol in University of Okayama not only by increasing dose, as Tateishi's method, but also by fixed dose, as Hess's method.[18] In the process of these experiments, clioquinol and its conjugates in serum and in nervous systems were determined in University of Tokyo by gas chromatography.[19]

## Experimental

**Materials**——Nine female beagle dogs, 8 months after birth, about 8 kg weight, were purchased from CLEA-JAPAN-INC. Entero-vioform (clioquinol 93%+sapamine 7%), obtained from CIBA-GEIGY Ltd., was packed in gelatin capsules (Enterovioform 200±10 mg/capsule). Milk was commercially available. Dogs were fed a solid ration (CD-1 made by CLEA-JAPAN-INC.).

**Administration and Sampling**——Group 1 (dog No. : 593, 611, 638): Each dog was orally administered with fixed dose of Entero-vioform (clioquinol 300 mg/kg) in gelatin capsules at 9: 00 every morning. Group 2 (dog No.: 604, 615, 623): Each dog was orally administered with powder Enterovioform, which was increased from 150 mg/kg/day to 300 mg/kg/day by 50 mg/kg on every three days, with 60 ml of milk at 9: 00 and 17: 00. The administration was stopped at 32nd, 33rd, and 34th day, for the beagle dogs became so weak. After increasing dose, 300 mg/kg/day was administered constantly from 42nd day. Group 3: None-treated three dogs were taken as controls. They were given such a ration which was equivalent to the average daily feed consumption by Groups 1 and 2. The temperature in the room controlled at 20±2°.

One ml of blood was sampled from vein of fore-limbs at 9: 00, 11: 00, 17: 00, and 19: 00, and if necessary, at more times. Blood was centrifuged and serum was stored. Just after death, serum, urine, and tissues were sampled from the beagle dogs and were immediately frozen at −70°.

**Appearance of Neurological Symptoms**——Weakness of the hind-limbs and hip swaying were observed as the appearance of neurological symptoms.[7,18]

**Determination of Clioquinol (CF), Its Glucuronide (CFG), and Its Sulfate (CFS)**——CF, CFG, and CFS in serum and in urine were determined by the method of Chen, *et al.*[19]

CF in nervous systems was determined as follows. 0.2—0.3 g of tissue was added with 7-bromo-5-chloro-8-hydroxyquinoline as an internal standard. The sample was homogenized with 0.7 ml of pyridine, and ultra sonicated. The homogenate was added with 2 ml of benzene, shaken and centrifuged. The upper phase was evaporated to dryness and dissolved in 3.0 ml of benzene. The solution was put on alumina column (2.0×0.3 cm i.d.). After washed with 3.0 ml of acetone, alumina was pushed out into a centrifuge tube, containing 2.0 ml of NaF-saturated water and 3.0 ml of benzene, and was vigorously shaken. After centrifuging, benzene phase was taken out and evaporated to dryness. The residue was added with 0.2 ml

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19) C.T. Chen, K. Samejima and Z. Tamura, *Chem. Pharm. Bull.* (Tokyo), **24**, 97 (1976).

of acetic anhydride–pyridine (1: 1) and heated at 60° for 30 min. The solution was evaporated to dryness and resolved in 0.1—0.2 ml of *n*-hexane, 1—5 μl of which was injected into a gas chromatograph equipped with electron capture detector.[19]

The extraction recovery of CF from nervous systems was examined as follows. CF in the tissues from rabbits, successively administered with Entero-vioform, was extracted according to the method described above. The extraction, evaporated to dryness, and the residue were dissolved in conc. $H_2SO_4$ and the content of iodine was analyzed with the protein bound iodine auto-analyzer.

## Results and Discussion

### Appearance of Neurological Symptoms

All beagle dogs administered with clioquinol showed the neurological symptoms. The dates of the appearance of symptoms varied more widely in Group 1 than in Group 2 (Table I).

Table I. Appearance of Neurological Symptoms

| Group | Dog No. | Day of appearance |
|---|---|---|
| 1 | 593 | 47 |
| | 611 | 19 |
| | 638 | 29 |
| 2 | 604 | 26 |
| | 615 | 29 |
| | 623 | 26 |

### Concentrations in Serum

It was expected that the maximum serum levels of CF in a day would appear two hours after the administration. Moreover the dogs in Group 2 received twice a day. Then blood sampling was performed at 9: 00, 11: 00, 17: 00, and 19: 00. The concentrations of CF,

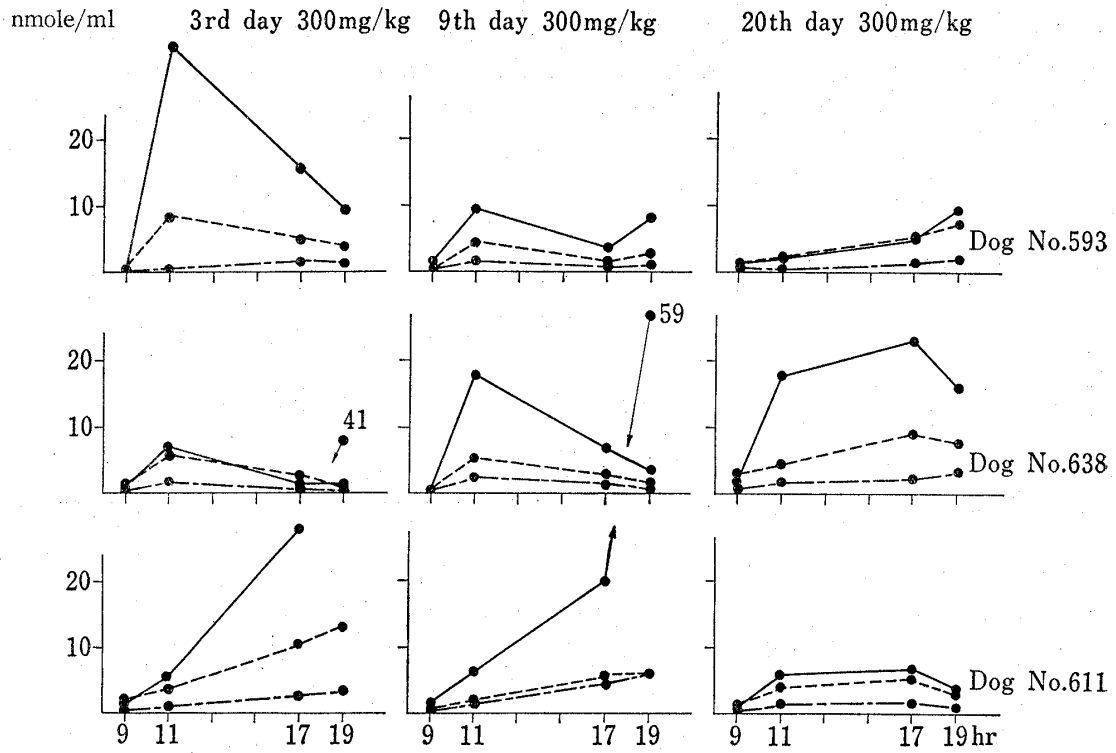



Fig. 1. Time Course of Serum Levels of Clioquinol (——), Its Sulfate (------), and Its Glucuronide (–·–·–), until the Appearance of Neurological Symptoms in Beagle Dogs (Group 1)

CFG, and CFS on day 3, 9, and 20 after the beginning of the daily continuous administration were analyzed (Fig. 1 and 2). Individual differences and daily variations of serum levels were large in Group 1, which received Entero-vioform in capsules once a day.

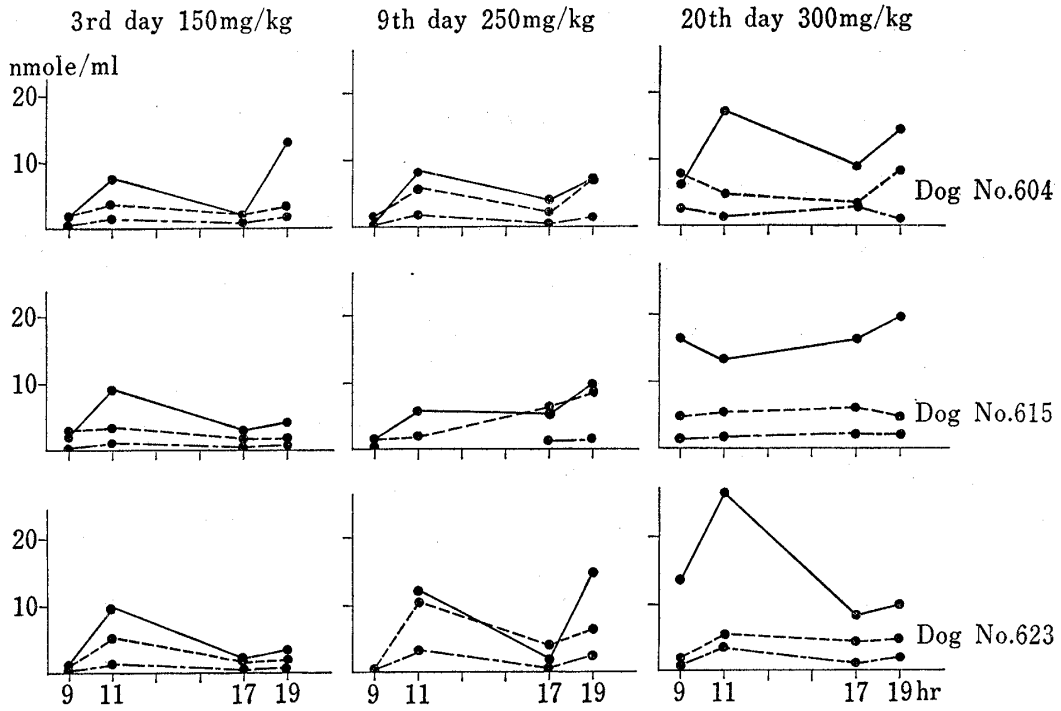



Fig. 2. Time Course of Serum Levels of Clioquinol (——), Its Sulfate (------), and Its Glucuronide (----) until the Appearance of Neurological Symptoms in Beagle Dogs (Group 2)

Serum levels of CF, CFG, and CFS in a day often showed "two phases" (Fig. 3). It was reported that serum levels of Persantin, a similarly insoluble drug in water, made two phases by having diet after the oral administration.[19] So the two phases in this experiment could have been caused by having diet.

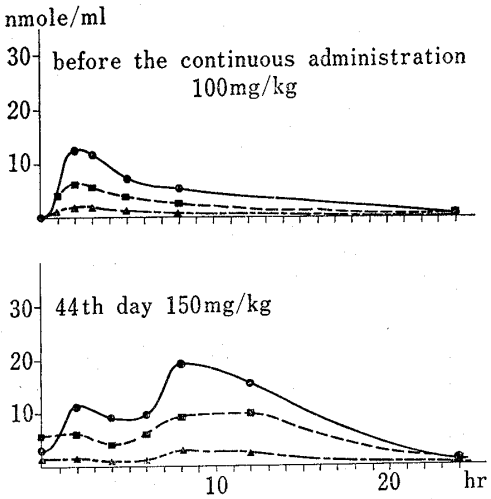
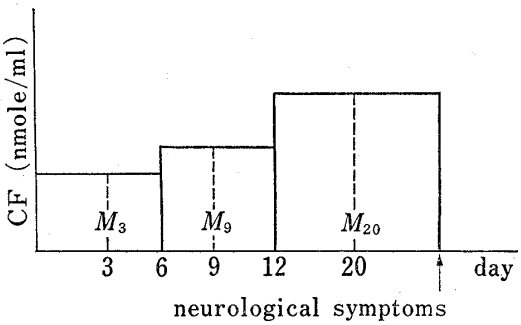



Fig. 3. Time Course of Serum Levels of Clioquinol (——), Its Sulfate (-----), and Its Glucuronide (----) after Oral Administration in the Beagle Dog (No. 615)




$C_i$=concentration at the time ($i$=9:00, 11:00,17:00, or 19:00)
$M_j$=mean concentration in the day ($j$=3rd, 9th, or 20th day)
$=\sum C_i/4$
integrated serum level$=\sum(M_j \times \text{day})$

Fig. 4. Integrated Serum Level

Moreover it is possible that the long term continuous administration of clioquinol could have changed the character of stomach or intestine concerning absorption of clioquinol.

From the concentrations of CF in serum in Fig. 1 and in Fig. 2, one may calculate the integrated serum levels of CF according to the formula in Fig. 4. The mean level of each day can be calculated from four time concentrations of CF. It may be supposed that the mean level of the 3rd day would be kept from the lst day to the 5th day, the mean level of the 9th day from the 6th day to the 12th day, and the mean level of the 20th day from the 13th day to the day of appearance of the symptoms. The samples are only six and this formula contains many hypotheses. But the difference in the integrated serum levels such calculated is smaller than those in total dose (Table II). From these data, it may be said that the integrated effects of exposure of nervous systems to CF was related to the appearance of neurological symptoms. The hypothesis would be supported by the experiments of cultivated fetal posterior ganglion of rats or mice.[20] The degeneration, swelling and filling with vacuoles of mitochondria in the axon required a shorter period in higher concentrations of CF, but a longer period in lower concentrations of CF.

Table II. Total Dose and Integrated Serum Levels of Clioquinol until the Appearance of Neurological Symptoms in Beagle Dogs

| Dog No. | Total dose g/kg | Integrated serum level (nmole/ml)·day |
|---|---|---|
| 593 | 13.8 | 270 |
| 611 | 5.4 | 303 |
| 638 | 8.4 | 272 |
| 604 | 6.0 | 224 |
| 615 | 6.9 | 311 |
| 623 | 6.0 | 268 |
| C.V. (%) | 40 | 11 |

**Concentrations in Nervous Systems**

The three beagle dogs were sampled at different stages and the tissues were analyzed for only CF (Table III). In No. 611, died of intestinal invagination 15 days after the appearance of the symptoms, CF was contained not only in peripheral nervous system but also in central nervous system. In No. 623, sacrificed 28 days of continuous administration and a day of suspension after the appearance of symptoms, the concentrations of CF in nervous systems were higher than those in serum at death. Moreover the concentrations in central nervous system were close to those of peripheral system. In No. 615, sacrificed 31 days of administration and 28 days of suspension after the appearance of symptoms, CF was not detected in serum at death, while the detectable quantities retained in some tissues of nervous systems. In urine of this beagle dog, large quantity of CFG was detected.

The high concentrations of CF in fat were detected not only of SMON patients[21] but also of No. 615 and of No. 623. These facts might be concerned with that the number of SMON patients is higher for middle or advanced age than for young, and the number for female is about twice as much as those for male.

The similarly high levels of CF in central nervous system to those in peripheral nervous system, attained by the continuous administration, are quite different from the distribution pattern of $^{125}$I-CF by a single administration.[17] The results demonstrate that CF pass,

20) T. Yonezawa, *Annual Report of SMON Research Commission*, 1972, 92.
21) Z. Tamura, M. Yoshioka, T. Imanari, J. Fukaya, J. Kusaka and K. Samejima, *Clin. Chim. Acta*, 47, 13 (1973).

Table III. Clioquinol Levels[a] in Nervous Systems

| | | 611 | 623 | 615 | Extraction[b] recovery (%) |
|---|---|---|---|---|---|
| Frontal lobe of cerebrum | | | 5.0 | 3.8 | |
| Cerebellum | | | 7.7 | 2.4 | 40 |
| Hypophysis | | 34.6 | 18.6 | t | |
| Cervical spinal cord | | 5.0 | 13.7 | t | 75 |
| Thoracic spinal cord | | | 6.4 | t | |
| Lumbar spinal cord | | 3.7 | 7.0 | t | |
| Spinal ganglions | | | 13.9 | t | |
| Sciatic nerve | | 14.8 | 15.8 | 3.2 | 75 |
| Optic nerve | | 13.4 | 10.3 | t | |
| Sural nerve | | | 10.1 | t | |
| Subcutaneous fat | | 14.1 | | | |
| Perirenal fat | | | 78.5 | | |
| Liver | | 19.7 | | | |
| Kidney | | 7.0 | | | |
| Serum | CF | 10.0 | 4.3 | 0 | |
| | CFS | 8.3 | 2.2 | 0.3 | |
| | CFG | 4.2 | 0.9 | 0 | |
| Urine | CF | | 5.4 | 0.3 | |
| | CFS | | large | 1.8 | |
| | CFG | | large | >10 | |

Units; nmole/g or nmole/ml.
t; trace.
611; 15 days after the appearance of symptoms.
623; 29 days after the appearance of symptoms.
615; 28 days after the suspension of clioquinol.
*a*) The values obtained with tissues were of CF and uncollected for extraction recovery.
*b*) The values were obtained with rabbits.

even slowly, into central nervous system from blood. In this case the blood-brain barrier may act as a resister.

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