Sex Difference of the Metabolic Disposition of Clioquinol in Rats

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The order of plasma levels of clioquinol and its conjugates in male rats after intraduodenal administration of 32.7 $\mu\text{mol/kg}$ dose of clioquinol was clioquinol sulfate (C-Sul) > clioquinol glucuronide (C-Glu) >> clioquinol, whereas that in female rats was C-Glu > C-Sul >> clioquinol. Total (urine + bile) recovery was almost the same among male and female rats. The percentage of excretion amounts of C-Sul (urine + bile) to the total excretion amounts for 24 h in male rats after intravenous administration of clioquinol was about twice that in female rats, while the percentage of excretion amounts of C-Glu to the total excretion amounts in male rats was smaller than that in female rats. In intravenous administration of 16.4 $\mu\text{mol/kg}$ dose of C-Glu, C-Sul and clioquinol other than C-Glu were found in bile and urine of male and female rats. The percentage of excretion amounts of C-Sul and C-Glu (urine + bile) to the total excretion amounts was similar among male and female rats, respectively. In intravenous administration of 16.4 $\mu\text{mol/kg}$ dose of C-Sul, C-Glu and clioquinol other than C-Sul were found in bile and urine of male and female rats, and the percentage of excretion amounts of C-Sul (urine + bile) to the total excretion amounts in male rats was 1.3 fold that in female rats. From the examination of the *in vitro* conjugation reaction, the formation rate of C-Glu in the small intestine was approximately same among male and female rats, whereas C-Glu and C-Sul formation rates in male rats were about twice those in female rats, respectively. From the similarity of the extent of the glucuronidation in the small intestine among male and female rats, one reason for the sex difference in the excretion of the two conjugated metabolites at the intraduodenal administration of clioquinol may be due to the difference in sulfation in the liver.

Keywords — clioquinol; clioquinol glucuronide; clioquinol sulfate; sex difference; plasma concentration; biliary excretion; urinary excretion; conjugation; rat

Introduction

Subacute myelo-optico-neuropathy (SMON) is now considered to be caused by the administration of clioquinol (5-chloro-7-iodo-8-quinolinol, chionoform).¹⁾ In several etiological researches, it was reported²⁾ that the ratio of appearance of the SMON in man was larger than that in woman. On the other hand, many studies on the neurotoxicity of clioquinol have been performed with various species of experimental animals.³⁾ However, concerning the sex difference in the disposition of clioquinol, only one report is available.⁴⁾ Sato *et al.* reported⁴⁾ that the proportion of clioquinol sulfate (C-Sul) excreted in urine of male rats was larger than that in female rats after oral administration of ¹³¹I labeled clioquinol, while the proportion of clioquinol glucuronide (C-Glu) in urine of female rats was larger than that in male rats. However, it has been reported^{3a,b)} that clioquinol ab-

sorbed was mostly metabolized to C-Glu and C-Sul in rats and a large amount of C-Glu was excreted in the bile. Therefore, to study the sex difference in disposition of clioquinol in rats, it is necessary to examine the excretion of the two conjugated metabolites in both urine and bile. Furthermore, it has been reported^{3a)} that C-Glu was partly converted to C-Sul, and C-Sul was partly to C-Glu in male rats after intravenous administration of C-Glu and C-Sul. However, whether the extent of the conversion is different among male and female rats is unclear.

In the present study, we have investigated biliary and urinary excretion of clioquinol and its two conjugated metabolites after intraduodenal administration of clioquinol and after intravenous administration of the two conjugated metabolites to male and female rats. Further, we have examined the *in vitro* conjugation rate of clioquinol in tissues of male and female rats.

Material and Methods

Chemicals and Reagents — Clioquinol was purchased from Sigma Chemical Co. (U. S. A.) and recrystallized twice from ethanol. Glucuronide of clioquinol and sulfate of clioquinol were the same as that used in our previous report.⁵⁾ Sodium salt of uridine-5'-diphosphoglucuronic acid (UDPGA) and disodium salt of adenosine-5'-triphosphate (ATP) were purchased from Boehringer Mannheim (W. Germany), and β -glucuronidase from Tokyo Zouki Co. (Tokyo). Other reagents and chemicals used were of reagent grade.

Experimental Animals — Male and female rats of Wistar strain, aged 10–11 week-old, were used. They had free access to water and MF, a solid feed made by Oriental Yeast Co. (Tokyo). The temperature of the animal quarters was controlled at 22–26 °C. All rats were fasted overnight before drug administration.

Intraduodenal Administration — Male and female rats were anesthetized with ether and polyethylene cannulas were inserted into the femoral artery and vein, bile duct and bladder. The cannula for administration was attached to the upper duodenum. The rats were left for 2 h after the surgery to recover from anesthesia. Then a dose of 32.7 $\mu\text{mol/kg}$ (*i.e.* 10 mg/kg) of clioquinol suspended in 0.5% sodium carboxymethylcellulose solution was administered into the upper duodenum through the cannula. Blood samples (*ca.* 0.5 ml) were collected through the artery cannula into heparinized tubes at 30, 60 and 120 min after administration and 0.2 ml of plasma was separated by centrifugation at 1620 *g* for 10 min. After each blood sampling, an equivalent volume of blood collected was transfused through the venous cannula. Bile and urine were collected at appropriate time intervals until 24 h after administration, and each was pooled. The body temperature of rats was maintained at 37–38 °C by heating with a lamp. The plasma, urine and bile samples were stored at –80 °C until analysis.

Intravenous Administration — Polyethylene cannulas were inserted into the femoral vein, bile duct and bladder of rats. A dose of 16.4 $\mu\text{mol/kg}$ (*i.e.* 5 mg/kg) of C-Glu or C-Sul

dissolved in 0.04 M NaOH solution was administered rapidly into the femoral vein of male and female rats. Bile and urine were collected at appropriate time interval until 24 h after administration, and each was pooled. All samples were stored at –80 °C until analysis.

In Vitro Conjugation Reaction — Male and female rats were killed by exsanguination. The liver was excised after perfusion with ice-cold normal saline. The small intestine (jejunal region, *ca.* 10 cm) was excised and its inside was washed once with the same solution as for liver. These tissues were homogenized on ice with 9 volumes of ice-cold 1.15% KCl solution. The homogenates were centrifuged at 9000 *g* for 20 min at 4 °C and the supernatant fraction was used for the experiment of conjugate formation.

Formation of C-Glu: The mixture, which consisted of 0.3 ml of the 9000 *g* supernatant fraction of either liver or small intestine, 0.3 ml of 0.1 M Tris-HCl buffer (pH 7.4) containing 3.3 $\mu\text{mol/ml}$ of UDPGA, and 2 ml of 0.1 M Tris-HCl buffer (pH 7.4), was preincubated at 37 °C for 5 min. Then 0.2 ml of 0.04 M NaOH solution containing 164 nmol/ml (*i.e.* 50 $\mu\text{g/ml}$) of clioquinol as a substrate was added to the mixture. The mixtures were incubated at 37 °C for 2, 4 and 6 min and the enzymatic reaction was terminated at each time by adding 0.2 ml of 3 M NaOH solution to the mixture followed by placing the mixture in a boiling water for 30 s. The mixture was immediately frozen in ethanol cooled with dry ice and then stored at –80 °C until analysis.

Formation of C-Sul: 3'-Phosphoadenosine-5'-phosphosulfate (PAPS) was generated according to the method of Kempen and Jansen⁶⁾ using a 44000 *g* supernatant fraction of the liver excised from an adult male rat. The mixture, which consisted of 0.3 ml of 9000 *g* supernatant fraction of either liver or small intestine, 0.5 ml of 0.4 M Tris-HCl buffer (pH 7.4) containing 0.25 M of disodium salt of ethylenediaminetetraacetic acid (EDTA-2Na), 1 ml of 0.4 M Tris-HCl buffer (pH 7.4) and 0.3 ml of PAPS, was preincubated at 37 °C for 5 min. Then 0.2 ml of 0.04 M NaOH solution containing 164 nmol/ml of clioquinol was added to the mixture.

The incubation was terminated at 2, 4 and 6 min by adding 0.2 ml of 6 M NaOH solution to the mixture followed by placing the mixture in boiling water for 30 s. The mixture was frozen in the same manner as above and then stored at -80°C until analysis.

Quantitation of Clioquinol and Its Metabolites — Concentrations of clioquinol and its conjugated metabolites, C-Glu and C-Sul, in plasma, bile and urine were determined by the gas chromatographic-mass spectrometric (GC-MS) method reported previously.⁵⁾ This method was based on the extraction of clioquinol from plasma, urine and bile with pyridine-benzene (1:9, v/v) mixture containing 5-chloro-7-bromo-8-quinolinol as an internal standard, selective hydrolysis, extraction of the two conjugates, acylation and GC-MS analysis. The separation was performed using a glass column (2 mm i.d. $\times$ 1 m) packed with 1% OV-17 on Chromosorb W, AW-DMCS, 80/100 mesh (Wako Pure Chemical Industries, Tokyo).

Amounts of C-Glu and C-Sul formed in the *in vitro* conjugation reaction were quantified with a slight modification of the method reported previously.⁷⁾ In brief, for the assay of C-Glu, 2 ml of 3 M acetate buffer (pH 5.0), 0.2 ml of distilled water and 0.3 ml of 0.3 M EDTA-2Na solution were added to the mixture. The aqueous phase was then washed once with 9 ml of pyridine-benzene mixture (1:9, v/v) and twice with 10 ml of benzene. To 3 ml of the aqueous phase, 0.3 ml of β -glucuronidase (3600 Fishman units) was added and incubated at 37°C for 2 h to liberate clioquinol. For the assay of C-Sul, washing of the aqueous phase was performed in the same

TABLE I. Plasma Concentration of Clioquinol and Its Conjugated Metabolites in Male and Female Rats after Intraduodenal Administration of $32.7\ \mu\text{mol/kg}$ Dose of Clioquinol

Time (min)		Concentration (nmol/ml)	
		Male	Female
30	Clioquinol	0.9 ± 0.1	0.1 ± 0.0
	C-Glu	1.6 ± 0.4	2.2 ± 0.2
	C-Sul	3.5 ± 0.9	0.8 ± 0.3
60	Clioquinol	0.7 ± 0.3	0.2 ± 0.0
	C-Glu	1.2 ± 0.2	2.8 ± 0.3
	C-Sul	2.2 ± 0.8	1.5 ± 0.4
90	Clioquinol	0.4 ± 0.1	0.2 ± 0.0
	C-Glu	0.7 ± 0.0	3.0 ± 0.2
	C-Sul	1.4 ± 0.4	1.3 ± 0.5

The value represents mean $\pm$ standard deviation of three rats.

manner as above. Then 0.3 ml of 6 M HCl solution was added to 3 ml of aqueous phase and hydrolyzed for 1.5 h at 45°C to liberate clioquinol. Liberated clioquinol was acylated and quantified by the GC-MS method according to the procedure reported previously.⁵⁾

Results

Intraduodenal Administration of Clioquinol

Plasma concentrations of clioquinol and its metabolites in male and female rats after intraduodenal administration of clioquinol are shown in Table I. The concentration of clioquinol was quite low in both male and female rats at each time. In male rats, the concentration of

TABLE II. Biliary and Urinary Excretions of Clioquinol and Its Conjugated Metabolites for 24 h after Intraduodenal Administration of $32.7\ \mu\text{mol/kg}$ Dose of Clioquinol to Male and Female Rats

	Male ($49.8 \pm 9.6\%$)			Female ($46.5 \pm 5.3\%$)		
	Clioquinol	C-Glu	C-Sul	Clioquinol	C-Glu	C-Sul
Bile	0.8 ± 0.6	76.2 ± 2.8	1.2 ± 0.9	0.6 ± 0.3	82.5 ± 4.1	9.3 ± 2.6
Urine	0.2 ± 0.0	1.8 ± 0.8	19.8 ± 3.3	0.8 ± 0.5	5.0 ± 1.5	1.9 ± 0.9
Total	1.0 ± 0.9	78.0 ± 2.1	21.0 ± 3.6	1.4 ± 0.8	87.5 ± 2.6	11.2 ± 3.3

The value in parenthesis represents the percent recovery of the dose for 24 h and the other value is the percent of the total recovery. The data represent mean $\pm$ standard deviation of three rats.

C-Sul was higher than that of C-Glu at each time, whereas, in female rats, the concentration of C-Glu was higher than that of C-Sul.

Data of biliary and urinary excretion in intraduodenal administration of clioquinol to male and female rats are summarized in Table II. The mean percentage of the total excretion amounts (sum of clioquinol, C-Glu and C-Sul in bile and urine) to the dose was 49.8% in male rats and 46.5% in female rats for 24 h. In both male and female rats, the excretion amounts of unchanged clioquinol were negligible in urine and in bile. The percentage of excretion amounts of C-Glu to the total excretion amounts in bile of male rats was remarkably larger than that in urine. This was also the case in female rats. In male rats, the percentage of excretion amounts of C-Sul to the total excretion amounts in urine was much larger than that in bile. However, in female rats, the percentage of excretion amounts of C-Sul to the total excretion amounts in bile was larger than that in urine. The ratio of the sum of C-Sul amounts in both urine and bile

to the total excretion amounts in male rats was approximately twice that in female rats.

Intravenous Administration of Conjugated Metabolites

Data of biliary and urinary excretion in intravenous administration of C-Glu to male and female rats are summarized in Table III. The mean percentage of the total excretion amounts to the dose was 101.2% in male rats and 93.9% in female rats. C-Sul and free form (clioquinol) were found other than C-Glu in bile and urine of both male and female rats. The excretion amounts of clioquinol were negligible in both male and female rats. The mean percentage of C-Glu excretion amounts (bile plus urine) to the total excretion amounts was almost same among both sexes, and that of C-Sul excretion amounts was also same. In male rats, the percentage of C-Glu excretion amounts in bile was remarkably larger than that in urine and the percentage of C-Sul in urine was larger than that in bile. While, in female rats, the percentage of C-Glu excretion amounts in bile was larger than

TABLE III. Biliary and Urinary Excretions of Clioquinol and Its Conjugated Metabolites for 24 h after Intravenous Administration of 16.4 μ mol/kg Dose of C-Glu to Male and Female Rats

	Male (101.2 $\pm$ 0.8%)			Female (93.9 $\pm$ 16.6%)		
	Clioquinol	C-Glu	C-Sul	Clioquinol	C-Glu	C-Sul
Bile	0.0 $\pm$ 0.0	87.5 $\pm$ 4.0	1.5 $\pm$ 0.8	0.4 $\pm$ 0.1	51.0 $\pm$ 2.6	6.2 $\pm$ 1.3
Urine	0.0 $\pm$ 0.0	0.6 $\pm$ 0.2	10.4 $\pm$ 2.3	0.6 $\pm$ 0.1	35.6 $\pm$ 1.5	5.2 $\pm$ 0.1
Total	0.0 $\pm$ 0.0	88.1 $\pm$ 4.1	11.9 $\pm$ 2.2	0.9 $\pm$ 0.2	86.6 $\pm$ 1.2	11.4 $\pm$ 1.2

The value in parenthesis represents the percent recovery of the dose for 24 h and the other value is the percent of the total recovery. The data represent mean $\pm$ standard deviation of three rats.

TABLE IV. Biliary and Urinary Excretions of Clioquinol and Its Conjugated Metabolites for 24 h after Intravenous Administration of 16.4 μ mol/kg Dose of C-Sul to Male and Female Rats

	Male (74.9 $\pm$ 8.3%)			Female (79.3 $\pm$ 14.9%)		
	Clioquinol	C-Glu	C-Sul	Clioquinol	C-Glu	C-Sul
Bile	0.5 $\pm$ 0.2	36.7 $\pm$ 3.5	0.0 $\pm$ 0.0	0.8 $\pm$ 0.4	16.5 $\pm$ 8.6	12.6 $\pm$ 6.3
Urine	1.5 $\pm$ 0.1	2.4 $\pm$ 1.0	58.9 $\pm$ 2.6	1.7 $\pm$ 0.9	1.3 $\pm$ 1.1	66.9 $\pm$ 11.4
Total	1.9 $\pm$ 0.1	39.1 $\pm$ 2.6	58.9 $\pm$ 2.6	2.5 $\pm$ 0.8	17.8 $\pm$ 8.4	79.5 $\pm$ 8.2

The value in parenthesis represents the percent recovery of the dose for 24 h and the other value is the percent of the total recovery. The data represent mean $\pm$ standard deviation of three rats.

that in urine and the percentage of C-Sul in bile was similar to that in urine.

Data of biliary and urinary excretions in intravenous administration of C-Sul to male and female rats are summarized in Table IV. The mean percentage of the total excretion amounts to the dose was 74.9% in male rats and 79.3% in female rats. C-Glu and free form (clioquinol) were found other than C-Sul in bile and urine of both male and female rats. The excretion amounts of clioquinol were negligible in both male and female rats. In male rats, the greater part of C-Sul was excreted in urine and C-Glu was in bile. In female rats, C-Sul was mainly excreted in urine and partly was in bile, while the greater part of C-Glu was excreted in bile. The ratio of C-Sul excretion percentage (urine plus bile) in male rats to that in female rats was 0.7, and the ratio of C-Glu excretion percentage in male rats to that in female rats was 2.2.

In Vitro Conjugation Reaction

Rates of C-Glu and C-Sul formation with 9000 *g* supernatant fraction of the liver and small intestine are shown in Table V. Each conjugation reaction proceeded with a constant rate during the incubation under the present conditions. The rate of C-Glu formation in the small intestine of male rats was similar to that of female rats. The rate of C-Glu formation in the liver of male rats was approximately twice that in female rats. On the contrary, the rate of C-Sul formation in the liver of male rats was approximately twice that of female rats.

TABLE V. Enzymatic Formation of C-Glu and C-Sul in 9000 *g* Supernatant Fraction of Liver and Small Intestine of Male and Female Rats

	Rate of conjugation		
	C-Sul ($\mu\text{mol/g/min}$)	C-Glu ($\mu\text{mol/g/min}$)	
	Liver	Liver	Intestine
Male	75.6 $\pm$ 13.5	28.3 $\pm$ 3.9	24.0 $\pm$ 5.1
Female	39.9 $\pm$ 7.8	13.5 $\pm$ 2.6	25.0 $\pm$ 8.8

The value represents the mean $\pm$ standard deviation of three to four experiments.

Discussion

The data described here demonstrate that the metabolic disposition of clioquinol is different among male and female rats. The order of plasma levels of clioquinol and the two conjugated metabolites after intraduodenal administration of clioquinol was C-Sul > C-Glu >> clioquinol in male rats and C-Glu > C-Sul >> clioquinol in female rats. This order may reflect the fact that the percentage of C-Glu excretion amounts (urine plus bile) to the total excretion amounts in male rats was smaller than that in female rats, and that of C-Sul to the total excretion amounts in male rats was larger than that in female rats.

From the data shown in Table I, it seems that the absorption rate of clioquinol in male rats was larger than that in female rats. Although it has been reported^{3b)} on the first-pass metabolism in rats, it may be that the difference of the absorption rate was not the result of a difference in the extent of the first-pass metabolism, since absorption of clioquinol from a suspension of clioquinol prepared with 0.05% sodium carboxymethylcellulose solution was remarkably slow compared with that from solution of clioquinol prepared with the bile acid. Therefore, we consider that the absorption rate from the suspension was so small that the saturation in the first-pass metabolism did not arise.

It has been reported that clioquinol was mostly metabolized to C-Glu and C-Sul and these conjugated metabolites were excreted in urine and bile.^{3a,b)} Further, in the preliminary experiments, we confirmed that the percentage of the total recovery (sum of clioquinol, C-Glu and C-Sul in bile and urine) from 24 to 48 h after the intraduodenal administration of clioquinol was negligible, less than 1% of dose. The similarity of the total recovery for 24 h in male and female rats after intraduodenal administration of clioquinol suggests that the extent of the intestinal absorption of clioquinol may be almost the same among both sexes of rats.

It has been reported^{3a,8)} that the two conjugated metabolites were hydrolyzed by enzymatic processes in liver and kidney and by a nonenzymatic process, and C-Glu was converted

partly to C-Sul and C-Sul was partly to C-Glu in male rats. In the present study of intravenous administration of the two conjugated metabolites (Tables III and IV), we found that this conversion was observed not only in male rats but also in female rats and the extent of the conversion from C-Sul to C-Glu at C-Sul administration was larger than that from C-Glu to C-Sul at C-Glu administration in both male and female rats. Further, we found that the extent of the conversion from C-Sul to C-Glu in male rats at C-Sul administration was larger than that in female rats, although the extent of the conversion from C-Glu to C-Sul after C-Glu administration was similar among both sexes of rats. However, the sex difference in the excretion observed in the intraduodenal administration of clioquinol (Table II) can not account for these results. On the other hand, it has been reported⁹⁾ that 2, 4-dinitrotoluene was mainly metabolized to 2, 4-dinitrobenzyl alcohol glucuronide and the transportation of the glucuronide in bile of male rats was faster than that of female rats. Our results from the excretion experiments (Tables III and IV) also suggest a sex difference in the transportation rate of clioquinol metabolites in bile.

It has been reported^{3b)} in the *in situ* studies that about 60% of clioquinol was metabolized to C-Glu during absorption of clioquinol from the intestinal tract in male rats. Our results (Table V) from the *in vitro* conjugation reaction using the small intestine also indicate that clioquinol is predominantly conjugated to C-Glu in the small intestine. In addition, the similarity of the *in vitro* activity of glucuronidation in the intestine suggests that the extent of the intestinal metabolism from clioquinol to C-Glu was almost same among male and female rats. Further, from the results shown in Table V, it appears that the rest of clioquinol escaped from the intestinal metabolism may be conjugated to C-Sul and C-Glu in the liver of male and female rats. The results of the *in vitro* conjugation reaction indicated that C-Glu and C-Sul formation rates in male rats were larger than those in female rats, respectively (Table V). As *in vitro* glucuronidation and sulfation experiments were performed on the optimized medium for each reaction in

the present conditions, it is unclear which conjugation reaction is more predominant in the liver. However, one reason for the sex difference in the excretion of the two conjugated metabolites at intraduodenal administration of clioquinol may be due to the difference of sulfation in the liver, since it is considered that the extent of the glucuronidation of clioquinol during absorption of clioquinol from the intestinal tract was almost same among male and female rats.

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