RELATION BETWEEN TIME COURSES OF PHARMACOLOGICAL
EFFECTS AND OF PLASMA LEVELS OF CAMAZEPAM AND ITS
ACTIVE METABOLITES IN RATS *

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Camazepam (CZ), a benzodiazepine, was biotransformed to more than ten metabolites. After intravenous and oral administration of these metabolites to rats, CZ, tempazepam (TZ), oxazepam (OZ), and hydroxy CZ (M₁) were found to possess pharmacological activities. The brain-to-plasma concentration ratios of CZ and these active metabolites were essentially constant with time after oral administration of CZ. Thus the brain, target organ, was kinetically included in the plasma compartment. The extent of binding of these compounds to plasma protein was independent of concentration tested.

Plasma levels of an unchanged drug and its active metabolite(s), and muscle relaxation effect (the impairment of rota rod performance) were measured at 0.5 to 8 h after oral administration of 2 to 3 doses of CZ, TZ, and OZ to rats. When the effect and plasma level data were computer-fitted to a simple Hill equation or a modified Hill equation including competitive factors, the modified Hill equation was found to be adequately applicable to the concentration–effect relation. The parameter values thus obtained could predict the contribution of the administered drug and its active metabolite(s) to the observed pharmacological effect after administration.

Keywords — benzodiazepine; camazepam; temazepam; oxazepam; plasma concentration; pharmacological effect; rat

INTRODUCTION

In the previous papers, we reported the relation between pharmacokinetics and pharmacodynamics of drugs whose metabolites are not present in the body or are pharmacologically inactive. However, when metabolites also contribute to pharmacological effects together with their parent drug, the kinetic studies on the effect–disposition relation may be very complicated. In fact, there are many drugs that are converted to active metabolites. For example, it is well known that metabolites of benzodiazepines elicit pharmacological activities together with their parent drugs.

Caccia et al. reported that camazepam (CZ), a benzodiazepine, is metabolized to two main metabolites, temazepam (TZ) and oxazepam (OZ), which are pharmacologically active. However, we have recently found that CZ is converted to more than ten metabolites besides TZ and OZ in rats. They still possess the diazepine ring so that some of them may be pharmacologically active like TZ and OZ.

In the present paper, we describe a search for active metabolites and the relation between plasma levels and pharmacological effect of CZ and its active metabolites in rats.

MATERIALS AND METHODS

Materials — CZ, TZ, and OZ, labeled with ¹⁴C in 2-position of the diazepine ring, were synthesized in our laboratories. Their radio-
chemical purities were shown to be more than 98% by thin-layer chromatography (TLC). The specific radioactivities of CZ, TZ, and OZ were 10.6, 11.5, and 5.7 μCi/mg, respectively. The un-labeled compounds (except M₀ and M₁₀) shown in Fig. 1 were also synthesized.

Administration of Test Compounds — Test compounds were dissolved in 10% HCO-60 saline for i.v. injection, and suspended in 0.5% aqueous carboxymethyl cellulose sodium (CMC-Na) for oral administration in a volume of 5 ml/kg. Male Sprague-Dawley rats (5–6 weeks unless stated otherwise, Shizuoka Animal Firm Co. Ltd.) were used. Test compounds were administered i.v. via the jugular or the tail vein and orally with a stomach tube to rats.

Assay of Plasma and Brain Levels of Test Compounds — Blood was withdrawn 5 min after i.v. dosing and 0.5, 1, 2, 4, 6, and 8 h after oral administration from the jugular vein. CZ and its metabolites in plasma and brain homogenate obtained after ¹⁴C-CZ dosing were extracted with ethylacetate and separated by two-dimensional TLC; solvent systems were CHCl₃-acetone (7:3) and isopropanol-benzene (1:9). Each zonal silica gel corresponding to the authentic compound was scraped off and assayed for ¹⁴C in a Pachard Tri-Carb liquid scintillation spectrometer. TZ and/or OZ in plasma and brain homogenate after administration of ¹⁴C-TZ and ¹⁴C-OZ were measured by the method for CZ with a slight modification. Plasma and brain levels of hydroxy CZ (M₈) after its i.v. injection were determined with a high performance liquid chromatograph (ALC/GPC204, Waters Associates, USA) after benzene extraction. Operating conditions were: column, stainless steel column (4.6 × 250 mm packed with Nucleosil 7C 18; mobil phase, methanol-acetonitrile-water (10:1:7); flow rate, 1 ml/min; detection, ultraviolet (UV) absorbance at 254 nm; tᵣ of M₈ was 13.5 min.

Plasma Protein Binding — ¹⁴C-CZ, ¹⁴C-TZ, ¹⁴C-OZ, and M₈ were added individually to each 3–5 ml aliquot of heparinized pool plasma obtained from 3–5 rats receiving no drug to make the concentrations of 0.05–10 μg/ml. About 1 ml of the sample plasma was placed in an ultrafiltration apparatus (MPS-1, Amicon Far East Ltd., Japan) and ultrafiltrated at a room temperature. The labeled compounds and M₈ in the filtrate and the plasma before filtration were determined as described above.

Assay of Pharmacological Effects — 1) Muscle Relaxation (a Rota Rod Test): Rats were placed on horizontal rods (i.d. = 9 cm) rotating at 8 rpm. Control rats remained on the rods for more than 3 min. Animals were placed on the rods 4 min after i.v. injection of test compounds and 0.5, 1, 2, 4, 6, and 8 h after oral administration. The intensity of the pharmacological effect was defined as the ratio of the number of animals that fell off the rods within 2 min versus the number of animals used. Six to eight and ten to seventeen animals were used at each i.v. and oral dose, respectively. ED₅₀ values were calculated by a linear regression of log dose-effect data from at least 4 doses of test compounds.

2) Anti-pentylenetetrazole (PTZ) Effect: PTZ dissolved in saline was injected at an i.p. dose of 70 mg/kg 4 min after i.v. preadministration of test compounds, and at an s.c. dose of 100 mg/kg at each assay time after oral pre-dosing. These i.p. and s.c. doses of PTZ proved to cause clonic convulsion within 5 and 30 min, respectively, in more than 95% of control rats. Thus the animals, pretreated with test compounds, that showed no clonic convulsion within these times were considered affected. Six to eight and ten animals were used at each i.v. and oral dose, respectively. ED₅₀ values were calculated as described above.

3) Anti-conflict Effect: A anti-conflict test was carried out by the method of Cook and Davidson, using well-trained rats weighing 300–400 g. Briefly, experimental sessions consisted of 12 min period of VI 30 s (non-punishment) and 3 min period of FR 10 (punishment) components. CZ, both TZ and OZ, and the other compounds were administered orally 1.5, 0.5, and 1.0 h, respectively, before the test-
ing. Anti-conflict effect was defined as a percent change of lever responses during the punished FR 10 component (total time = 12 min) after administration of test compounds. Four to six animals were used at each dose. The doses that require a 50% increase of lever responses compared with pre-drug values were estimated by a linear regression of log dose-effect data from at least 4 doses.

Model Equation — If CZ, TZ, OZ, and M₅ are pharmacologically active, and if these metabolites of CZ are formed in the following order: CZ → M₅ → TZ → OZ, then the pharmacological effect after administration of these compounds except OZ is expected to be produced not only by the administered compounds but also by their active metabolite(s) formed. We now try to construct model equations that should relate their pharmacological effects to concentrations of these compounds in a pharmacokinetic compartment including sites of action, essentially on the basis of the Hill equation.⁷

If an administered compound and its active metabolite(s) exert their effect independently according to the concentration-effect relation based on a simple Hill equation, then the intensity of observed effect after oral administration is shown by Eq. 1.

\[
E_N = \sum_{i=1}^{N} \epsilon_i = \sum_{i=1}^{N} \left(1 + \left[\frac{E_{50,i}}{C_i} \right]^H \right)
\]  

where \( E_N = \) a fractional maximal effect at a given time after oral dosing of \( N \)-th compound; \( \epsilon_i = \) a partial effect caused by \( i \)-th compound; \( C_i \) and \( E_{50,i} = \) a plasma level at a given time and the 50% effective plasma level of \( i \)-th compound, respectively; \( N \) or \( i = 1, 2, 3, \) and 4 are for OZ, TZ, M₅, and CZ, respectively; \( H = \) Hill coefficient.

Benzodiazepines are known to exert their pharmacological effect by binding to their common receptors. Thus, when they (a parent drug and its active metabolite(s) formed) are present simultaneously near their receptors, they compete with each other for the receptors. As a result, it is possible that the pharmacological effect produced by the respective compound is less when the other compound(s) is present than when the other(s) is absent. Taking this into consideration, we construct the modified Hill equation (Eq. 2) which should be applied to the concentration-effect relation of the parent compound and its metabolite(s).

\[
E_N = \sum_{i=1}^{N} \epsilon_i = \sum_{i=1}^{N} \left(1 + \left[\frac{1 + \sum_{j \neq i}^{N} C_j/ E_{50,j}}{C_i} \right] \right) \left[\frac{E_{50,j}}{C_i} \right] H
\]  

where \( j = 1, 2, 3, \) and 4 are for OZ, TZ, M₅, and CZ, the other terms are defined in Eq. 1.

Estimation of Model Parameters — Pharmacological effect data were fitted by a nonlinear least squares program to Eq. 1 or 2 with weighting values of unity on a digital computer (Okitac 4300b, Oki Industry Co., Japan): the program was based on the algorithm of Berman et al.⁸ Plasma level data of CZ and its active metabolites were directly substituted for \( C_i \) (or \( C_j \)) in both equations. Better fitness of the observed effect data to a model equation was evaluated by sum of squared deviation as well as visual inspection.

RESULTS

1. Search for Active Metabolites

CZ and more than ten metabolites were found in plasma and brain after oral administration of \(^{14}\)C-CZ to rats; Fig. 1 shows that these metabolites are desacarbamoyl, desmethyl, and/or hydroxy products. We examined whether these metabolites are pharmacologically active in rats (Table I). After oral administration of CZ, TZ, OZ, and several desmethyl metabolites, only CZ, TZ, and OZ produced the pharmacological activities such as muscle relaxation, anticonvulsion, and anti-conflict effects. Next, CZ and its eleven metabolites were injected i.v., and M₅ as well as CZ, TZ, and OZ was found to produce effects (see Table I).
FIG. 1. Metabolic Pathways of Camazepam

TABLE I. $ED_{80}$ (mg/kg) of Camazepam and Its Metabolites after Intravenous and Oral Administration in Rats

<table>
<thead>
<tr>
<th>Compound</th>
<th>Oral</th>
<th>Intravenous</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Muscle relaxation (Rota rod)</td>
<td>Protection against pentylenetetrazole clonic convulsion</td>
</tr>
<tr>
<td>Camazepam</td>
<td>52</td>
<td>34</td>
</tr>
<tr>
<td>$\text{M}_4$</td>
<td>8.7</td>
<td>6.7</td>
</tr>
<tr>
<td>$\text{M}_4'$</td>
<td>(2.4–14.6)</td>
<td>(5.1–8.7)</td>
</tr>
<tr>
<td>$\text{M}_5$</td>
<td>&gt;160</td>
<td>–</td>
</tr>
<tr>
<td>Oxazepam</td>
<td>23</td>
<td>19</td>
</tr>
<tr>
<td>$\text{M}_7$</td>
<td>(9–42)</td>
<td>(14–26)</td>
</tr>
<tr>
<td>$\text{M}_7'$</td>
<td>&gt;160</td>
<td>–</td>
</tr>
<tr>
<td>$\text{M}_8$</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>$\text{M}_9$</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>$\text{M}_9''$</td>
<td>&gt;160</td>
<td>–</td>
</tr>
<tr>
<td>$\text{M}_{10}$</td>
<td>–</td>
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</tr>
</tbody>
</table>

$^a$ Requires 50% increase of lever responses compared with pre-drug values. $^b$ 95% confidence limits.
FIG. 2. Plasma Levels of Camazepam, Temazepam, Oxazepam, and M₅ versus Time after Oral Administration of Camazepam, Temazepam, and Oxazepam to Rats

The number in each figure indicates the dose in mg/kg. Each concentration value represents mean ± S.E. (n = 5-15). — • — , camazepam; — △ — , temazepam; — ○ — , oxazepam; — □ — , M₅.
2. Determination of a Compartment Which Includes the Sites of Action

On the basis of the pharmacological results in the previous section and of the metabolic pathways shown in Fig. 1, the following is postulated: (1) After oral administration of CZ, CZ and its active metabolites (TZ, OZ, and M₅) appear in plasma and brain, and all of them exert the pharmacological effect. (2) After oral dosing of TZ, TZ and OZ formed are present in plasma and brain, and both elicit the effect. (3) After oral administration of OZ, OZ appears in plasma and brain and exerts the activity by itself. Thus, the plasma levels of these administered compounds and their possible active metabolite(s) were measured as a function of time after oral dosing of CZ, TZ, and OZ to rats (Fig. 2). Fig. 3 shows the brain-to-plasma concentration ratios (B/P ratio) of CZ and its three active metabolites after oral administration of ¹⁴C-CZ to rats. Except for the ratios of TZ and OZ at the first sampling time (0.5 h), the other B/P ratios of these compounds were essentially constant with time after oral administration of ¹⁴C-CZ. In addition, the B/P ratios of TZ and OZ formed after oral dosing of ¹⁴C-TZ were also time-independent except for that of OZ at 0.5 h and their mean values were close to the corresponding values shown in Fig. 3. Thus the brain, target organ, could be included kinetically in the plasma compartment.

The extent of binding of these compounds to rat plasma protein was found independent of concentration tested: the mean binding percentages of these four compounds ranged from 79 to 86. Thus, the total (free + bound) plasma level data were used as concentration in a plasma compartment.

3. Fitting of the Pharmacological Data to Functional Equations

Fig. 4 shows the intensity of muscle relaxation as a function of time after oral administration of 2-3 doses of OZ, TZ, and CZ to rats. On fitting of these effect data to the functional equations shown in Materials and Methods, it seemed difficult to obtain the converged results because the plasma level-time curves of CZ and M₅ are very close to each other after administration of all dose levels of CZ. To solve this problem, we used the i.v. data. The mean plasma levels (±S.E.) of CZ and M₅ were 1.10 (±0.17) and 5.51 (±0.34) μg/ml (n=3-4), respectively, at 5 min after i.v. equiactive doses of CZ and M₅, corresponding to ED₅₀s. Though these values should be also their 50% effective plasma levels after oral dosing, the difference between the experimental conditions of i.v. and oral administration must be taken into consideration. These values are, therefore, utilized only to determine the relative value of the pharmacological potency between CZ and M₅: EC₅₀ of M₅ = 5.01 × EC₅₀ of CZ. On computer-fitting, this relation was applied to Eqs. 1 and 2 so that EC₅₀ of M₅ was excluded from the estimated parameters.

Finally, we simultaneously fitted the effect data at all dose levels of all compounds to Eq. 1

![Graph](image-url)
FIG. 4. Muscle Relaxant Effect (Impairment of Rota Rod Performance) versus Time after Oral Administration of Camazepam, Temazepam, and Oxazepam to Rats

The effect on the ordinate at a given time on the abscissa was defined as the number of animals affected versus the number of animals tested (see the detailed definition in Materials and Methods). The numbers near the curves show the dose in mg/kg. The curves represent the model-predicted values based on Eqs. 1 (-----) and 2 (---). The parameter values (±S.D.) estimated by fitting to Eq. 2 are: EC$_{50}$ (µg/ml) for oxazepam, temazepam, and camazepam, and Hill coefficient = 1.13 ± 0.06, 0.179 ± 0.011, 0.311 ± 0.030, and 2.05 ± 0.13, respectively; the derived parameter value, EC$_{50}$ for M$_5$, = 5.01 × (EC$_{50}$ for camazepam) = 1.56. The corresponding parameter values (±S.D.) by Eq. 1 are: 1.22 ± 0.14, 0.295 ± 0.054, 2.29 ± 0.71, 1.40 ± 0.19 (estimated), and 11.5 (derived). Ten to seventeen animals were used in each dose.

FIG. 5. Contribution of the Administered Compound and Its Active Metabolite(s) to the Observed Pharmacological Effect

The lines are calculated by Eq. 2 with the known parameter values and concentration values. The solid circles represent the observed values which are the same as those shown in Fig. 4.
or 2. The estimated parameter values and the model-predicted curves are shown in Fig. 4 and its legend. The better model (either Eq. 1 or 2) was selected according to the sum of squared deviations (SS) between the observed and the model-predicted values. SS by Eq. 2 was about 9 times smaller than that by Eq. 1 after CZ dosing whereas the difference between SS by Eqs. 1 and 2 was only within 2 times after administration of TZ and OZ. In addition, a visual inspection supports these trends. Thus, it is suggested that Eq. 2 is more adequately applicable to the simultaneous characterization of the concentration–effect relation of CZ and its active metabolites.

4. Evaluation of the Contribution of CZ and Its Active Metabolites to the Observed Effect

By applying the estimated parameter values as well as the plasma level data to each $e_j$ in Eq. 2, we estimated the time-courses of the effect intensity caused by each compound (Fig. 5). The observed effect after CZ dosing is caused mainly by CZ itself with the higher dose, but greatly by TZ with the lower dose; OZ as a metabolite contributes negligibly and somewhat to the observed effect after administration of CZ and TZ, respectively.

DISCUSSION

Of more than ten metabolites of CZ, pharmacological effects in rats were observed after oral and/or i.v. administration of TZ, OZ, and M$_5$ as well as CZ. And these compounds are considered to possess pharmacological potency because they produced the effects at as early as 5 min after i.v. injection when their metabolites are present negligibly in plasma and brain.

Though the target organ for benzodiazepines is the brain, we used concentrations of CZ and its active metabolites in the plasma compartment to relate them to the muscle relaxant effect because the brain-to-plasma concentration ratios of these compounds were essentially constant with time after oral dosing. However, this B/P ratio of metabolically formed TZ and OZ is significantly lower at the first sampling time (0.5 h) than at subsequent times (1–8 h). Though this fact seems to make the above approach somewhat unclear, it did not cause a serious problem in the kinetic characterization of the concentration–effect relation of CZ and its active metabolites because the contribution of the effect caused by these metabolites to the observed effect was found very small at 0.5 h (see Fig. 5).

Techniques of drug concentration–effect analysis have been often based on unified models which describe kinetic and dynamic data simultaneously (see, for example, ref. 9). In the present study, we could not construct an adequate pharmacokinetic model for plasma levels of CZ and its active metabolites because of existence of nonlinear disposition (see Fig. 2). Thus, these plasma level–time data were directly substituted for $C_j$ or $C_j$ in Eqs. 1 and 2 to which the pharmacological data were computer-fitted. After nonlinear least squares fitting, the model-predicted values (the curves shown in Fig. 4) and SS thus obtained indicate that Eq. 2 is much better than Eq. 1 for describing the concentration–effect relation of CZ and its active metabolites after CZ dosing. Though, strictly speaking, Eq. 2 (rather than Eq. 1) is also applicable to the relation after administration of TZ, the small contribution of OZ as a metabolite to the observed effect (see Fig. 5) makes the better applicability of Eq. 2 unclear. Therefore, Eq. 2, a modified Hill equation including competitive factors, is more adequately applicable to the concentration–effect relation where at least two active substances contribute evenly (or no one does negligibly) to the observed (total) effect by competitively binding to their receptors. The administration of CZ is the case. In fact, CZ and these active metabolites are known to have affinities to benzodiazepine receptors (ref. 10, 12 and our unpublished data).

The important finding obtained in the present study may be about the extent of contribution of the administered compound and its active metabolite(s) formed to the observed effect, as shown in Fig. 5. The contribution of OZ as a metabolite to the observed effect after oral
dosing of CZ and TZ was much smaller than that predicted from ED$_{50}$ values. In fact, EC$_{50}$ of OZ estimated by the fitting to Eq. 2 is six times that of TZ, whereas ED$_{50}$ of OZ is only 2—3 times that of TZ. This discrepancy is mainly caused by that, though the B/P ratios of TZ and OZ are similar, the maximal plasma level of unchanged OZ is about two times higher than that of unchanged TZ after oral dosing of the same doses of OZ and TZ to rats (see Fig. 2). Thus, the muscle relaxant effect by OZ as a metabolite is much smaller than that by TZ as long as the plasma level of OZ is comparable to or lower than that of TZ. Caccia et al. found that anti-
PTZ convulsant effect in rats after oral administra-
tion of CZ is mostly caused by its active metabolites, TZ and OZ, indicating that CZ itself is much less active. However, we confirmed that CZ itself produces muscle relaxation mainly together with TZ. A possible explanation for this discrepancy of the results between Caccia et al. and us may be the difference in subtypes of the benzodiazepine receptor: this receptor consists of at least two subtypes, i.e., type I receptor mediates antianxiety and anticonvulsant effects; type II, the other benzodiazepine-like effect. Because the affinity of CZ for the cerebral benzodiazepine receptors measured by an in vitro displacement test is only 2—3 times of that of TZ, the muscle relaxant effect of CZ is much more potent than that expected from its relative affinity to TZ for receptors. In other kinetic study on the relation between the time courses of plasma levels and of the increase of delta waves (electroencephalogram (EEG), a measure of sedative effect) after oral administra-
tion of CZ and TZ to rhesus monkeys, the 50% effective plasma level, EC$_{50}$, of CZ was found to be comparable to EC$_{50}$ of TZ (our unpublished data). Thus CZ tends to produce muscle relaxant and sedative effects (both probably mediated by type II receptor) more than anticonvulsant effect (mediated by type I).

Though Hill equation is known to be applicable to the concentration-effect relation of drugs, little has been so far reported on a kinet-
ic approach to a case where a drug exerts pharmacological effect together with its various active metabolites. In the present study, we success-
fully established, via a modified Hill equa-
tion, the relation between pharmacological effect and plasma levels of CZ in the presence of its active metabolites. Though this equation including competitive factors was not derived by a mathematical manipulation exactly based on the pharmacokinetic and -dynamic processes, it is found to be a possible empirical equation applicable to the concentration-effect relation of such a drug. However, if kinetic and dynamic processes, i.e., drug concentration receptor occupation, pharmacological response, are well established on the basis of functional equations for each process, then a more detailed character-
ization of disposition-effect relation of CZ and its active metabolites may become possible.

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