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Characterization of urinary metabolites of a new benzofuroquinoline derivative, 3,9-bis(*N,N*-dimethylcarbamoyloxy)-5 *H*-benzofuro[3,2-*c*]-quinoline-6-one (KCA-098), in dogs

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The metabolism of 3,9-bis(*N,N*-dimethylcarbamoyloxy)-5 *H*-benzofuro[3,2-*c*]-quinoline-6-one (KCA-098), a new inhibitor of bone resorption and stimulator of bone formation, was examined after oral administration to dogs. Nine metabolites and the unchanged KCA-098 were isolated by extraction and HPLC from dog urine. The structures of these metabolites were characterized by LC/MS or LC/MS/MS, and/or were confirmed by comparison with corresponding authentic standards. The presumed main metabolic pathways were hydrolysis, hydroxylation, and *N*-demethylation of the *N,N*-dimethylcarbamate ester group.

1. Introduction

A new benzofuroquinoline derivative with weak estrogen activity, 3,9-bis(*N,N*-dimethylcarbamoyloxy)-5 *H*-benzofuro[3,2-*c*]quinoline-6-one (designated as KCA-098) was developed from coumestrol as a lead compound (Table). KCA-098 has the unique effects of inhibiting bone resorption and stimulating bone formation [1–3], and has been suggested to be useful for the treatment of bone diseases. As described in a previous paper [4], the existence of four crystalline forms of KCA-098 was confirmed by X-ray powder diffraction, IR spectroscopy, and thermal analysis, and all crystalline forms were found to be poorly soluble in water and to have low bioavailability in rats. It is important to clarify the metabolic fate of KCA-098 used for pharmaceutical preparations in order to improve its gastrointestinal absorption. In this study, the metabolism of KCA-098 was investigated after oral administration to dogs, and structures of the urinary metabolites were confirmed by liquid chromatography/mass spectrometry (LC/MS or LC/MS/MS). Tentative metabolic pathways of the compound in dogs are presented and discussed.

2. Investigations and results

2.1. HPLC-profiles of metabolites

Peak profiles of the metabolites were determined by use of a reversed-phase liquid chromatography designed to optimize the resolution of peaks near the void volume. Fig. 1 shows typical HPLC chromatograms (conditions A, B, C) obtained after administration of KCA-098. Under condition A, one major metabolite peak was observed at 14.3 min, and was designed as **M-1**. Under condition B, six major metabolite peaks appeared at 15.2, 26.3, 37.4, 41.2, and 43.5 min and were designed as **M-2**, **M-3**, **M-4**, **M-5**, **M-6**, and **M-7**, respectively. In condition C, three additional metabolite peaks that were not detected under other HPLC conditions were found at 16.7, 18.7, and 38.2 min, and these peaks were designed as **M-8**, **M-9**, and **M-10**, respectively. None of the ten metabolites were detected in predrug urine.

2.2. Identification and chemical characterization

2.2.1. High polarity metabolite

The HPLC retention time of the metabolite **M-1** (detectable only under condition A) was not identical to any of the authentic standards shown in the Table. The HPLC method (condition A) was used to collect and further purify this peak for structural analysis. The fraction obtained by enzymatic hydrolysis of **M-1** with sulfatase showed no **M-1** peak, but a new peak having the same retention time as **M-2** appeared. Therefore, **M-1** is a sulfate conjugate of **M-2**. Besides, a later result showed **M-2** to be the hydrolysis compound (authentic standard I) of KCA-098. These results suggest that **M-1** is a sulfate conjugate of I.

2.2.2. Medium-polarity metabolite

HPLC separation (condition B) resulted in six major metabolite peaks, i.e., **M-2**, **M-3**, **M-4**, **M-5**, **M-6**, and **M-7**. Retention times for **M-2**, **M-4**, and **M-7** matched those of the authentic standards I, II, and V, respectively. Peak fractions for **M-2**, **M-3**, **M-4**, **M-5**, **M-6**, and **M-7** were manually collected and subsequently analyzed by APCI LC/MS.

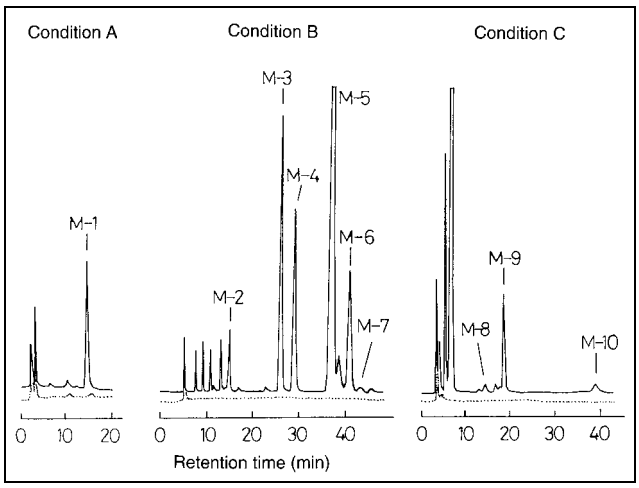
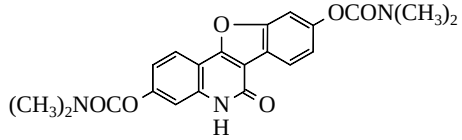
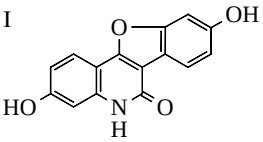
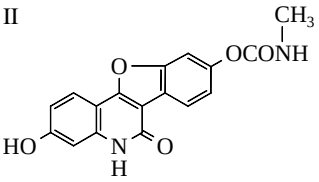
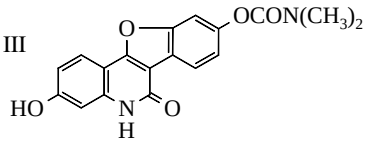
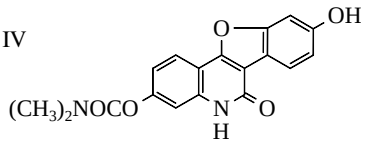
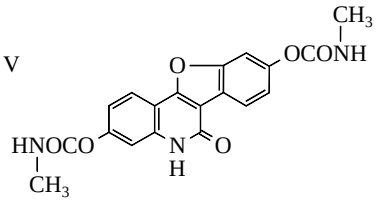


Fig. 1: HPLC chromatograms of urine extract after oral administration of KCA-098 to dogs. HPLC was performed under conditions A, B, and C. The dotted line represents a blank urine sample

Table: MS and ^{1}H NMR analysis of authentic standards

Compd.	Mass spectrum (m/z)	^{1}H NMR (DMSO, d_6, δ)
KCA-098 		410 $[M+H]^+$, 339, 268 2.94 (s, 6H), 3.07 (s, 3H), 3.09 (s, 3H), 7.12 (dd, J = 8.7, J = 2.2, 1H), 7.23 (dd, J = 8.4, J = 2.0, 1H), 7.24 (d, J = 2.2, 1H), 7.67 (d, J = 2.0, 1H), 8.02 (d, J = 8.4, 1H), 8.03 (d, J = 8.7, 1H), 12.07 (s, 1H)
I 		268 $[M+H]^+$ 6.79 (dd, J = 8.7, J = 2.2, 1H), 6.88 (d, J = 2.2, 1H), 6.89 (dd, J = 8.4, J = 2.1, 1H), 7.12 (d, J = 2.1, 1H), 7.78 (d, J = 8.4, 1H), 7.81 (d, J = 8.7, 1H), 9.91 (s, 1H), 10.29 (s, 1H), 11.69 (s, 1H)
II 		325 $[M+H]^+$, 268 2.71 (d, J = 4.5, 3H), 6.82 (dd, J = 8.3, J = 2.0, 1H), 6.90 (d, J = 2.0, 1H), 7.17 (dd, J = 8.3, J = 2.0, 1H), 7.60 (d, J = 2.0, 1H), 7.70 (brq, J = 4.5, 1H), 7.86 (d, J = 8.3, 1H), 7.95 (d, J = 8.3, 1H), 11.75 (s, 1H)
III 		339 $[M+H]^+$, 268 2.94 (s, 3H), 3.08 (s, 3H), 6.82 (dd, J = 8.3, J = 2.0, 1H), 6.91 (d, J = 2.0, 1H), 7.19 (dd, J = 8.3, J = 2.0, 1H), 7.61 (d, J = 2.0, 1H), 7.70 (brq, J = 4.0, 1H), 7.86 (d, J = 8.3, 1H), 7.95 (d, J = 8.3, 1H), 10.40 (br, 1H), 11.78 (s, 1H)
IV 		339 $[M+H]^+$, 268 2.94 (s, 3H), 3.07 (s, 3H), 6.94 (dd, J = 8.3, J = 2.0, 1H), 7.10 (dd, J = 8.3, J = 2.0, 1H), 7.17 (d, J = 2.0, 1H), 7.22 (d, J = 2.0, 1H), 7.85 (d, J = 8.3, 1H), 8.00 (d, J = 8.3, 1H), 10.00 (br, 1H), 11.97 (brs, 1H)
V 		382 $[M+H]^+$, 325, 268 2.70 (d, J = 4.5, 6H), 7.13 (d, J = 8.6, 1H), 7.22 (d, J = 8.4, 1H), 7.26 (s, 1H), 7.68 (s, 1H), 7.75 (brq, J = 4.5, 1H), 7.82 (brq, J = 4.5, 1H), 8.02–8.06 (m, 2H), 12.04 (s, 1H)

M-2: The metabolite **M-2** fraction displayed a molecular ion peak of m/z 268 $[M+H]^+$, which was 142 mass units lower than the $[M+H]^+$ ion of KCA-098 at m/z 410 (Fig. 2), and was shown to have the molecular weight of authentic standard I by APCI LC/MS. The APCI LC/MS data obtained from **M-2** is in good agreement with that obtained from the synthetic authentic standard I. These results suggest that **M-2** is formed by hydrolysis of the dimethylcarbamate ester at the C-3 and C-9 positions of KCA-098.

M-3: The APCI LC/MS spectrum of the **M-3** fraction showed a molecular ion peak of m/z 355 $[M+H]^+$, which was 55 mass units lower than the $[M+H]^+$ ion of KCA-098 at m/z 410 (Fig. 2). The spectrum also displayed mass spectral fragment ion peaks of m/z 325 $[M+H\text{-}(CH_2O)]^+$ and m/z 268 $[M+H\text{-}(CON(CH_3)CH_2OH)]^+$, with the loss of a hydroxymethyl group and a hydroxymethylcarbamoyl group, respectively. These results indicate that **M-3** is formed by mono-oxidation of the methyl group of the dimethylcarbamate ester at the C-3 or C-9 position and by hydrolysis of the other dimethylcarbamate ester of KCA-098. Incubation of the **M-3** peak fraction with 6N HCl by heating decreased the HPLC area of **M-3**, and two new peaks appeared, corresponding to the retention times of **M-2** and **M-4**. These two peaks were chromatographically identical to that of authentic standards I and II, respectively. From these results, we suggest that **M-3** is formed by hydrolysis of the dimethylcarbamate ester at the C-3 position of KCA-098 and by oxidation of the methyl group of the other dimethylcarbamate ester group at the C-9 position.

M-4: The metabolite **M-4** fraction displayed a molecular ion peak of m/z 325 $[M+H]^+$, which was 85 mass units lower than the $[M+H]^+$ ion of KCA-098 at m/z 410 (Fig. 2), and was shown to have the molecular weight of authentic standard II by APCI LC/MS. The spectrum also displayed a mass spectrum fragment ion peak of m/z 268 $[M+H\text{-}(CONCH_3)]^+$ with the loss of a mono-methylcar-

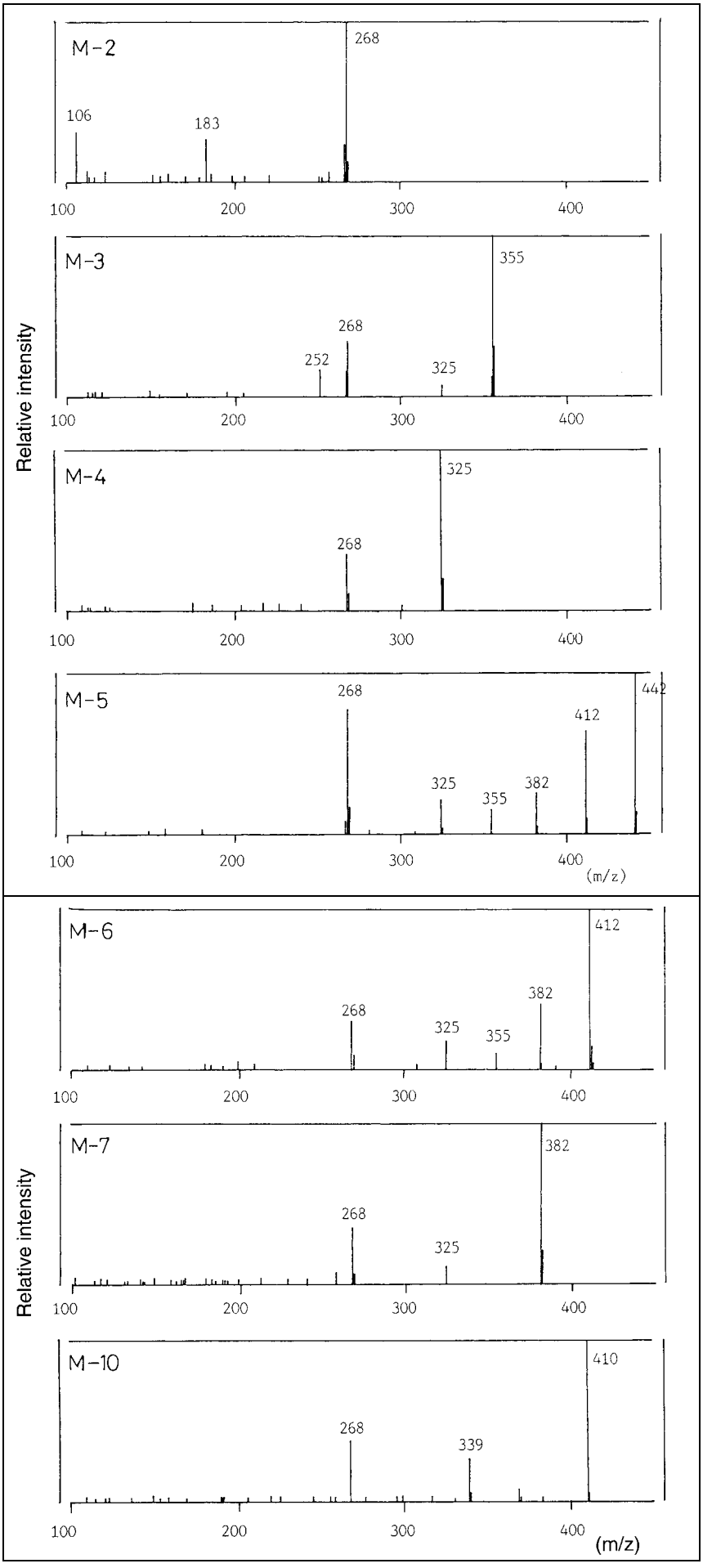


Fig. 2: APCI LC/MS spectra of metabolites isolated from dog urine after oral administration of KCA-098

bamoyl group; and the authentic standard II showed a similar pattern. These data suggest that **M-4** is formed by mono-demethylation of the methyl group of the dimethylcarbamate ester at the C-9 position and by hydrolysis of the other dimethylcarbamate ester of KCA-098.

M-5: **M-5** was observed as a major metabolite in urine after administration of KCA-098. The APCI LC/MS spectrum showed an $[M+H]^+$ ion at m/z 442, which was 32 mass units higher than the $[M+H]^+$ ion of KCA-098 at m/z 410 (Fig. 2), suggestive of the possibility that this metabolite is a bis-oxidation compound of KCA-098. Fragment ions were subsequently observed at m/z 412 $[M+H\text{-}(CH_2O)]^+$, 382 $[M+H\text{-}(CH_2O)_2]^+$, 355 $[M+H\text{-}(CON(CH_3)CH_2O)]^+$, 325 $[M+H\text{-}(CON(CH_3)CH_2O)\text{-}(CH_2O)]^+$, and 268 $[M+H\text{-}(CON(CH_3)CH_2O)_2]^+$. Moreover, the ESI LC/MS and LC/MS/MS spectra showed an $[M+H]^+$ ion at m/z 442. The product ion spectrum for the m/z 442 ion of **M-5** showed fragment ions at m/z 383 $[M+H_2\text{-}(CH_2O)_2]^+$, 355 $[M+H\text{-}(CON(CH_3)CH_2O)]^+$, and 268 $[M+H\text{-}(CON(CH_3)CH_2O)_2]^+$ (Fig. 3). This results were in agreement with those obtained by the APCI LC/MS data. Incubation of the **M-5** peak fraction with 6N HCl by heating resulted in the disappearance of **M-5**, concomitant with an increase in peaks corresponding to authentic standards I, II and V. Thus **M-5** appears to be formed by bis-oxidation of one of the methyl groups of each dimethylcarbamate ester at the C-3 and C-9 positions of KCA-098.

M-6: The APCI LC/MS spectrum of the **M-6** fraction showed a molecular ion peak of m/z 412 $[M+H]^+$, which was 2 mass units higher than the $[M+H]^+$ ion of KCA-098 at m/z 410 (Fig. 2). The spectrum also displayed mass spectral fragment ion peaks of m/z 382 $[M+H\text{-}(CH_2O)]^+$, 355 $[M+H\text{-}(CONCH_3)]^+$, 325 $[M+H\text{-}(CON(CH_3)CH_2O)]^+$, and 268 $[M+H\text{-}(CON(CH_3)CH_2O)\text{-}(CONCH_3)]^+$.

Incubation of the **M-6** peak fraction with 6N HCl by heating resulted in the disappearance of **M-6**, concomitant with an increase in peaks corresponding to authentic standards I, II and V. These results suggest that **M-6** is formed by monodemethylation and mono-oxidation of one of the methyl groups of each dimethylcarbamate ester. Unfortunately, the positions of mono-demethylation and mono-oxidation could not be determined from these data.

M-7: The minor metabolite **M-7** fraction displayed a molecular ion peak of m/z 382 $[M+H]^+$, which was 28 mass units lower than the $[M+H]^+$ ion of KCA-098 at m/z 410 (Fig. 2), and was shown have the molecular weight of authentic standard V by APCI LC/MS. The spectrum also displayed mass spectra fragment ion peaks of m/z 325 $[M+H\text{-}(CONHCH_3)]^+$ and 268 $[M+H\text{-}(CONHCH_3)_2]^+$; and the authentic standard V showed a similar pattern. These data indicate that **M-7** is formed by di-demethylation of KCA-098.

2.2.3. *Low-polarity metabolite*

HPLC separation (condition C) resulted in three metabolite peaks, i.e., **M-8**, **M-9**, and **M-10**, which were not detected under conditions A or B. Retention times for **M-8**, **M-9**, and **M-10** matched those of the authentic standards IV, III, and unchanged KCA-098, respectively. Therefore, we presume that **M-8** and **M-9** are formed by hydrolysis of the dimethylcarbamate ester at the C-9 and C-3 positions, respectively. The APCI LC/MS spectrum of the **M-10** fraction showed a molecular ion peak of m/z 410 $[M+H]^+$, which was the same ion peak as KCA-098 (Fig. 2). The spectrum also displayed mass spectral fragment ion peaks of m/z 339 $[M+H\text{-}(CON(CH_3)_2)]^+$, 268 $[M+H\text{-}(CON(CH_3)_2)_2]^+$, and these fragment ion peaks also appeared with the KCA-098 authentic standard, indicating that **M-10** was unchanged KCA-098 (Fig. 4).

3. Discussion

KCA-098, a carbamate ester derivative, has two *N*,*N*-dimethylcarbamate groups at the C-3 and C-9 positions. Several drugs containing a phenolic group show a limited and variable bioavailability following oral administration due to extensive first-pass metabolism in the gut and/or liver by conjugation reactions [5, 6]. Bioreversible derivatization of the phenolic group to produce prodrugs has in some cases proved to be a valuable approach to reduce the extent of first-pass metabolism, as exemplified with naltrexone [7], β-estradiol [8], and various carbamate esters of the dopamine agonist (−)-3-(3-hydroxyphenyl)-*N*-propylpiperidine [9]. Moreover, Hansen et al. [10] reported that the *N*,*N*-dimethylcarbamate ester may be a potentialy useful prodrug for the protection of a parent compound against first-pass metabolism. Bambuterol is a bis-dimethylcarbamate prodrug of the adrenoreceptor agonist terbutarline [11], and its metabolites and metabolic pathways have been studied in detail [12–14]. Lindberg et al. [12] reported that seven metabolites between bambuterol and terbutaline could be identified in rat liver microsomes,

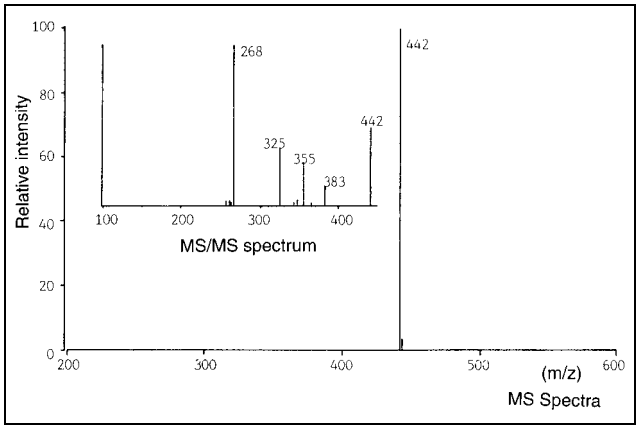


Fig. 3: ESI LC/MS and LC/MS/MS spectra of **M-5** isolated from dog urine after oral administration of KCA-098

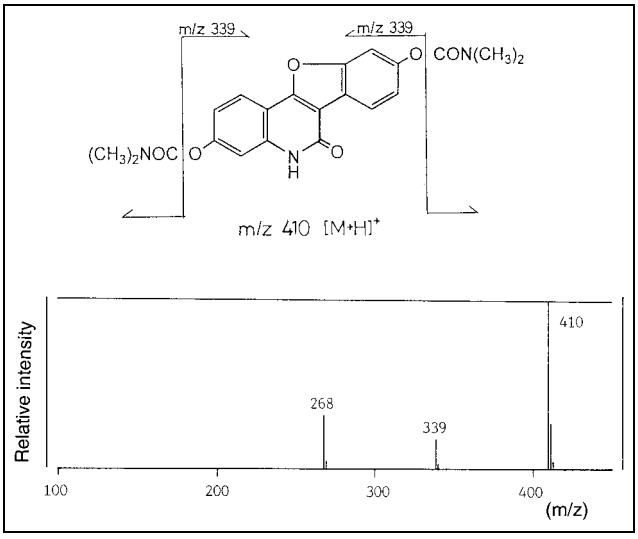
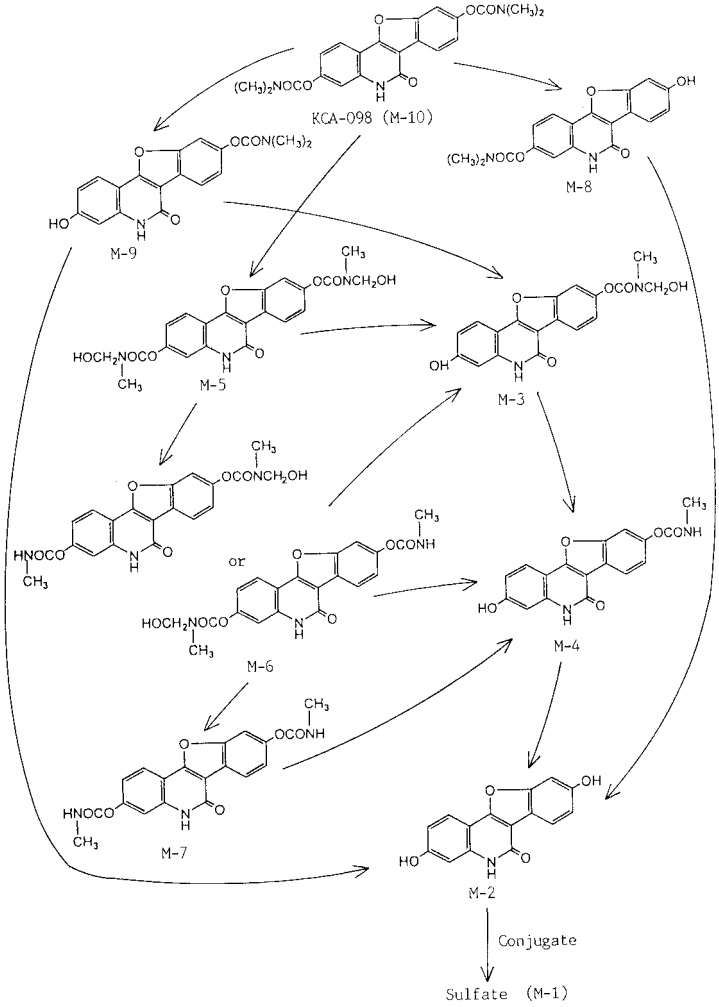


Fig. 4: Mass fragmentation of KCA-098 authentic standard and mass spectrum

Scheme



which were formed as a result of hydroxylation, demethylation, and hydrolytic reactions. Because KCA-098, also a carbamate ester derivative, has two *N*,*N*-dimethylcarbamate groups at the C-3 and C-9 position, the metabolism of KCA-098 was expected to be most likely similar to that of bambuterol. In this study, ten metabolites were recognized in dog urine after oral administration of KCA-098. Metabolites **M-2**, **M-3**, **M-4**, **M-8**, and **M-9** were characterized by hydrolysis of the *N*,*N*-dimethylcarbamate ester group, and these metabolites may be formed by the interaction with butylcholinesterase in blood [14]. Metabolites **M-3**, **M-5**, and **M-6** were formed by the hydroxylation on one or two *N*-methyl groups, and these metabolites may be formed by hydroxylation with liver cytochrome P-450 enzymes [10, 12]. The existence of *N*-hydroxymethyl metabolites of 45191-S in dog plasma have been reported [15]. Relatively stable hydroxylated metabolites have also been reported to be formed from other carbamates [16, 17]. Therefore, **M-5**, a major hydroxylated metabolite, may be stable in dog urine. It seems that **M-4**, **M-6**, and **M-7** are *N*-demethylation metabolites that are formed in cytochrome P-450-catalyzed oxidation reactions [18, 19]. The sulfate of the hydrolyzed metabolite (authentic I) is formed by conjugation. Thus, the tentative metabolic pathway of KCA-098 in dogs can be proposed as depicted in the Scheme. Even though each pathway should be confirmed with *in vitro* metabolic experiments, the metabolic pathway via **M-5** is thought to be the major route with **M-8** as the minor one, based upon the results of HPLC chromatograms in Fig. 1.

In conclusion, unchanged compound and nine metabolites isolated from dog urine were separated by HPLC after oral administration of KCA-098 to dogs. The structure of the metabolites was determined by APCI LC-MS and compared with that of authentic compounds. The presumed major metabolic pathways in dogs are hydroxylation, demethylation, and hydrolysis of the *N*,*N*-dimethylcarbamate ester group, and the major metabolite is presumed to be the *N*-methyl hydroxylated compound **M-5**.

4. Experimental

4.1. Materials

The authentic standards of KCA-098 and its metabolites synthesized at Kissei Pharmaceutical Co., Ltd. were used to identify metabolites in this study. The MS and ^{1}H NMR spectra data of these compounds are shown in the Table. Sulfatase type H1 was purchased from Sigma Chemical Company. Reagents for extraction solvents were of analytical grade and other solvents were of HPLC grade.

4.2. Animals

Male beagle dogs were obtained from Clear Japan, Inc., and used at 17 months of age. Their body weights ranged from 10 kg to 13 kg. All animals were acclimatized for more than one month to a room temperature of 23 ± 2 °C and a relative humidity of $55 \pm 10\%$ and were fed commercial food (DS; Oriental Yeast Co., LTD., Tokyo, Japan) and 500 ml of tap water daily throughout the study. The animals were fasted overnight before and 6 h after dosing, but were given water ad libitum.

4.3. Drug administration

KCA-098 was suspended in 0.5% CMC sodium solution to prepare a 20 mg/ml dosing solution. The dose suspension was administered orally to dogs via sound at a dose of 100 mg/kg. Urine samples were collected from dogs 0–24 h after administration and were stored frozen at approximately −20 °C before being analyzed.

4.4. Determination of the metabolites in urine

Urine samples (0–24 h collection) were made acidic with 0.1N HCl, and extracted with a mixture of benzene and ethyl acetate. After evaporation of the solvent under a stream of nitrogen gas, residues were dissolved in a small volume of HPLC mobile phase for HPLC analysis. The major urinary metabolite peaks were co-chromatographed with authentic standards for comparison of retention times, and were collected under the same HPLC conditions. The mobile phase was dried under a stream of nitrogen gas, and the dried samples were further analyzed by LC/MS and/or LC/MS/MS.

Chemical hydrolysis of metabolite fractions collected by HPLC was carried out for 5 h at 60 °C with an excess amount of 6N HCl. The hydrolysates were injected into the HPLC apparatus after neutralization. Aliquots of urine were incubated with sulfatase to determine the possible presence of sulfate-conjugated metabolites. The fractions collected by HPLC were incubated with sulfatase type H1 in acetate buffer (pH 5.0) at 37 °C for 15 h. After incubation, the reaction mixture was added to acetonitrile and shaken for 10 min, and supernatant was collected after centrifugation for 10 min at 3000 × g. This supernatant was subjected to HPLC.

4.5. Analytical procedures

4.5.1. High performance liquid chromatography

The HPLC system was equipped with a Tri Rotar pump (Japan Spectroscopic), fitted with a fluorometer (RF-353, Shimazu). Three sets of conditions were employed because of the polarity of metabolites.

Condition A: A Wakosil-II column (150 mm × 4.6 mm ID, Wako Chemical Co., LTD.) was employed using the following mobile phase, with a flow rate of 0.7 ml/min: 30 mM ammonium acetate (pH 4)/acetonitrile, (9 : 1) v/v. Separation was monitored with a fluorometer (Ex 355 nm, Em 380 nm).

Condition B: The same column of condition A was employed using the following mobile phase, with a flow rate of 1 ml/min: 30 mM ammonium acetate (pH 4)/acetonitrile, (8 : 2) v/v. Separation was monitored with a fluorometer similar to condition A.

Condition C: An Inertsil ODS-2 (150 mm × 4.6 mm ID, GL Science) was employed using the following mobile phase, with a flow rate of 0.75 ml/min: methanol/water, (51 : 49) v/v. Separation was monitored with a fluorometer (Ex 345 nm, Em 375 nm).

4.5.2. Mass spectrometry

LC/MS spectra were obtained on a Hitachi M-200 double-focusing mass spectrometer with an atmospheric pressure chemical ionization (APCI) interface and an L-6200 pump (Hitachi). Nebulization heater temperature and desolvation heater temperature were set at 280 °C and 410 °C, respectively; and drift voltage and electron multiplier voltage were 150 V and 1200 V, respectively. LC/MS/MS spectra were obtained on a TSQ-7000 mass spectrometer with electron-spray ionization (Thermo Quest USA), operated in positive-ion mode. LC/MS/MS experiments were conducted using argon as a collision gas, and a collision energy of −25 eV.

4.5.3. Nuclear magnetic resonance spectroscopy

NMR spectra were obtained with a GX-270 spectrometer (270 MHz, Jeol). Each sample was dissolved in deuterated dimethylsulfoxide. Tetramethylsilane (TMS) was used as internal standard.

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