ENTEROHEPATIC CIRCULATION OF CLOQUINOYL IN THE RAT

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The existence of the enterohepatic circulation (EHC) of cloquinol was confirmed by using paired rats, donor and recipient, which were connected to each other with a bile duct-to-duodenum cannula. The concentrations of cloquinol and its metabolites appearing in the plasma of the recipient following intraduodenal 10 mg/kg dose of cloquinol to the donor were fairly low. However, within 24 h after the administration ca. 12% of the dose was reexcreted in the bile of the recipient as cloquinol glucuronide and ca. 2% in the urine as cloquinol sulfate. From these results and the data of biliary excretion in our previous paper, it was found that the glucuronide was absorbed with a role on the EHC. Further, both in vitro and in situ results suggested that cloquinol glucuronide excreted in the biles may be absorbed partially to return to the parent drug in the intestinal tract and partially as such without deconjugation.

Keywords: cloquinol; cloquinol glucuronide; cloquinol sulfate; cloquinol enterohepatic circulation; cloquinol glucuronide absorption; closed rat intestinal loop; everted gut sac

INTRODUCTION

Cloquinol (chinoform, 5-chloro-7-iodo-8-quinolinol) is now regarded as a possible etiological agent in subacute myelo-optico-neuropathy (SMON). This drug was mostly converted to cloquinol glucuronide (C-Glu) and cloquinol sulfate (C-Sul) in man and animals, and the conjugates were excreted in urine and bile. In the previous paper, we reported that, in rats, cloquinol was metabolized mainly to C-Glu and C-Sul, and that a large amount of the C-Glu was excreted in the bile. From this finding, the existence of the enterohepatic circulation (EHC) of cloquinol and/or the metabolite was suggested. A previous study on EHC of cloquinol using paired rats showed that radioactive material excreted in bile after administration of C cloquinol entered into EHC. However, detail on the nature of this absorbed radioactive material and reabsorption process remained unclear.

The purpose of the present study is to confirm the existence of EHC of cloquinol and/or its metabolites in rats and to investigate the extent of the EHC and process of the reabsorption.

MATERIALS AND METHODS

Chemicals and Animals—Cloquinol, kindly supplied by Tanabe Seiyaku Co. (Osaka), was recrystallized once from ethanol. C-Glu was the same as that used previously. Sodium carboxymethylcellulose (CMC-Na) was obtained from Wako Pure Chemical Industries (Tokyo) and neomycin sulfate from Sigma Chemical Co. (St. Louis, Mo.). All other solvents and reagents used were of reagent grade.

Male Wistar rats weighing 260–280 g

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(Japan Laboratory Animals Co., Tokyo) were used in the present study and fasted overnight before use.

**Enterohepatic Circulation Study** — Paired rats, donor and recipient, were anesthetized with ether, followed by insertion of one end of a polyethylene cannula into the bile duct of the donor and the opposite end into the duodenum of the recipient. After the rats had recovered from anesthesia, clioquinol (10 mg/kg dose, i.e. 32.7 µmol/kg dose) suspended in 0.5% CMC-Na aqueous solution with ultrasonic was administered through a polyethylene cannula inserted into the duodenum of the donor. Blood (ca. 0.3 ml) was collected in heparinized tubes through a polyethylene cannula inserted into the femoral artery of the recipient 1, 2, 3, 5, 7, 9, 12, and 24 h after administration. Plasma was separated from blood by centrifugation at 3000 rpm for 10 min. Urine and bile were also collected through each of the polyethylene cannulae inserted into the bladder and bile duct, respectively, of the recipient at appropriate time intervals up to 24 h after administration. Plasma, urine and bile samples were stored at -20°C until analysis. Blood transfusion to the recipient after each blood sampling, bile supply to the donor to replace lost bile, and maintenance of the body temperature (37 – 38°C) of rats were carried out in the same way as described previously.3)

**Permeation Study with Everted Gut Sac** — Everted gut sacs, 6 cm-length, were prepared from ileum region of rat intestine according to the method of Wilson and Wiseman.6) Each sac was filled with 1 ml of drug free Krebs–Ringer bicarbonate buffer (pH 7.4) and immersed in a 50-ml Erlenmeyer flask containing 4.5 ml of the same buffer, followed by saturating with a mixed gas of 95% O₂ and 5% CO₂. After about 10-min preincubation to raise the temperature of the buffer to 37°C in an incubator (Incubator Personal Ace, Taiyo Scientific Industrial Co., Tokyo), 0.5 ml of the same buffer containing 1 mg (2.1 µmol) of C-Glu was added into the flask and incubation was immediately begun at the rate of 80 strokes/min. Five, 10, 15, 30, or 45 min after beginning of incubation, 0.2 ml each of the outside fluid (mucosal fluid) and inside fluid (serosal fluid) was taken for analysis. After the remaining serosal fluid was discarded, the gut was rinsed with 30 ml of normal saline, blotted with filter paper and weighed. The mucosal fluid, serosal fluid and gut samples were stored at -20°C until analysis. In preliminary examinations on stability, C-Glu in Krebs-Ringer bicarbonate buffer (pH 7.4) at 37°C was stable at least up to 60 min for this experiment.

**In Situ Intestinal Absorption Study** — Preparations of closed intestinal loop and cannulation of mesenteric vein for rat were carried out according to the procedure described previously.3) Dissolved C-Glu (7.9 mg/kg dose, i.e. 16.4 µmol/kg dose) in 10% diluted rat bile solution, which was diluted with water, was injected into the loop of ileac region of small intestine. After injection (injected volume: 1 ml), mesenteric venous blood from the loop was collected at 10-min intervals for 60 min. Plasma was separated in the same manner as described in enterohepatic circulation study and stored at -20°C until analysis. The blood lost from the vein was continuously replaced by intravenous infusion, via the femoral vein, of heparinized blood collected from other rats.

Similar experiments with C-Glu (7.9 mg/kg dose) were carried out using the loop of the same region of rat pretreated with neomycin sulfate of single oral dose of 50 mg/kg twice a day for 4 d.

**Analysis of Clioquinol and Its Metabolites** — Concentrations of clioquinol and its metabolites, C-Glu and C-Sul, in plasma, urine, bile, mucosal fluid and serosal fluid were determined according to the gas chromatographic-mass spectrometric method as described previously.3,8,9) The concentrations in gut sac were determined by the following manner. Gut sac, 0.45–0.52 g wet weight, was homogenized with eight volumes of a mixture of water and pyridine (1:1) under cooling in an ice bath, and concentrations of clioquinol and its metabolites in the homogenate were determined by the same method9) using 0.1 ml of sample volume.
RESULTS AND DISCUSSION

Enterohepatic Circulation Study

To examine the existence of EHC of clioquinol and/or its metabolites, “Linked” experiments in which bile of the donor after intraduodenal administration of clioquinol (10 mg/kg dose) was allowed to flow directly into duodenum of the recipient were performed. Plasma concentration-time courses of clioquinol and its metabolites, C-Glu and C-Sul, and biliary and urinary excretion rates of the metabolites in the recipient are shown in Fig. 1. Clioquinol and the two metabolites were detected in the first blood sample withdrawn from the recipient 1 h after the administration to the donor. Their plasma concentrations reached maximum 9 h after the administration, but their mean concentrations were quite low and less than 0.8 nmol/ml. During 24 h period 12% of the dose given to the donor was excreted in the bile of the recipient as C-Glu, and the excreted amounts of C-Sul and unchanged clioquinol were negligibly small. Whereas 2% was excreted in the urine as C-Sul; C-Glu and unchanged clioquinol were almost not detected. The previous study using 14C-clioquinol showed that approximately 35% of the radioactivity administered at a single oral dose (5 mg/kg) to rats was excreted in the bile and that EHC of clioquinol and its metabolite was present in the “Linked” experiments by paired rats. Results of our present study supported these findings.

Based on the biliary excretion data in rats as described previously, it was estimated that 39% (sum of C-Glu, C-Sul and unchanged clioquinol) of the dose given to the donor flowed into the duodenum of the recipient up to 24 h, and that 97, 2 and 1% of this total inflow amount were C-Glu, C-Sul and unchanged clioquinol, respectively. Therefore, it was estimated that 36% of this total inflow amount was reexcreted in the bile and urine of the recipient, because 14% of the dose given to the donor was excreted as C-Glu and C-Sul in these of the recipient mentioned above. It was, further, suggested that C-Glu excreted in bile plays a role on the EHC and the process of the EHC included the absorption of C-Glu and/or clioquinol produced by deconjugation of that.

Permeation Study of C-Glu with Everted Gut Sac

Concentration-time courses of C-Glu and clioquinol in the mucosal fluid, serosal fluid and gut sac obtained after adding C-Glu to the mucosal fluid are shown in Fig. 2. Clioquinol was detected little in the mucosal fluid, serosal fluid and gut sac at each time. On the other hand, increase of C-Glu concentration was observed clearly in the serosal fluid and gut sac. C-Sul was not detected in any samples. These results suggest that C-Glu may be permeable to the gut wall.

In Situ Intestinal Absorption Study of C-Glu

FIG. 1. Plasma Concentration–Time Courses of Clioquinol and Its Metabolites (a), and Biliary (b) and Urinary (c) Excretion Rates of the Metabolites in Recipient Rats

●: clioquinol, ○ and □: clioquinol glucuronide, △ and ■: clioquinol sulfate. Each point with vertical bar represents the mean values with standard deviation of three rats.
To confirm the in vitro finding, absorption of C-Glu from the intestinal tract of both pretreated and nontreated rats with neomycin sulfate was studied using in situ loop technique with complete mesenteric venous blood collection. In case of C-Glu injection to the nontreated rats as illustrated in Fig. 3(a), both C-Glu and clioquinol were found in the mesenteric venous plasma at the first interval of 10 min and both rates of appearance were gradually increased with time. The fact of appearance of clioquinol in the venous plasma suggests that C-Glu is hydrolyzed at least in part to clioquinol by intestinal flora, because it was shown in a bacteriological study\textsuperscript{3} that among intestinal bacteria tested, especially \textit{E. coli} hydrolyzed C-Glu to clioquinol. Ratio of the mean amount of C-Glu to that of clioquinol in the venous plasma at each time interval was in the range of 4.1 \textendash{} 7.4 while that in clioquinol injection\textsuperscript{3} was in the range of 1.2 \textendash{} 1.6. On the other hand, in case of rats pretreated with neomycin sulfate to suppress the intestinal flora, as shown in Fig. 3(b), C-Glu was found clearly in the mesenteric venous plasma collected at each time interval, whereas the amount of clioquinol was a little. Ratio of the mean amount of C-Glu to that of clioquinol in the venous plasma at each time interval was in the range of 12.7 \textendash{} 28.3. These results suggest that C-Glu may be absorbed intact from intestinal tract.

In case of C-Glu injection to the nontreated rats (Fig. 3(a)), cumulative amounts of clioquinol and C-Glu appearing in the mesenteric venous plasma up to 60 min were 47.1 and 231.8 nmol, respectively, and in case of C-Glu injection to the pretreated rat (Fig. 3(b)), 11.4 and 178.9 nmol, respectively. In Fig. 3(a), the amount of C-Glu appearing in the venous plasma can be interpreted as the sum of that of C-Glu absorbed intact and that of C-Glu from intestinal reconjugation of clioquinol produced by deconjugation in the intestinal tract. Ratio of the cumulative amount of C-Glu (172.4 nmol) appeared in the venous plasma to that of clioquinol (128.8 nmol) in the clioquinol injection reported in the previous paper\textsuperscript{5} was 1.3. And the ratio may be considered to indicate the degree of the appearance in the venous plasma of C-Glu formed by intestinal metabolism of clioquinol. Consequently, the cumulative amount of C-Glu (61.2 nmol) from the intestinal metabolism of clioquinol will be estimated to be 1.3 fold of the amount of clioquinol (47.1 nmol) appearing in the venous plasma, and thereafter, 170.6 nmol among the cumulative amount (231.8 nmol) of C-Glu in Fig. 3(a) will be considered as the amount of C-Glu absorbed intact. This amount corresponds to about 60\% of total

\begin{figure}[h]
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\includegraphics[width=\textwidth]{figure2.png}
\caption{Concentration-Time Courses of Clioquinol Glucuronide (C-Glu) and Clioquinol in Mucosal Fluid (a), Serosal Fluid (b) and Gut Sac (c) after Adding C-Glu to Mucosal Fluid

\textbullet{}: clioquinol, \textbullet{}: C-Glu. Initial concentration of C-Glu in mucosal fluid was 0.42 \textmu{}mol/ml. All everted gut sacs were prepared from ileum region of rat intestine. Each point with vertical bar represents the mean values with standard deviation of three experiments of each.}
\end{figure}
cumulative amount (sum of clioquinol and C-Glu) appearing in the venous plasma. In similar calculations, cumulative amount of C-Glu absorbed intact is estimated to be 164.1 nmol in C-Glu injection to the pretreated rats with neomycin sulfate shown in Fig. 3(b). This amount agreed closely with the estimated amount (170.6 nmol, Fig. 3(a)) of C-Glu absorbed intact mentioned above.

In conclusion, existence of EHC of biliary metabolite of clioquinol, C-Glu, was confirmed in the paired rats. Further, the in vitro and in situ results suggest that not only C-Glu excreted in bile is deconjugated in intestinal tract and absorbed as clioquinol, but also C-Glu will be absorbed intact from small intestine.

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