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Original Article

Metabolism of Procymidone in Female Rabbits

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To examine the metabolic fate of procymidone [*N*-(3,5-dichlorophenyl)-1,2-dimethylcyclopropane-1,2-dicarboximide, Sumilex®, S-7131], female New Zealand White rabbits were given a single oral dose of [carbonyl-^{14}C]procymidone at 125 mg/kg and their urine, feces, and blood were collected. The radiocarbon was rapidly eliminated from the body, the total ^{14}C excretion within 3 days after administration being 95.3% (urine: 72.1%; and feces: 23.2%). ^{14}C level in the blood was maintained from 1–6 hr with a rapid decrease thereafter. The main metabolites in female rabbits were glucuronide conjugates of 3 hydroxylated-procymidone metabolites, which were not found in rats or mice, generated by the following metabolic reactions: 1) oxidation of one of the methyl groups to carboxylic acid *via* hydroxymethyl, 2) cleavage of the imide linkage, and 3) glucuronide formation of the 3 hydroxylated-procymidone metabolites. The glucuronyltransferase activity toward one of the hydroxylated-procymidone metabolites were examined *in vitro* with addition of hepatic glucuronyltransferase of female rabbits or rats. There appeared to be such activity toward hydroxylated-procymidone metabolites only in female rabbits and no activity in female rats, suggesting the difference of the conjugation activity caused the species difference of procymidone metabolism between female rabbits and rats.

INTRODUCTION

Procymidone [*N*-(3,5-dichlorophenyl)-1,2-dimethylcyclopropane-1,2-dicarboximide, Sumilex®, S-7131] is a fungicide which is used for control of plant diseases such as gray mold and sclerotinia rot.[1–3]

Its metabolism in rats and mice was studied by Mikami *et al.*[4] and Shiba *et al.*,[5] respectively. In both animals, procymidone was found to be rapidly and almost completely excreted after oral administration. The main metabolic reactions were established to be oxidation of the methyl groups to hydroxymethyl or carboxylic acid derivatives and hydrolysis of the imide bond.

For toxicological studies, rodents (rats and mice) are frequently used, but, rabbits and dogs are more commonly applied for some developmental and chronic investigations. To cast light on species difference in metabolism of procymidone, a metabolic study in rabbits was therefore undertaken.

The present report deals with the metabolic fate (^{14}C excretion, ^{14}C level in blood and biotransformation) of [carbonyl-^{14}C]procymidone following oral administration of a single dose in female rabbits and includes a comparison of glucuronyltransferase activity between female rabbits and rats.

MATERIALS AND METHODS

1. Designation of Compounds

The abbreviations used in this report are summarized in Table 1.

2. Chemicals

Procymidone and its metabolites were prepared by Sumitomo Chemical Co., Ltd., Japan. All the other chemicals used were of reagent grade.

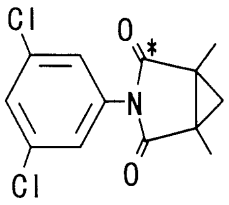
[^{14}C]Procymidone (radiochemical purity, >98%; and chemical purity, >99%) labeled in the carbonyl group with a specific activity of 2.26 GBq/mmol (61.0 mCi/mmol) was synthesized in our laboratory (Fig. 1). The labeled compound was purified by TLC with chloroform as the solvent prior to use.

3. Thin-layer Chromatography (TLC)

Pre-coated silica gel 60 F 254 chromatoplates (Art. 5715, 20×20 cm, 0.25 mm layer thickness, E. Merck, FRG) were used for purification of [carbonyl-^{14}C]-procymidone and for analysis of metabolites. The solvent systems used were as follows: A) toluene/ethyl formate/formic acid (5 : 7 : 1), B) ethyl acetate/2-propanol/acetic acid (11 : 8 : 1), C) butanol/water/acetic

Table 1 Procymidone and its metabolites.

Compound	Abbreviation
N-(3,5-Dichlorophenyl)-1,2-dimethylcyclopropane-1,2-dicarboximide	Procymidone
N-(3,5-Dichlorophenyl)-1-hydroxymethyl-2-methylcyclopropane-1,2-dicarboximide	Procymidone-OH
2-(3,5-Dichlorophenylcarbamoyl)-2-hydroxymethyl-1-methylcyclopropane-1-carboxylic acid	Procymidone-NHOH
2-(3,5-Dichlorophenylcarbamoyl)-1-hydroxymethyl-2-methylcyclopropane-1-carboxylic acid	Procymidone-NH'OH
N-(3,5-Dichlorophenyl)-1-carboxy-2-methylcyclopropane-1,2-dicarboximide	Procymidone-COOH
2-(3,5-Dichlorophenylcarbamoyl)-1-methylcyclopropane-1,2-dicarboxylic acid	Procymidone-NH$(COOH)_2$
2-(3,5-Dichlorophenylcarbamoyl)-2-methylcyclopropane-1,1-dicarboxylic acid	Procymidone-NH'$(COOH)_2$
2-(3,5-Dichlorophenylcarbamoyl)-1,2-dimethylcyclopropane-1-carboxylic acid	Procymidone-NHCOOH



* Labeled position

Fig. 1 The chemical structure of [carbonyl-^{14}C] procymidone.

acid (6 : 1 : 1). The values in parentheses represent the volumetric proportions.

Rf values for standard compounds were reported previously.[4] Unlabeled standards on TLC plates were detected by viewing under UV light (254 nm). The radioactive metabolites on TLC plates were detected by autoradiography using SB-5 films (Kodak, U.S.A.) or imaging plates (Fuji Photo Film Co., Ltd., Tokyo, Japan).

4. *Animal Conditions*

The in-life portion of the study was conducted under the following environmental conditions : room temperature, 23±2°C ; relative humidity, 55±10% ; ventilation, >10 air exchanges per hour ; and artificial lighting from 8 : 00 am to 8 : 00 pm. Rabbits had free access to pelleted diet (LRC-4, Oriental Yeast Co., Ltd., Tokyo, Japan) and water, which had been passed throughout a water filtration device (Type : PTS-3, 5D-C-1, AMF, Tokyo, Japan), through the study.

Three female New Zealand White (NZW) rabbits weighing about 3 kg (Kitayama LABES Co., Ltd., Nagano, Japan) and aged 17 weeks old were orally dosed with the ^{14}C-labeled compound at 125 mg (9.25 MBq)/3 ml 0.5% Methyl Cellulose 400cP (Wako, Osaka, Japan) solution/kg using a plastic syringe (10 ml volume, Terumo, Tokyo, Japan) equipped with a nelaton catheter (No. 10, Izumo Rubber Manuf. Co., Ltd., Tokyo, Japan) and were housed individually in aluminium metabolic cages (Clea Japan Inc., Tokyo, Japan).

Feces and urine from each rabbit were collected 1, 2, and 3 days after administration of the ^{14}C-labeled compound. Blood (3-4 ml) of each rabbit were collected from auricular vein 1, 3, 6, 9, 12, 24, 48, and 72 hr after administration of the ^{14}C-labeled compound.

5. *Radioanalysis*

Radioactivity was quantified by liquid scintillation counting (LSC) with a Tri-Carb® 2500TR Liquid Scintillation Counter (Packard, U.S.A.) or a LS6000LL Liquid Scintillation Counter (Beckman, U.S.A.) giving disintegrations per minute (dpm) by the external standard method.

Duplicate aliquots of 0-1 day, 1-2 day, and 2-3 day urine were dissolved directly in 10 ml of EMULSFIER SCINTILLATOR PLUS (Packard) and the radioactivity was counted by LSC.

The 0-1 and 1-2 day feces were extracted three times with 3-fold of acetonitrile and the extracts were directly radioassayed by LSC. Other feces (2-3 day) were homogenized with a 4-fold of water. Radioactivity in fecal homogenates and unextractable fecal residues was quantified by a dissolving method. After addition of 1 ml aliquots of SOLUENE®-350 (Packard), the samples (100 mg) were left at room temperature for 3 days and then treated with H_2O_2 for 10 min, followed by quantification of radioactivity by LSC with 10 ml HIONICFLUOR (Packard).

Radioactivity in the blood was quantified by a combustion method.[6] Air dried samples were combusted with a Tri-Carb® 307 Sample oxidizer (Packard) with ^{14}C trapped as $^{14}CO_2$ in a scintillation solution of 8 ml CARBO-SORB® and 12 ml PERMAFLUOR®E+ (Packard) prior to LSC. To quantify metabolites in the blood, the blood (1, 3, 6, 9, 12, and 24 hr) of individual rabbits were extracted three times with 3-fold of acetonitrile and the extracts were directly radioassayed by LSC. Blood residues were quantified by a dissolving method.

6. *Quantification of Metabolites in Urine, Feces, and Blood*

The metabolites in urine, feces, and blood were identified by two-dimensional TLC analysis with solvent systems A (first) and B (second) and TLC co-chromatography using the available authentic standards.

The metabolites were separated by TLC and quantified with a BAS2000 and a MacBAS (Fuji Photo Film Co., Ltd.). The 0–1 and 1–2 day fecal extracts, the 0–1 day and 1–2 day urine and each extract of 1, 3, 6, 9, 12, and 24 hr blood were separately subjected to TLC analysis using solvent systems A and B to quantify metabolites.

7. Identification of Conjugates in Urine

Three conjugated metabolites in urine were isolated by TLC in solvent system C and then subjected to enzyme hydrolysis, conducted according to the methods of Saito *et al.*[7] Each conjugate was incubated with β-glucuronidase (bovine liver, Type B-1, sulphatase free, Sigma, MO, U.S.A.) or sulfatase (limpet, type IV, Sigma) in 1 ml of 0.2 M acetate buffer (pH 5.0) at 37°C overnight. Saccharo-1,4-lactone (Sigma), an inhibitor of β-glucuronidase, was added to the incubation mixture in the case of hydrolysis by the sulfatase. After the reaction solution was dried *in vacuo* at the end of incubation with an evaporator, the released aglycones were dissolved in methanol and identified by TLC in solvent system A.

8. In Vitro Assay of Glucuronyltransferase Activity toward Procymidone-OH

Female rat and rabbit liver microsomes were prepared by the methods of Saito *et al.*[7] and the included glucuronyltransferase activity toward procymidone-OH which is one of the 3 hydroxylated-procymidone metabolites was determined by the method of Saito *et al.*[8] The reaction mixture contained 5 mM $MgCl_2$, 10 μM [glucuronyl-^{14}C]UDP-Glucuronic Acid (NEN, U.S.A.), with or without 500 μM procymidone-OH and 0.1 mg of the rat or rabbit microsome in 0.1 M Tris-HCl (pH 7.4) buffer (100 μl), and incubation was for 5 min at 37°C. The glucuronide derivative was then extracted with ethanol and subjected to TLC using solvent system C.

RESULTS

1. ^{14}C Excretion

The time course of ^{14}C excretion into urine and feces over 3 days after a single oral administration of [carbonyl-^{14}C]procymidone at 125 mg/kg is shown in Fig. 2. ^{14}C excretion was rapid and essentially complete, total ^{14}C recovery 3 days after the administration being 95.3% (urine: 72.1%; and feces: 23.2%).

2. ^{14}C Levels in Blood

Data for ^{14}C levels in blood are shown in Fig. 3 and Table 3. A high plateau level was maintained from 1–6 hr with a rapid decrease thereafter. Biological half-life of ^{14}C in blood from 6 to 72 hr after administration was 12 hr. The maximal ^{14}C level in blood was 21.1 μg procymidone equivalent per g-blood (ppm).

3. Metabolites in Excreta and Blood

Metabolites in excreta were quantified with 0–1 and 1–2 day urine and feces, because the radioactivity in 0–2 day urine and feces was >92% of the dosed ^{14}C and radioactivity in 2–3 day urine and feces was negligible (2.6% of the dosed ^{14}C). The parent compound and 8 metabolites were detected and quantified in urine and feces by TLC co-chromatography. The radioactivity in areas where no distinct concentration of radioactivity (metabolite) was detected on the TLC autoradiograms was summed and presented as "others."

3.1 Analysis of urinary metabolites

The 0–1 and 1–2 day urine samples were subjected to TLC analysis and at least 8 metabolites were detected. Then urinary polar metabolites (*Rf* values: 0.33, 0.27, and 0.22 with solvent system C) were separately subjected to enzyme hydrolysis with β-glucuronidase. The released aglycones were analyzed by TLC and identified as the mixture of 3 hydroxylated-procymidone

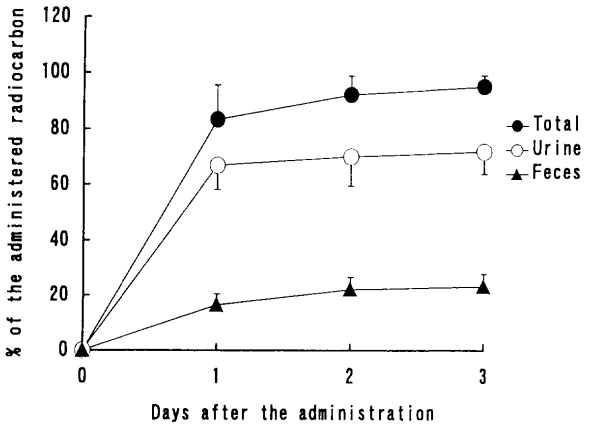


Fig. 2 Cumulative excretion of ^{14}C after a single 125 mg/kg oral administration of ^{14}C-procymidone to female rabbits.

The vertical bars represent the standard deviations of values for three rabbits.

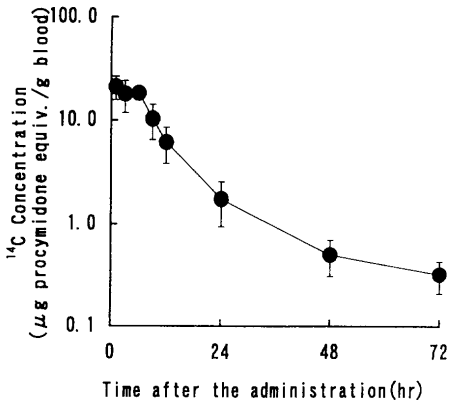


Fig. 3 Time course of decrease in ^{14}C-levels in the blood after a single 125 mg/kg oral administration of ^{14}C-procymidone to female rabbits.

The vertical bars represent standard deviations of values for three rabbits.

Table 2 Relative amounts of metabolites identified in the urine and feces collcted over 2 days after a single 125 mg/kg oral dose of [carbonyl-^{14}C]procymidone was given to female rabbits.

Metabolite	% of the dosed ^{14}C	
	Feces	Urine
Procymidone	8.7±1.77	N.D.
Procymidone-NHCOOH	0.2±0.06	0.2±0.02
Procymidone-OH	0.3±0.08	N.D.
Procymidone-NHOH	0.3±0.20	0.6±0.11
Procymidone-NH′OH	0.8±0.40	0.8[a)]
Procymidone-COOH	0.4±0.22	0.1±0.02
Procymidone-NH(COOH)$_2$	0.4±0.28	2.2±0.90
Procymidone-NH′(COOH)$_2$	0.3±0.10	10.2±1.20
Glucuronide of procymidone-OHs	N.D.	53.4±11.30
Others	1.0±0.43	3.0±0.44
Subtotal	12.4±0.83	70.5±10.66
Unextractable	9.8±4.16	—
Total	22.2±4.47	70.5±10.66

Data represent the mean values±SD for three rabbits, but values below the detection limit were excluded; [a)] the value for one rabbit. SD : standard deviation. N.D. : not detected. Procymidone-OHs : procymidone-OH, procymidone-NHOH and procymidone-NH′OH.

Table 3 ^{14}C levels and contents of procymidone and its metabolites in the blood of female rabbits given a single 125 mg/kg oral dose of [carbonyl-^{14}C]procymidone.

Metabolite	μg procymidone equiv./g blood					
	1 hr	3 hr	6 hr	9 hr	12 hr	24 hr
Procymidone	3.5	1.5	2.6	1.5	0.5	0.2
Procymidone-NHCOOH	0.4	0.7	0.8	0.3	0.2	0.2
Procymidone-OH	2.5	2.9	2.2	1.1	2.2	0.2
Procymidone-NHOH	0.8	0.8	0.5	N.D.	0.2	0.1
Procymidone-NH′OH	0.6	0.4	0.1	N.D.	0.2	0.1
Procymidone-COOH	7.1	7.3	8.2	4.4	N.D.	N.D.
Procymidone-NH(COOH)$_2$	N.D.	N.D.	0.2	N.D.	N.D.	N.D.
Procymidone-NH′(COOH)$_2$	1.3	0.8	N.D.	N.D.	0.5	0.1
Others	1.9	1.1	0.6	0.8	1.3	0.4
Subtotal	18.1	15.5	15.2	8.1	5.1	1.3
Unextractable	3.0	2.5	3.0	2.2	0.9	0.6
Total	21.1	18.0	18.2	10.3	6.0	1.9

N.D. : not detected.

metabolites (procymidone-OH, procymidone-NHOH, and procymidone-NH′OH). No aglycones were released by the hydrolysis with sulfatase. The urinary polar metabolites were considered to be glucuronides of 3 hydroxylated-procymidone metabolites designated as glucuronide of procymidone-OHs.

Table 2 shows the relative amounts (% of the dosed ^{14}C) of 0–2 day urinary and fecal metabolites. Main metabolites in 0–2 day urine were procymidone-NH′(COOH)$_2$ and glucuronide of procymidone-OHs, accounting for 10.2 and 53.4%, respectively. The amounts of the minor urinary metabolites were procymidone-NHCOOH, procymidone-NHOH, procymidone-NH′OH, procymidone-COOH, and procymidone-NH(COOH)$_2$, accounting for 0.2, 0.6, 0.8, 0.1, and 2.2%, respectively. Procymidone was not detected in the urine.

3.2 Analysis of fecal metabolites

The 0–1 and 1–2 day fecal extracts were subjected to TLC using solvent systems A and B and 7 metabolites were detected. Procymidone was predominant, accounting for 8.7%. Minor metabolites identified were procymidone-NHCOOH, procymidone-OH, procymidone-NHOH, procymidone-NH′OH, procymidone-COOH, procymidone-NH(COOH)$_2$, procymidone-NH′(COOH)$_2$, accounting for 0.2, 0.3, 0.3, 0.8, 0.4, 0.4, and 0.3%, respectively.

3.3 Analysis of metabolites in blood

Table 3 shows metabolite concentrations in blood over the first 24 hr after dosing. TLC analysis of acetonitrile extracts revealed the major metabolites to be procymidone-COOH, procymidone-OH, and intact procymidone. The concentration of latter reached a maximum within 1 hr after administration and was decreased rapidly with the biological half-life of procymidone from 6 to 24 hr after administration being 5 hr.

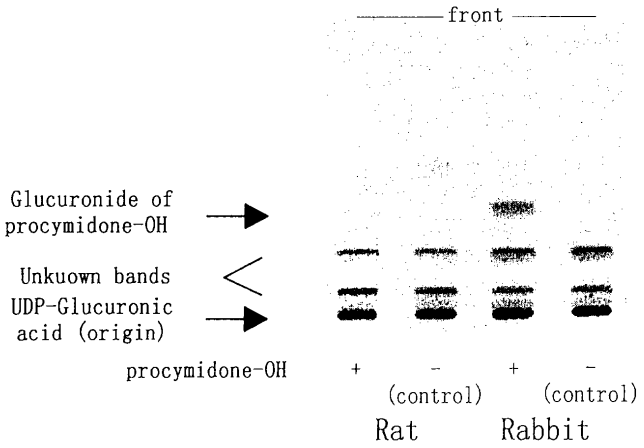


Fig. 4 TLC autoradiogram demonstrating products of *in vitro* glucuronidation of procymidone-OH.

The reaction mixture contained $MgCl_2$ (5 mM), ^{14}C-UDP-Glucuronic Acid (10 μM), with (+) or without (−) procymidone-OH (500 μM) and rat or rabbit microsomes (0.1 mg) in 0.1 M Tris-HCl (pH 7.4) buffer (100 μl).

4. Glucuronyltransferase Activity toward Procymidone-OH

Figure 4 provides evidence of *in vitro* glucuronidation of procymidone-OH by rabbit liver microsome. Procymidone-OH was representative of procymidone-OHs, because procymidone-OH was the major aglycone

(>70%) of urinary polar metabolites. The similar bands (UDP-glucuronic acid and 2 unknown bands) were observed, when procymidone-OH was excluded from the reaction mixture of both the rat and the rabbit microsomes (control). Only by the rabbit microsomes with procymidone-OH, the glucuronide of procymidone-OH is detected by TLC autoradiography. In contrast, no such reaction appeared under the same conditions with the rat liver microsome.

DISCUSSION

The present study demonstrated that procymidone was rapidly metabolized and completely eliminated from female rabbits within 3 days after administration of [carbonyl-^{14}C]procymidone. The ^{14}C excretion into the urine (72.1%) was significantly larger than that into the feces (23.2%). On the first day, the ^{14}C recovery was 83.5% (urine: 67.0%; feces: 16.5%). Plateau-like ^{14}C levels were observed in the blood for 1-6 hr and decreased thereafter with the biological half-life being 12 hr.

In the previous report,[5] ^{14}C excretion into excreta of male rats and male mice on the first day after administration of [^{14}C]procymidone at 100 mg/kg was 59% (urine: 54%; feces: 5%) and 92% (urine: 74%; feces: 18%) of the dosed ^{14}C, respectively. ^{14}C levels of the blood in male rats and male mice was maintained for 2-24 hr and 2-12 hr, respectively, and decreased thereafter with the biological half-life being 12 and 2 hr, respectively. Mikami *et al.*[4] reported that there was no sex related difference in rats. Therefore it was considered that ^{14}C is excreted somewhat faster in female rabbits than in rats, though excretion profile in female rabbits was similar to that in rats. As for ^{14}C excretion rate, it was similar to that in mice rather than that in rats.

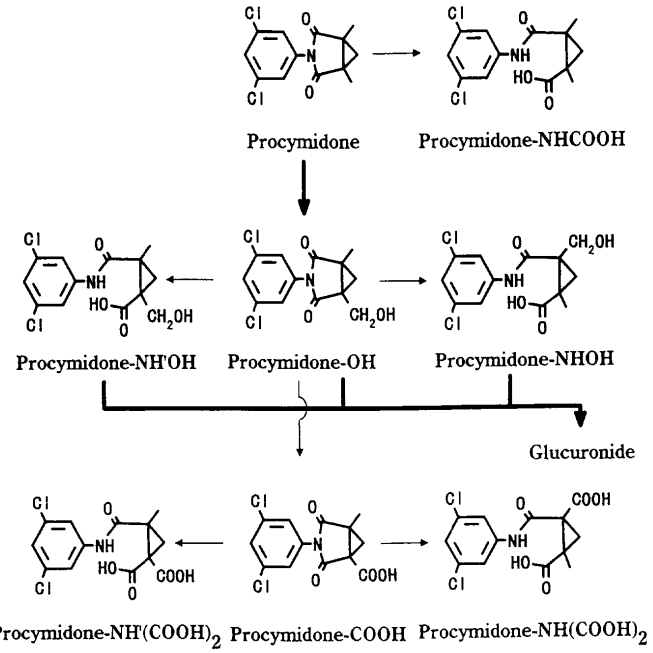


Fig. 5 Proposed metabolic pathways of procymidone in female rabbits.

The proposed metabolic pathways of procymidone in female rabbits are shown in Fig. 5. On the basis of the amounts of the metabolites identified in excreta, the major metabolic reactions were concluded to be as follows: 1) oxidation of one of the methyl groups to carboxylic acid *via* hydroxymethyl, 2) cleavage of the imide linkage, and 3) glucuronides formation of the 3 hydroxylated-procymidone metabolites.

The major metabolites in female rabbits were found to be glucuronides of procymidone-OHs (53.4%), whereas glucuronides of procymidone-OHs were not detected and procymidone-NH'$(COOH)_2$ was a major metabolite (47.0%) in male rats.[5] In the blood, there was no marked difference of the amounts of metabolites between rabbits and rats because glucuronides were not observed.

The difference between two species in glucuronide formation was confirmed by *in vitro* experiment showing an apparent glucuronyltransferase activity toward procymidone-OH in female rabbit, but no apparent activity in female rat. It seems that the oxidation activity of the methyl groups to carboxylic acid *via* hydroxymethyl in rats somewhat greater than that in rabbits though this difference was not confirmed. It is considered that the activity of glucuronide formation in rabbits were greater than the activity of carboxylic acid formation.

Our findings indicate that procymidone is readily absorbed from the gastrointestinal tract, distributed through blood, metabolized, and excreted completely in rabbits, but that there are considerable species differences in metabolic pathways between rats and rabbits.

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要　　約

雌ウサギにおけるプロシミドンの代謝

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プロシミドンの雌ウサギにおける体内動態を調べる目的で，^{14}C-プロシミドンを125 mg/kgの投与量で雌ウサギに単回経口投与し，尿，糞および血液を採取した．投与した^{14}Cは体内より速やかに排泄され，投与3日後の排泄率は95.3%（尿：72.1%，糞：23.2%）であった．血中の^{14}Cは1～6時間維持されたのち速やかに消失した．雌ウサギでは，最も主要な代謝物は3種のプロシミドン水酸化物のグルクロン酸抱合体であり，ラットやマウスとは異なっていた．主要な代謝反応としては 1）メチル基のヒドロキシメチル基を経由したカルボン酸への酸化，2）イミド結合の開裂，および 3）グルクロン酸によるプロシミドン水酸化物に対する抱合化の3種が推定された．さらにウサギとラットのグルクロン酸抱合活性に関する種間差を検討するため，主要な代謝物であるプロシミドン水酸化物1種に対するグルクロン酸抱合活性をそれぞれの動物の肝ミクロソームを用いて *in vitro* で調べた．雌ウサギの場合のみグルクロン酸抱合体が生成し，雌ラットでは生成しなかったことから，抱合活性の差がプロシミドンの体内動態に関するラットとウサギの種間差を派生させたものと思われた．