Transmethylation Reactions and Autoradiographic Distribution of Vitamin B₁₂: Effects of Clioquinol Treatment in Mice

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ABSTRACT—The catastrophic epidemic of subacute myelo-optic neuropathy (SMON) affected Japan around 1970 with thousands of victims. The cause was attributed to high doses of locally acting oxyquinolines. It has been speculated that oxyquinoline derivatives of the clioquinol type can disturb the retention of vitamin B₁₂ through chelation of Co²⁺. In the present paper, possible effects of clioquinol on the uptake and tissue distribution of [⁵⁷Co]-cyanocobalamin have been studied in mice. In vivo experiments showed markedly decreased accumulation of radiolabelled vitamin B₁₂ in the kidney and skin in animals that were pre-treated with clioquinol. The chloroform:water partition coefficients for [⁵⁷Co]-cyanocobalamin in the presence or absence of clioquinol were also determined. No statistically significant alterations in the partition coefficient for [⁵⁷Co]-cyanocobalamin in the presence of clioquinol was evident, indicating that clioquinol does not bind cobalt. In addition, transmethylation reactions in the CNS in mice treated with clioquinol were studied. Specific activities of methionine adenosyltransferase, and S-adenosylhomocysteine levels were not affected. In contrast, clioquinol treatment caused a significant increase in the levels of S-adenosylmethionine in the brain. The data of the present study show that clioquinol treatment can affect the accumulation of vitamin B₁₂ in the kidney and the skin but not in the brain. These results do not support the hypothesis that clioquinol causes its damage to the nervous system by a direct chemical interaction with vitamin B₁₂.

Keywords: S-Adenosylmethionine, L-Methionine S-adenosyltransferase, Clioquinol, Vitamin B₁₂, Subacute myelo-optic neuropathy (SMON)

The catastrophic epidemic of subacute myelo-optic neuropathy (SMON) affected mostly Japan with thousands of victims in the 50s, 60s and 70s (1, 2). The cause was found to be the use of high doses of "locally acting" oxyquinolines such as clioquinol (5-chloro-7-iodo-8-quinolinol; Enterovioform®). The symptoms of SMON include abdominal pains, sensory disturbances, motor paralysis and loss of visual acuity (2). For unknown reasons the number of affected individuals was much lower in other parts of the World outside Japan (3). The pathogenetic mechanisms behind SMON are, however, still unknown. Clioquinol is a powerful chelator of divalent cations and the chelates have high lipophilicity, which has lead to the speculation that clioquinol could work as a heavy metal transporter to the CNS (via clioquinol-heavy metal chelation), causing metal toxicity in nerve tissue (4, 5).

Several authors have noted the similarity between many of the SMON symptoms and the neurological manifestations of vitamin B₁₂ deficiency, and it has been suggested that clioquinol could chelate cobalt in vitamin B₁₂ (6), but this has never been studied (H. Shiraki and M. Xilinas, personal communication). Deficiency of vitamin B₁₂ is mostly due to failure of the stomach to produce an intrinsic factor secondary to undernourishment, pregnancy or tape worm infections. Signs of vitamin B₁₂ deficiency vary from one species to another but are always severe. Degeneration of the nervous system may occur, and the neuropathies after B₁₂ deficiency are often irreversible. In one study, the B₁₂ levels in the cerebrospinal fluid (CSF) from SMON patients were found to be normal, but this is not particularly remarkable since these studies were carried out a long time after diagnosis, and if a change would have occurred initially, the patients were probably B₁₂ normalized at the time of analysis (6). Despite the severe side-effects of the SMON-type, oxyquinoline preparations

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are still used for treatment of bacterial infections, especially in the 3rd World (7). Vitamin B₁₂ binds to the serum transport protein, transcobalamin II (TC II) and enters cells by receptor-mediated endocytosis. The internalized TC II-B₁₂ complex undergoes processing, which leads to TC II degradation and the release of free oxidized cobalamin [Cob(III)alamin]. This form undergoes reduction to a divalent state [Cob(II)alamin] before binding to methionine synthase.

The synthesis of *S*-adenosylmethionine (SAM) from methionine and ATP is catalyzed by methionine adenosyltransferase (MAT). The demethylated product of SAM is *S*-adenosylhomocysteine (SAH), which is metabolized to adenosine and homocysteine by SAH hydrolase. Through complex reactions catalyzed by the vitamin B₁₂- and a methyltetrahydrofolate-dependent enzyme, methionine synthetase (E.C. 2.1.1.13), homocysteine is converted to methionine (8, 9).

Reduced levels of SAM in the cerebrospinal fluid of patients with Parkinson's disease (10), Alzheimer's disease (11), subacute combined degeneration of spinal cord (12) and AIDS/HIV-dementia complex syndrome (10), as well as changes in the activity of MAT in patients with schizophrenia and bipolar disorders have been reported previously (13). It has been demonstrated that high levels of SAM reduce MAT catalytic activity (14) and decrease MAT gene transcription (15) by a negative feedback mechanism.

In the present study, we have explored the possibility that an oxyquinoline derivative, such as clioquinol, can cause disturbances in the metabolism and retention of vitamin B₁₂ through chelation of Co²⁺. With an autoradiographic method, using radiolabelled vitamin B₁₂, and other biochemical methods, we have studied vitamin B₁₂ distribution and transmethylation reactions in the CNS in mice treated with clioquinol.

MATERIALS AND METHODS

Chemicals

5-Chloro-7-iodo-8-hydroxy-quinolin (clioquinol) was a gift from Dr. M. Xilinas (Lundbeck, Copenhagen, Denmark). [⁵⁷Co]-Cyanocobalamin (spec. act. 370 Bq/μg cyanocobalamin) was purchased from Amersham Life Sciences (Amersham, England). Radiolabelled [methyl-³H]-methionine (191 mCi/mmol) was obtained from Du Pont (Mechelen, Belgium), and the ion exchange resin (Biorad AG 50w-x8) was purchased from Biorad Laboratories (Richmond, CA, USA). Ready Safe TM liquid scintillation fluid was obtained from Beckman (Fullerton, CA, USA). *S*-Adenosylmethionine *p*-toluene sulfonate, *S*-adenosylhomocysteine and all other compounds were purchased from Sigma Chemical Co. (St. Louis, MO,

USA).

Animal treatments

Male NMRI mice, with body weights of approximately 35 g, were obtained from B&K (Sollentuna, Sweden). Animals were housed in groups of 5 animals per cage with a 12-hr light dark cycle, at a constant temperature of 21°C. Six-week-old male mice were pre-treated with either saline 0.9% or clioquinol (50 mg/kg/day) by intraperitoneal (i.p.) injection for three days and subsequently injected i.p. with [⁵⁷Co]-vitamin B₁₂ in doses of 1 μCi per animal, given in a volume of 0.1 ml. At 48 hr after injection of the radiotracer, the animals were sacrificed.

For the determination of MAT, SAM and SAH, mice were injected i.p. with either 0.9% saline or clioquinol (50 mg/kg/day) for 7 days. At the times indicated in the text, animals were killed by decapitation after CO₂-asphyxiation. Brains were rapidly removed, and the cerebellum, cerebrum and brainstem were dissected from each hemisphere and frozen immediately on dry ice.

Whole-body autoradiography

After sacrifice, the mice were immersed in carboxymethyl cellulose (CMC) that was frozen in an ethanol bath (−78°C). Sagittal whole-body sections, 20-μm-thick, were cut and collected on tape. The tapes containing the sections were freeze-dried at −10°C. The autoradiographic technique has been described in detail by Ullberg (16). Autoradiographs were made by apposition of the sections against X-ray films (Hyperfilm, Amersham Life Science). After exposure for 5 months, the films were developed. The autoradiographs were analyzed by computerized densitometry using a previously described system (17).

Scintillation counting

In parallel to the autoradiographic studies, quantitative measurements of the distribution of radioactive vitamin B₁₂ were performed. Two series of adult mice received intraperitoneal injections of saline (0.9%) or clioquinol (50 mg/kg). The injections were given every 24 hr for 3 days, followed by intraperitoneal injection of 0.1 ml [⁵⁷Co]-cyanocobalamin on the fourth day. The animals were sacrificed 48 hr after the last injection, and specimens from the brain, liver and kidney were dissected. The tissue specimens were weighed and assayed for radioactivity in a gamma spectrometer (Wallac Co., Turku, Finland). The values obtained with the assays were calculated to give the concentration of radioactivity per gram of the tissue.

Determination of chloroform:water partition coefficients

The chloroform:water partition coefficient of labelled [^{57}Co]-cyanocobalamin in the absence or presence of clioquinol was determined. The amount of radioactivity was 1 $\mu\text{Ci/ml}$. Clioquinol was dissolved in chloroform to a concentration of 100 mM. Two milliliters of chloroform, with or without clioquinol, and 2 ml of water were mixed vigorously for 10 min. The samples were then kept for 2 days at room temperature in capped test tubes. The amount of radioactivity in 100- μl aliquots in both the organic and the aqueous phase was then determined in a Packard Auto-gamma spectrophotometer. The ratio of the cpm's in the chloroform phase to the cpm's in the aqueous phase was considered the chloroform:water partition coefficient.

Preparation of brain homogenates

For the assay of MAT activity, dissected brain regions were resuspended in 0.01 M phosphate buffer, 1 mM EDTA, 0.25 M sucrose (pH 7.4), homogenized by ten strokes with an Ultra-Turrax, and frozen at -72°C until the assays were performed. Protein concentrations were assayed by the method of Markwell et al. (18).

For the estimation of the concentrations of SAM and SAH, the frozen tissues were homogenized in 17 volumes (v/w) of 0.4 M perchloric acid (HClO_4) containing 3.2 mM $\text{Na}_2\text{S}_2\text{O}_5$ and 10 mM EDTA. After centrifugation ($9000 \times g$, 10 min, 4°C), the supernatant was decanted and filtered (simple glass filter) and 4 N KOH added to adjust the pH to 7.0. After 15 min on ice, the samples were centrifuged ($8000 \times g$, 10 min, 4°C) to remove the precipitate. The supernatants were stored for 20 min at 4°C until separation on columns.

Assay of SAM and SAH by reversed phase HPLC-UV

Bakerbond[®] columns (Millinckrodt Baker Co., Deventer, Netherlands) were activated by application of 3 ml of methanol and 3 ml water. Then, 2-ml aliquots of samples were applied, followed by 0.5 ml of water and 0.2 ml of 0.175 M acetic acid. Thereafter, SAM and SAH were eluted with 1 ml of 0.175 M acetic acid and 1 ml of 25% methanol in 0.175 M acetic acid. Finally, the samples were diluted with 0.5 ml of water and stored at -20°C until HPLC analyses.

The HPLC-system consisted of a M-45 Solvent Delivery System (Waters Associates, Inc., Milford, MA, USA), a Rheodyne 7125 injector with a 0.25-ml sample loop, a cartridge guard column and a stainless steel column, connected to a variable wavelength absorbancy detector (LDC spectroMonitor Model 1204A) operated at 260 nm. The mobile phase consisted of a phosphate buffer (0.05 M, pH 3.5) containing 3.1 mM octanesulfonic acid (OSA) and 12.5% methanol. The solid phase was a

Nucleosil[®] 5 C_{18} column for HPLC (Macherey-Nagel, Düren, Germany). SAM and SAH standards were diluted in 12.5% methanol in 0.175 M acetic acid. The flow rate of the mobile phase was 1 ml/min. The turnover time per sample was 25 min, and the concentrations were calculated by reference to peak heights of external standards.

Assay of MAT catalytic activity

MAT activity was assayed by a radiometric method according to previously described procedures (15, 19), measuring the rate of formation of *S*-adenosyl[methyl- ^3H]methionine from ATP and L-[methyl- ^3H]methionine.

Statistical analyses

The results are given as means $\pm$ S.E.M. and for statistical analysis, Student's unpaired *t*-test was used. *P*-values less than 0.05 were considered statistically significant.

RESULTS

Autoradiography

The objective of this study was to investigate if clioquinol treatment can cause alterations of the accumulation of radiolabelled vitamin B_{12} in tissues of mice. The tissue distribution of vitamin B_{12} was analyzed with semi-quantitative densitometry in autoradiographs of whole-body cryosections of mice after an i.p. injection of labelled vitamin B_{12} . The accumulation of radioactive vitamin B_{12} was highly tissue-specific (Fig. 1). The most pronounced accumulation was observed in the kidneys and skin of both control mice and animals treated with clioquinol. Using this autoradiographic technique, data on the relative amount of [^{57}Co]-vitamin B_{12} was obtained from a number of tissues (Table 1). With regard to the activity in kidney, the levels were above the level that can be accurately quantitated, i.e., the films were "over-exposed" with respect to this particular organ. In control animals, considerable accumulation was observed in the liver, pancreas and brown adipose tissue. In contrast, skeletal muscle, intestine and the CNS displayed very low accumulation of [^{57}Co]-vitamin B_{12} . The mucosa of the large intestines showed much higher radioactive concentration than the mucosa of the small intestines (data not shown).

In sections of animals treated with clioquinol, the general distribution pattern of [^{57}Co]-vitamin B_{12} was similar to that in sections of control mice. However, a highly significant decrease in radioactive accumulation was observed in the skin ($P < 0.01$). Also, in the kidney a relative decrease was apparent (Fig. 2); however, the magnitude of this change was not quantifiable.

Fig. 1. An autoradiograph of a 20- μ m-thick whole-body cryosection of an adult mouse 48 hr after an injection with [^{57}Co]-vitamin B $_{12}$. Notice the massive accumulation of radiolabelled vitamin B $_{12}$ in the kidney.

Table 1. Analysis of [^{57}Co]-cyanocobalamin distribution in whole-body autoradiographs of mice after treatment with saline or clioquinol

Tissue	Control (OD)	Clioquinol (OD)
Liver	69.5 $\pm$ 11.1	71.8 $\pm$ 9.3
Forebrain	13.3 $\pm$ 2.0	15.3 $\pm$ 1.0
Brainstem	9.2 $\pm$ 1.6	11.5 $\pm$ 2.3
Kidney	(100.0 $\pm$ 15.0)	(75.7 $\pm$ 27.7)
Adipose tissue	56.3 $\pm$ 7.7	52.7 $\pm$ 5.2
Skeletal muscle	9.5 $\pm$ 3.3	6.8 $\pm$ 2.0
Intestine	12.8 $\pm$ 3.4	10.0 $\pm$ 1.8
Skin	69.8 $\pm$ 2.3	37.3 $\pm$ 4.8*
Pancreas	62.3 $\pm$ 12.3	83.5 $\pm$ 2.5

The data represent relative optical density (OD) reported as the mean $\pm$ S.E.M. of 5 mice in each group (* $P < 0.01$).

Scintillation counting

In order to validate the autoradiographic study, the accumulation of radiolabelled vitamin B $_{12}$ was quantified by scintillation counting for some organs. Three different tissues were dissected after injection with [^{57}Co]-vitamin B $_{12}$: brain, kidney and liver. The kidneys of control mice had the highest accumulation per gram tissue (wet weight). In control mice the amount of radiolabelled vitamin B $_{12}$ trapped in the kidney was approximately 10 times higher than that in liver and approximately 100

times higher than that in the brain. When tissues from mice treated with clioquinol were compared with tissues from control animals, a statistically significant reduction in the kidney accumulation was evident ($P < 0.01$) (Table 2); i.e., the levels of radioactivity in kidneys from mice treated with clioquinol were approximately 30% of those observed in controls.

Partition coefficients

It has been reported that clioquinol is able to form hydrophobic chelates with divalent metal ions. Hence, the partition coefficient for such a chelate is dramatically changed as compared to the metal ion itself. In the present investigation, the partition coefficients (chloroform:water) for [^{57}Co]-vitamin B $_{12}$ was determined in the absence or presence of clioquinol. No shift in the partition coefficient was evident when the radioactive vitamin was incubated in the presence of clioquinol. The ratios of radioactivity between the chloroform phase and the water phase (cpm in chloroform/water) were approximately 0.0002, both in the absence and presence of clioquinol, indicating that no hydrophobic chelates were formed between clioquinol and the cobalt in [^{57}Co]-vitamin B $_{12}$.

Estimation of MAT, SAM and SAH

As a complement, some other steps of the "one-carbon cycle" were also studied after clioquinol treatment since it

Fig. 2. Autoradiographs corresponding to whole-body cryosections of adult mice treated with clioquinol (Cli) or control animals treated with saline (Sal). Thereafter the animals were injected with [^{57}Co]-vitamin B $_{12}$ 48 hr prior to sacrifice.

Table 2. Distribution of radiolabelled vitamin B $_{12}$ ([^{57}Co]-cyano-cobalamin) in mouse tissues after clioquinol treatment

Treatment	Brain	Liver	Kidney
Control	9.4 $\pm$ 0.9	97 $\pm$ 8	895 $\pm$ 207
Clioquinol	8.4 $\pm$ 1.5	85 $\pm$ 21	252 $\pm$ 61*

The data represent thousands cpm/g tissue (wet weight) reported as the mean $\pm$ S.E.M. of 5 mice in each group (*P<0.01).

Table 3. Activities of methionine adenosyltransferase in mice treated with clioquinol for 7 days and controls

Brain region	MAT specific activity (pmol/mg/min)	
	control	clioquinol
Cerebrum	2.540 $\pm$ 0.14	2.280 $\pm$ 0.11
Brainstem	3.380 $\pm$ 0.55	4.040 $\pm$ 0.21
Cerebellum	5.540 $\pm$ 0.8	5.860 $\pm$ 0.16

The data represent mean $\pm$ S.E.M. of 5 mice in each group.

has been shown that these steps are affected by vitamin B $_{12}$ deficiency. The specific activity of MAT was estimated in brain tissue from control mice and mice treated with clioquinol. However, no difference was observed between the two groups (Table 3). With regard to SAM, the levels were significantly higher in cerebrum tissue of the clioquinol-treated group compared with the controls (Fig. 3). The concentrations of SAH were not changed by clioquinol treatment (Fig. 4). The SAH levels in cerebellum were below the level of detectability with our HPLC system.

DISCUSSION

The results of the present autoradiographic study are in agreement with the findings of Flodh (20) on the distribution of radiolabelled vitamin B $_{12}$ in adult male mice after i.p. injection. For instance, in Flodh's study, it is reported that the kidneys represent the major storage depot of injected vitamin B $_{12}$ in mice. Moreover, the relative accumulation of radiolabelled vitamin B $_{12}$ was essentially constant from 4 hr after injection up to 2 weeks. Hence, it is reasonable to assume that the distribution is

Fig. 3. Levels of *S*-adenosylmethionine (SAM) in tissue homogenates of brain regions from mice treated with clioquinol or saline (controls). The error bars represent standard error of the mean of 5 mice in each group. SAM levels were significantly higher in fore-brain with clioquinol-treated group in comparison to controls (** $P < 0.001$). □ Control, ▨ Clioquinol.

Fig. 4. Levels of *S*-adenosylhomocysteine (SAH) in tissue homogenates of brain regions from mice treated with clioquinol or saline (controls). The error bars represent standard error of the mean of 5 mice in each group. None of the differences between mice treated with clioquinol and controls reached statistical significance. □ Control, ▨ Clioquinol.

“stabilized” after 48 hr, which was the time point chosen for the present study.

In this study, the effect of clioquinol on the accumulation of radiolabelled vitamin B₁₂ in male mice was studied. Clioquinol treatment did not cause any obvious effect on brain (nor liver) influx of vitamin B₁₂ after 48 hr. This suggests that the clioquinol given to the mice did not cause any major chelation of cobalt derived from vitamin

B₁₂. Accumulation of vitamin B₁₂ in the kidneys was decreased by clioquinol treatment. These results were obtained with both the autoradiographic method and the method employing direct measurements on dissected tissue specimens. The accumulation in kidney is presumably governed by specific mechanisms distinct from hydrophobic trapping, since other fat tissues had a considerably lower accumulation of radiolabelled vitamin. It may be speculated that clioquinol alters specific transport mechanisms of vitamin B₁₂ in the kidneys. Interestingly, high levels of radiolabelled clioquinol have been observed in the kidneys after administration in several species (21, 22).

Moreover, studies on the partition coefficient for vitamin B₁₂ in chloroform:water indicate that clioquinol does not form hydrophobic complexes with cyanocobalamin or cobalt ions from cyanocobalamin. This suggests that cobalt ions from vitamin B₁₂ do not form chelates with clioquinol.

The data of the present investigation do not support the hypothesis that SMON is a consequence of vitamin B₁₂ depletion in the CNS as a result of cobalt chelation. This does not rule out the possibility that clioquinol affects B₁₂ turnover in the CNS after long-term chronic treatment. Thus, under conditions when the CNS supply of B₁₂ is low, the reduced stores of this vitamin in the kidneys might worsen the situation.

A study by Tjälve and Ståhl (4) strongly suggests that substances, such as clioquinol, that form hydrophobic chelates with divalent cations have the ability to cause a general tissue accumulation of metal ions. Some of these chelates seem to be preferentially trapped in the CNS, e.g., nickel-clioquinol. These authors speculate that the high incidence of SMON in Japan may be a consequence of abnormal metal pollution in the environment. Hence, patients treated with clioquinol in Japan would experience a massive accumulation of metal ions in the nervous system. Moreover, it is known that clioquinol enhances intestinal uptake of dietary zinc in patients suffering from *acrodermatitis enteropathica* (Dr. G. Michaelsson, personal communication). Clioquinol treatment could result in an enhanced Zn²⁺ trapping in most tissues, possibly with a preference for the brain. The Zn²⁺ chelating properties of clioquinol might be of relevance for Alzheimer's disease (23).

In the current study we estimated SAM and SAH to determine if clioquinol treatment affects the balance of the one-carbon cycle. We observed an increase in SAM levels in the brain. One may speculate that this increase in SAM may be the result of a decrease in the capacity of downstream enzymes; i.e., of *S*-adenosylmethionine decarboxylase (SAMD) or SAM-dependent methyl transferases. Moreover, increased synthesis of SAM presum-

ably also could cause such effect, not due to increased MAT activity but due to an increase in methionine concentration.

In conclusion, the data of the present study show that treatment with clioquinol causes a decrease in the accumulation of radiolabelled vitamin B₁₂, particularly in the kidneys and skin. Several times it has been proposed that clioquinol may cause severe side-effects in the CNS via interactions with cobalt in vitamin B₁₂. However, no changes in the amount of vitamin in the CNS were evident after clioquinol treatment. Accordingly, the data of the present study indicate that the cobalt in vitamin B₁₂ is tightly bound and thus cannot be trapped by clioquinol. Hence, the present data suggest that the brain damage produced by this drug is not the result of a direct chemical interaction with vitamin B₁₂.

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