Percutaneous Absorption of Clonazepam in Rabbit

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The percutaneous (p.c.) absorption of clonazepam (CZP), an antiepileptic drug, was investigated in rabbits. CZP was efficiently absorbed from a gel ointment (0.5% CZP, 1 g, 9 cm^2) with Azone® and therapeutic plasma concentrations were maintained for 27 h. The bioavailability of CZP from the gel ointment was 47.2±3.1%, which was significantly larger than that (3.3±0.5%) after the ointment without Azone® or that (12.5±3.6%) after oral administration. About a half of CZP in the ointment with Azone® was absorbed during a 24 h application. The maximum and minimum plasma concentrations and the area under the plasma concentration-time curve gradually increased during repeated application of the ointment (2% CZP, 0.25 g/d, 2.25 cm^2), probably due to the accumulation of drug in the skin and body. The efficient absorption and sustained plasma concentration of CZP after application suggest that a once a day p.c. administration regimen is possible by using the ointment with Azone®.

Keywords clonazepam; percutaneous absorption; gel ointment; bioavailability; kinetic parameter; repeated application; rabbit

Clonazepam (CZP), one of the members of the benzodiazepine class of compounds, has been increasingly used in the treatment of seizure disorders. CZP is usually prescribed along with other common anticonvulsant drugs such as phenytoin, phenobarbital and succinimide. Pharmacokinetics of CZP have been studied in human,[1,2] monkey,[3] and dog.[2,4] The studies in man indicate that the elimination half-life ($t_{1/2}$) is relatively long; the $t_{1/2}$ of the β phase of the elimination curve ranges between 19 and 60 h,[1] or 18.7 and 39 h.[2] In addition, extensive biotransformation of CZP occurs in man and less than 0.5% of the dose is recovered in the urine as the intact drug in the 0—24 h excretion period.[2] The longer $t_{1/2}$ of CZP makes it possible to prescribe a once or twice a day oral administration regimen in man.

Epilepsy, involving sudden and transitory episodes of abnormal phenomena of motor, sensory, autonomic or psychic origin, is a chronic condition and long-term treatment is the rule. Therefore, it is necessary to develop a convenient and effective formulation which can be applied to patients easily and has the long action. We therefore attempted to develop percutaneous ointments containing CZP, aimed at systemic delivery of the antiepileptic drug. The pharmaceutical availability of the ointments was evaluated using rabbits. In addition, some *in vivo* parameters were determined in comparison with those after oral administration of the drug.

Experimental

Reagents and Animals CZP and Azone® were supplied by Sumitomo Chemical Co., Ltd. and Nelson Research and Development Co., respectively. Diazepam, an internal standard for high-performance liquid chromatography (HPLC), was a generous gift of Takeda Pharmaceutical Co., Ltd. Hiviswako 104, a gel base for ointments, was obtained from Wako Pure Chemical Industries, Ltd. All other chemicals used were of reagent grade. Male Japanese white rabbits, weighing 2.5 to 3.5 kg, were used throughout this experiment. The animals had free access to RC4 diet (Oriental Yeast Co., Ltd.) and water before the experiment. The animals for oral administration studies were fasted for 24 h before the experiment, with water being freely available.

Intravenous (i.v.) Administration CZP was administered intravenously in a 0.2 mg/kg dose after dissolving in a mixture of *N,N*-dimethylformamide and saline (30:70, v/v). After administration, blood samples (0.5—1.0 ml) were collected periodically for 24 h. The plasma was separated immediately by centrifugation.

Oral Administration CZP, which was suspended in 2% acacia (CZP 1.0 mg/ml), was administered orally in a 1.0 mg/kg dose through a rubber stomach tube and immediately 3 ml of water was instilled. Blood samples were collected periodically for 24 h after dosing. The plasma was separated immediately centrifugation.

Preparation of Ointments CZP was dissolved in diethylene glycol monoethyl ether (carbitol). The solution was mixed with a gel base containing water, propylene glycol, diisopropyl adipate and diisopropanolamine and/or absorption enhancers, Azone® and sorbitan monooleate. Details of the ointment composition are listed in Table I.

Percutaneous (p.c.) Administration On the first day of the experiment, the hair of the back area was carefully removed with an electric clipper without damaging the stratum corneum. On the next day, 1.0 or 0.25 g of ointment was uniformly spread over the shaved back skin (3.0×3.0 or 1.5×1.5 cm area, respectively, designated by attaching an adhesive tape with a cut-out area) and immediately occluded with a sheet of aluminum foil and fixed with one adhesive tape. The ointment remained in contact with the skin for 24 h. In the case of repeated p.c. application of 2.0% CZP ointment (Rp. 3), the ointment was applied for 24 h by the same method as in the single administration study, then the unabsorbed ointment was wiped off with absorbent cotton soaked in warm water. The application of

TABLE I. Formulae of CZP Ointments and Application Protocol

Rp.	CZP (% (w/w))	Solvent (% (w/w))		Base (% (w/w)) Hiviswako 104	Azone (% (w/w))	SM[c] (% (w/w))	Application		
		Carbitol[a]	PG[b]				Ointment (g)	Dose (mg/rabbit)	Area (cm^2)
1	0.5	20.0	20.0	1.0	—	—	1.0	5.0	9.0
2	0.5	20.0	20.0	1.0	4.0	4.0	1.0	5.0	9.0
3	2.0	39.0	10.0	1.0	4.0	4.0	0.25	5.0	2.25

a) Diethylene glycol monoethyl ether. *b*) Propylene glycol. *c*) Sorbitan monooleate. The remainder was diisopropanolamine (1.1% (w/w)), diisopropyl adipate (2.0% (w/w)) and water.

ointment was repeated for 3 d with daily different application sites. The application protocol is listed in Table I.

Determination of CZP Remaining in Ointment after Application Twenty-four hours after application of 0.5% CZP ointment (Rp. 2, 1.0 g, 3.0 × 3.0 cm area), unabsorbed ointment was wiped off with gauze. The gauze was shaken in 3.0 ml of 2 M glycine buffer, pH 10, and the drug in the mixture was extracted with a mixture of benzene and dichloromethane (9 : 1, v/v, 5.0 ml). The organic layer was concentrated in vacuum. Five milliliters of a mixture of 0.9% NaCl–10 mM phosphate buffer, pH 7.4, and acetonitrile (1 : 1, v/v) was added to the concentrated sample and vortexed. The mixture was shaken with 2.0 ml of petroleum ether to remove Azone®. The aqueous phase was used as the HPLC sample. To correct for the loss of drug during the operation, CZP in the ointment wiped up immediately after application was determined by the same method.

Determination of CZP CZP in plasma sample was determined by the method of Hishida *et al.*[5] with slight modifications. To a 0.2—0.5 ml aliquot of a plasma sample, 100 ng of diazepam (internal standard) dissolved in acetonitrile (100 ng/0.1 ml) and 5.0 ml of the solvent were added. After vortexing for 30 s, the mixture was centrifuged. The clear supernatant (4.8 ml) obtained was evaporated in vacuum. The residue was dissolved in 0.12 ml of mobile phase solution and filtered through a Chromatodisc filter (0.45 μm, Biofield Co., Ltd.). The filtrate (90 μl) was injected into a column (4.6 mm i. d. × 15 cm) packed with Inertsil ODS (5 μm particle size, Gasukuro Kogyo Co., Ltd.) using Shimadzu LC-6A liquid chromatograph equipped with a SPD-6AV UV-VIS detector. The mobile phase was a mixture of 1% trimethylamine adjusted to pH 3.0 with phosphoric acid and acetonitrile (3 : 2, v/v), pumped at a flow rate of 1.0 ml/min. Detection was at 254 nm. The limit of detection of CZP in plasma was approximately 7 ng/ml.

Analysis of Data Kinetic parameters were calculated by using the least-squares fit program, MULTI.[6] The plasma concentration data after i.v. administration were fitted to the equation:

$$C_t = Ae^{-\alpha t} + Be^{-\beta t}$$

where C_t is the drug concentration at time t, and A, α, B and β are the biexponential equation constants.

The area under the plasma concentration-time curve (AUC), the mean residence time (MRT), the area under the first moment curve ($AUMC$) and the variance of the residence time (VRT) were calculated by means of the following equations[7]:

$$AUC = \int_0^t C_p dt + \int_t^\infty C_t \cdot e^{-k_e t} dt$$

$$AUMC = \int_0^t t \cdot C_p dt + \int_t^\infty t \cdot C_t e^{-k_e t} dt$$

$$MRT = AUMC/AUC$$

$$VRT = \left(\int_0^t t^2 \cdot C_p dt + \int_t^\infty t^2 \cdot C_p \cdot e^{-k_e t} dt \right) \Big/ AUC - MRT^2$$

where C_t and k_e are the plasma concentration at the last sampling point and the terminal elimination rate constant calculated using the last 3—4 data points, respectively, according to the least-squares regression analyses.

The means of all data are presented with their standard deviation (mean ± S.D.). Statistical analysis was performed by using the non-paired Student's t test, and a p value of 0.05 or less was considered to be significant.

Results

Plasma Concentration of CZP after Single i.v. Administration The plasma concentration-time curve for CZP after a single i.v. administration (0.2 mg/kg) is shown in Fig. 1. The plasma decay curve after dosing showed biexponential kinetics. Pharmacokinetic parameters calculated by using the 2-compartment open model are listed in Table II. The half-life ($t_{1/2,\beta}$) of the β phase was about 5.9 h, indicating relatively slow elimination of the drug.

Oral and p.c. Administrations Plasma CZP concentration–time profiles after oral and p.c. administrations are shown in Fig. 2 and the kinetic parameters obtained are listed in Table III. The plasma levels reached therapeutically effective concentrations (20—70 ng/ml)[8] 1 h after oral administration. However, at 24 h after dosing the level in

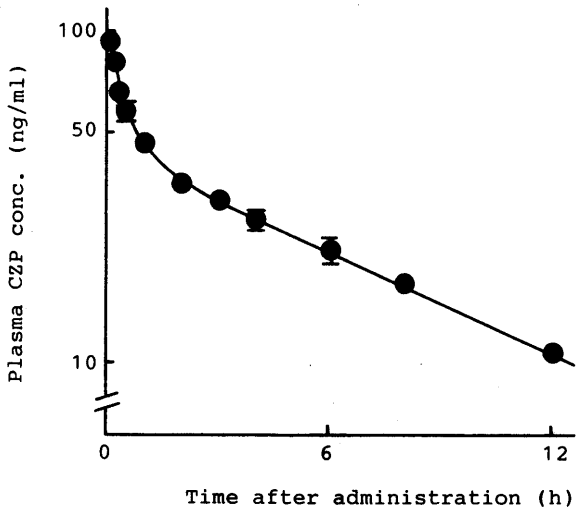

Fig. 1. Plasma Concentration of CZP after Intravenous Administration

Each point represents the mean ± S.D. of 4 experiments. Dose, 0.2 mg/kg.

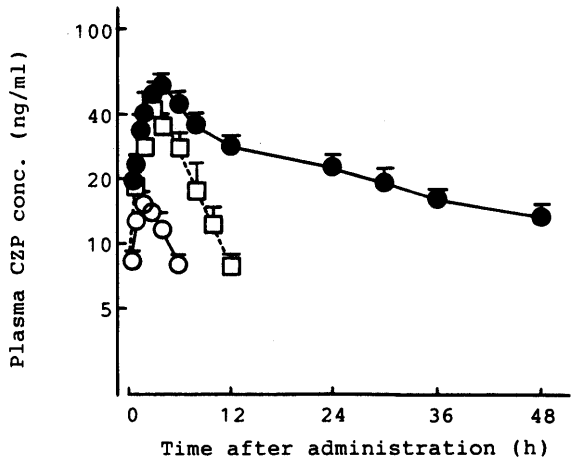

Fig. 2. Plasma Concentration of CZP after Oral and Percutaneous Administrations

Each point represents the mean ± S.D. of 4—5 experiments. The applied dose of ointment was 1.0 g/9 cm². □, oral (1.0 mg/kg); ○, p.c. (Rp. 1); ●, p.c. (Rp. 2).

TABLE II. Pharmacokinetic Parameters after Intravenous Administration

Parameter	Mean ± S.D.
A (ng/ml)	60.15 ± 2.60
α (h^{-1})	2.69 ± 0.67
B (ng/ml)	43.35 ± 3.25
β (h^{-1})	0.117 ± 0.009
k_{10} (h^{-1})	0.263 ± 0.019
k_{12} (h^{-1})	1.345 ± 0.361
k_{21} (h^{-1})	1.194 ± 0.296
V_1 (l/kg)	1.936 ± 0.104
AUC (ng·h/ml)	490.4 ± 26.6
MRT (h)	8.17 ± 0.57
VRT (h^2)	11.4 ± 0.3

k_{10}, elimination rate constant; k_{12} and k_{21}, distribution rate constants; V_1, distribution volume of central compartment. Each value represents the mean ± S.D. of 4 experiments.

TABLE III. Pharmacokinetic Parameters of CZP after Percutaneous and Oral Administrations

Parameter	Oral	Percutaneous		
		Rp. 1	Rp. 2	Rp. 3
C_{max} (ng/ml)	42.5 ± 4.8	16.2 ± 1.0	55.9 ± 1.8[c]	49.6 ± 3.7[d]
T_{max} (h)	2.0	1.5	3.0	2.0
AUC (ng·h/ml)	306.2 ±32.7	121.1 ±16.3	1808.2 ±117.3[c]	801.3 ±170.4[d]
Bioavailability[a]	12.5 ± 3.6	3.3 ± 0.5	48.2 ± 3.1[c]	21.9 ± 4.6[e]
Duration[b]	6.25± 1.44	0	27.1 ± 2.3	6.1 ± 0.6[e]
MRT (h)	6.80± 0.93	6.45± 1.12	45.43± 3.54[c]	29.60± 12.46[e]
VRT (h^2)	51.7 ± 4.7	33.8 ±13.0	—	—

a) Bioavailability (%)$=\frac{AUC_{p.c.}\ dose_{i.v.}}{AUC_{i.v.}\ dose_{p.c.}}$. *b*) Time for which a therapeutically effective concentration (20—70 ng/ml) was maintained. *c*) $p<0.02$ compared with Rp. 1. *d*) $p<0.05$ and *e*) $p<0.02$ compared with Rp. 2.

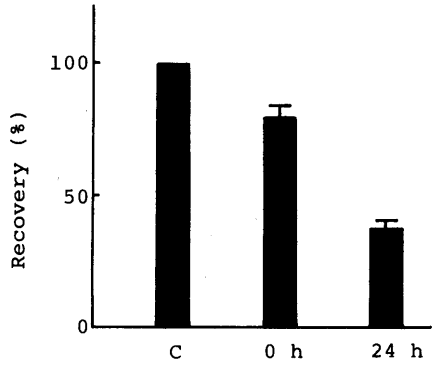

Fig. 3. The Recovery of CZP from Ointment after Application

Each bar represents the mean ± S.D. of 4 experiments. C, amount of drug from ointment (1.0 g) unapplied.

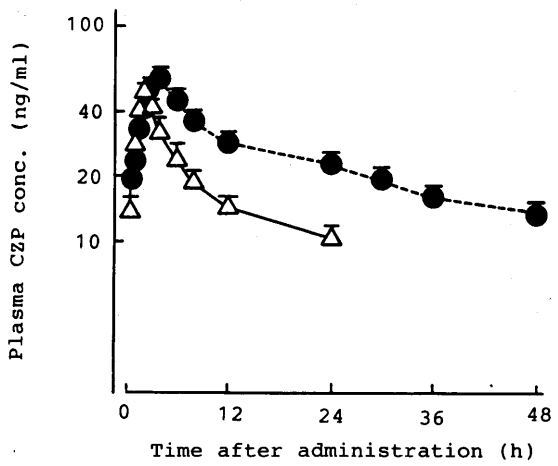

Fig. 4. Plasma Concentration of CZP after a Single Percutaneous Administration (Rp. 3)

Each point represents the mean ± S.D. of 4 experiments. The applied dose was 0.25 g/2.25 cm^2 (Rp. 3, △) and 1 g/9 cm^2 (Rp. 2, ●).

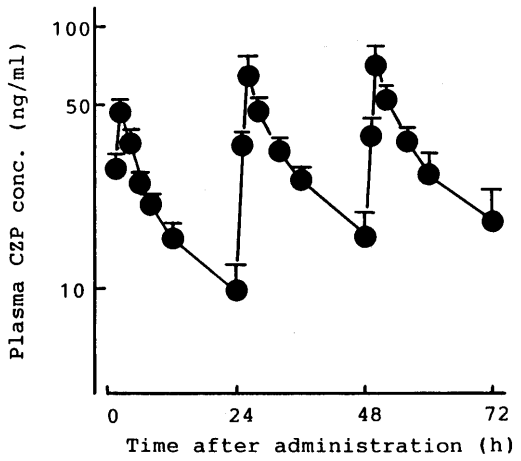

Fig. 5. Plasma Concentration of CZP after Repeated Percutaneous Administrations

Each point represents the mean ± S.D. of 4 experiments. The applied dose (Rp. 3) was 0.25 g/2.25 cm^2 daily.

plasma was below the detection limit. The absorption of the drug was relatively rapid and the time (T_{max}) to reach the peak plasma concentration was about 3 h after dosing, although the calculated bioavailability was low (12.5 ± 3.6%).

After p.c. application of the 0.5% CZP ointment without Azone® (Rp. 1), plasma levels were very low and fell below the limit of detection 8 h after dosing. The maximum drug concentration (C_{max}) was 16.2 ± 1.0 ng/ml, and the plasma level did not attain a therapeutically effective concentration. When the 0.5% CZP ointment with Azone® (Rp. 2) was applied, the bioavailability was 48.2%, which was 15 times larger than that (3.3%) after Rp. 1 and was also significantly larger than that after oral administration ($p<0.02$). The period over which therapeutic plasma concentrations were maintained after application of Rp. 2 was over 24 h, suggesting that a therapeutically effective level may be maintained by a once a day p.c. application regimen.

Amount of CZP Remaining in Ointment after Application The recoveries of CZP from the ointments unapplied and applied for 24 h to rabbit skin were 78.7 and 37.4%, respectively, as depicted in Fig. 3. These values suggested some loss (about 21%) of the drug during the extraction and wiping processes and consequently it appeared that about 53% of the drug in the ointment was absorbed into the skin and circulation during the experimental period. The latter value agreed roughly with the bioavailability (48.2%) after application of the ointment for 24 h.

Single p.c. and Repeated p.c. Administrations in a Small Application Area For practical purposes, a small area (1.5 × 1.5 cm area) of application site and a small amount (0.25 g) of ointment, the CZP content of which was enhanced to 2%, were used in this experiment. The plasma

TABLE IV. Pharmacokinetic Parameters of CZP after Repeated Percutaneous Administrations

Day	C_{max}	C_{min}	AUC_{0-24} (ng·h/ml)
1	47.51±4.79	9.82±1.85	460.7±32.2
2	63.98±3.68[a)]	15.92±1.94[a)]	711.8± 9.6[a)]
3	70.70±4.19[a,b)]	17.95±1.21[a)]	779.7±30.6[a,c)]

a) $p<0.02$ compared with day 1. *b)* $p<0.01$ and *c)* $p<0.02$ compared with day 2.

concentration–time curve for CZP after a single application of the ointment (Rp. 3) is shown in Fig. 4, together with the curve obtained previously with 0.5% CZP ointment (Rp. 2). The parameters calculated are presented in Table III. Although the C_{max} after the 2% ointment was approximately similar to that of the 0.5% ointment, the elimination from plasma was relatively fast, probably due to the lack of prolonged absorption of the drug, as suggested by the AUC value. The period of maintenance of therapeutic plasma concentrations was decreased to about 6 h, whereas the period was about 27 h for the 0.5% ointment (Rp. 2).

The plasma CZP concentrations and kinetic parameters during repeated p.c. application of the 2% CZP ointment are shown in Fig. 5 and Table IV, respectively. Significant increases in the C_{max}, the minimum plasma level (C_{min}) and AUC were observed with repeated application, in comparison with those after the first dosing, suggesting the gradual accumulation of the drug. On the third dosing, the plasma concentrations were roughly maintained within the therapeutic range over the entire application period.

Discussion

The pharmacokinetic properties and plasma levels of CZP after p.c. application have not been fully reported up to the present. Since CZP is highly effective in the treatment of status epilepticus, it may be useful for patients to develop a new delivery system of the drug. We therefore examined the p.c. absorption of CZP in the absence and presence of enhancers in comparison with oral administration of the drug.

In the absence of enhancers, good absorption of CZP through rabbit skin was not observed. This may be due to the low thermodynamic activity of CZP, as suggested by its low solubility and high melting point (236.5—238.5 °C).[9] The p.c. absorption of a drug varies with its physicochemical properties,[10] such as molecular weight, melting point, solubility and partition coefficient between oil and water. The addition of absorption enhancers to the ointment significantly enhanced the p.c. penetration of CZP and consequently the therapeutic plasma concentration was maintained over a long period. This demonstrated the effectiveness of p.c. application of CZP with a suitable enhancer such as Azone®.[11]

The bioavailability after p.c. application of the ointment (Rp. 2) was 47.0%, the value being larger than that of oral administration. This suggests that the 0.5% ointment with enhancer is a useful and effective ointment in the treatment of seizure disorders. However, a small amount of preparation and a small area of application site are among the goals of a p.c. drug delivery system. Thus 2% CZP ointment was prepared and 0.25 g of the ointment was applied to rabbit back skin. The absorption of the drug after a single application of the 2% ointment (2.25 cm^2) was considerably less than that after the 0.5% ointment (9 cm^2) and a concentration above 20 μg/ml was maintained for only 6 h. This may relate to the low solubility of CZP and possible partial precipitation in 2% ointment. When the 2% ointment was applied to the skin repeatedly, the plasma CZP concentrations were gradually enhanced. This phenomenon can probably be explained in term of accumulation of the drug in the body. The difference between the parameters obtained after dosing of the 0.5% and 2% ointments may results mainly from the difference of the area onto which the skin patch was applied. However, the increased content of carbitol in the 2% ointment may also contribute to the decrease in these values, probably owing to the increased viscosity. The total amount of drug in the blood, in transdermal pharmacokinetics experiments, is parallel to the area of application.[12] Therefore, the period (6 h) for which a therapeutic plasma level can be maintained after dosing of the 2% ointment could probably be extended by the enlargement of the area of application.

In conclusion, CZP was efficiently absorbed from the gel ointment in the presence of absorption enhancers, Azone® and sorbitan monooleate, and therapeutic CZP concentrations were maintained for 27 h with the 0.5% ointment (1 g, 9 cm^2) and 6 h with 2% ointment (0.25 g, 2.25 cm^2). These results suggest that a once a day p.c. administration regimen for epileptic patients is possible by using the ointment.

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