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A Gas Chromatographic-Mass Spectrometric Method for the Evaluation of Bioconversion of Indomethacin Prodrugs into the Parent Drug[1)]

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The administration of a deuterium-labeled drug together with a prodrug made it possible to determine the bioconversion rate of the prodrug into the parent compound accurately in dogs. Deuterated indomethacins were prepared from acetaldehyde *p*-methoxyphenylhydrazone in four steps. Indomethacin in blood plasma was derivatized into the hexafluoroisopropyl ester and determined by means of selected ion monitoring employing indomethacin-d_9 as an internal standard. When a single dose of indomethacin or oxametacin was orally given to dogs, the time courses of plasma level of indomethacin were remarkably different from one another. This result implied that the bioconversion rate of oxametacin into indomethacin could not be directly estimated from the area under the drug concentration–time curve (AUC) value. When a mixture of indomethacin-d_4 and oxametacin was administered to three dogs, the ratios of the AUC value of the non-labeled indomethacin derived from oxametacin to that of the labeled indomethacin in the dogs were found to be almost equal. This approach was also applicable to the estimation of acemetacin. It has been demonstrated that the bioavailabilities of oxametacin and acemetacin can be fairly accurately evaluated with a small number of animals by the combined use of labeled indomethacin and selected ion monitoring.

Keywords——indomethacin; oxametacin; acemetacin; prodrug; bioavailability; gas chromatography–mass spectrometry; selected ion monitoring; stable isotope

The bioavailability of a prodrug is usually evaluated by comparison of the area under the drug concentration–time curve (AUC) values obtained when the prodrug and its parent drug are administered. However, when marked individual variations in the absorption of the parent drug from the gastrointestinal tract exist, the bioconversion rate of the prodrug is not directly reflected in the plasma level of the drug.[2–7)] Elimination of factors due to individual variations in the absorption may provide a reasonable bioconversion rate of the prodrug. The need to evaluate the bioavailabilities of oxametacin (1-(*p*-chlorobenzoyl)-2-methyl-5-methoxy-3-indolylacethydroxamic acid)[4,8,9)] and acemetacin (1-(*p*-chlorobenzoyl)-2-methyl-5-methoxy-3-indolylacetoxyacetic acid)[5,6,10,11)] prompted us to develop a new method for estimating the bioconversion rates of these prodrugs by comparison of the AUC values of deuterium-labeled indomethacin and non-labeled indomethacin derived from the prodrug. The present paper describes the preparation of deuterated indomethacin[12)] and its use for estimation of the bioconversion rates of oxametacin and acemetacin by means of gas chromatography–mass spectrometry (GC–MS).

Materials and Methods

Reagents——*p*-Methoxyphenylhydrazine·HCl was purchased from Aldrich Chemical Co. (Milwaukee, WI), acetaldehyde and levulinic acid were from Wako Pure Chemical Co. (Tokyo), trifluoroacetic anhydride and *p*-chlorobenzoyl chloride from Tokyo Kasei (Tokyo) and 1,1,1,3,3,3-hexafluoroisopropanol, methanol-d_4, acetone-d_6, *p*-chlorobenzyl chloride-d_4, acetic acid-d_4, toluene-d_8, NaOD, and D_2SO_4 from E. Merck AG (Darmstadt). Indomethacin and oxametacin were kindly supplied by Teikoku Hormone Mfg. Co. (Tokyo). All other reagents used were of analytical-reagent grade.

Gas Chromatography–Mass Spectrometry (GC–MS)——A Shimadzu LKB-9000B gas chromatograph–mass spectrometer connected on-line with a Shimadzu GC–mass pack 500-FDGT computer was used. A coiled glass column (1 m × 3 mm i.d.) was packed with 2% OV-1 on Gas Chrom Q (80—100 mesh). The flow rate of carrier gas (helium) was 30 ml/min. The temperatures of the column, injection port, and ion source were kept at 215, 270, and 270°C, respectively. The accelerating voltage, ionization voltage, and trap current were 3.5 kV, 70 eV, and 60 μA, respectively.

Derivatization for GC–MS——The hexafluoroisopropyl (HFIP) ester was prepared by treatment of samples with hexafluoroisopropanol (0.3 ml) and trifluoroacetic anhydride (0.05 ml) at 50°C for 1 h.

Syntheses of Authentic Samples——Melting points were taken on a micro hot-stage apparatus and are uncorrected.

Acetaldehyde N^1-(*p*-Chlorobenzoyl)-*p*-methoxyphenylhydrazone-d_4 (I): *p*-Chlorobenzoyl chloride-d_4 (0.5 g) was added dropwise to a stirred solution of acetaldehyde *p*-methoxyphenylhydrazone (1.0 g) in pyridine (4 ml) over a period of 1 h, and the whole was stirred for a further 6 h. The reaction mixture was then poured into ice-water. The precipitate was collected by filtration and dried under reduced pressure at room temperature to give I (1.7 g) as yellowish crystals. MS *m/z*: 306 ([M$^+$]), 143 ([M−163]$^+$).

Acetaldehyde N^1-(*p*-Chlorobenzoyl)-*p*-methoxyphenylhydrazone·HCl-d_4 (II): HCl gas was passed into a solution of I (1.0 g) in MeOH (0.6 ml)–toluene (13.5 ml) over a period of 1 h under ice-cooling. The crystalline precipitate was collected by filtration and washed with toluene (100 ml) to give II (0.8 g) as colorless needles. mp 145°C.

1-(*p*-Chlorobenzoyl)-2-methyl-5-methoxy-3-indolylacetic Acid-d_4 (III): A mixture of II (0.34 g) and levulinic acid (0.15 g) in AcOH (4 ml) was heated at 80°C for 3 h. After removal of acetic acid with the aid of an N_2 gas stream, the residue was recrystallized from aq. acetone to give III (0.26 g) as colorless needles. mp 143°C. MS (HFIP (derivative) *m/z*: 511 ([M$^+$]) (d_4: *ca.* 100%), 368 ([M−143]$^+$), 316 ([M−195]$^+$), 143 ([M−368]$^+$).

1-(*p*-Chlorobenzoyl)-2-methyl-5-methoxy-3-indolylacetic Acid-d_9 (IV): DCl gas generated from D_2SO_4 and NaCl was passed into a solution of I (53 mg) in methanol-d_4 (0.04 ml)–toluene-d_8 (1.4 ml) over a period of 1 h under ice-cooling. The precipitate was washed with toluene to give the deuterium chloride salt. A solution of levulinic acid (0.2 g) in acetone-d_6 (1 ml) and methanol-d_4 (2 ml) was refluxed for 5 h and evaporated to dryness. A mixture of the deuterium chloride salt and deuterated levulinic acid was dissolved in acetic acid-d_4 (3 ml) and refluxed at 80°C for 3 h. After removal of the solvent with the aid of an N_2 gas stream, the residue was subjected to preparative thin-layer chromatography using AcOEt/iso-PrOH/10% NH_4OH (3: 2: 2) as a developing solvent. The adsorbent corresponding to the spot at *Rf* 0.45 was eluted with acetone. Recrystallization of the eluate from aq. acetone gave IV (10 mg) as colorless needles. MS (HFIP derivative) *m/z*: 516 ([M]$^+$) (d_5: 0.8%, d_6: 3.1%, d_7: 23.4%, d_8: 21.5%, d_9: 40.0%, d_{10}: 0.5%, d_{11}: 0.7%), 373 ([M−143]$^+$), 321 ([M−195]$^+$), 143 ([M−373]$^+$).

Administration of Oxametacin, Acemetacin, and Indomethacin to Dogs——A single dose of indomethacin and oxametacin was given orally to CSK beagle dogs (body weight 11.6—17.6 kg). Administration of either oxametacin or acemetacin combined with indomethacin-d_4 was similarly carried out in the following manner: 1) a single dose of indomethacin (2.8 μmol/kg) was given to three dogs, 2) a single dose of oxametacin (2.8 μmol/kg) to three dogs, 3) a mixture of oxametacin (2.8 μmol/kg) and indomethacin-d_4 (2.8 μmol/kg) to four dogs, 4) a mixture of oxametacin (14.0 μmol/kg) and indomethacin-d_4 (2.8 μmol/kg) to three dogs, 5) a mixture of acemetacin (2.8 μmol/kg) and indomethacin-d_4 (2.8 μmol/kg) to four dogs.

Determination of Indomethacin in Plasma——Blood (1 ml) was withdrawn from a superficial vein at intervals (0.5, 1, 1.5, 2, 3, 4, 5, 8, and 24 h) and centrifuged at 3000 rpm for 15 min. The internal standard (indomethacin-d_9) was added to the plasma, and the whole was adjusted to pH 4.7 with acetate buffer solution and extracted with AcOEt (5 ml × 3). The extract was redissolved in EtOH and applied to a piperidinohydroxypropyl Sephadex LH-20 (PHP-LH-20) column.[13] The column was washed with EtOH (10 ml), then indomethacin was eluted with 1% AcOH/EtOH (10 ml). The eluate was derivatized into the HFIP ester with hexafluoroisopropanol (0.3 ml) and trifluoroacetic anhydride (0.05 ml), and then subjected to GC–MS.

Results and Discussion

Several methods have been developed for the determination of indomethacin in biological fluids.[14–20] However, problems of degradation by alkali[16,21,22] and narrow useful pH range[16,23,24] led to unsatisfactory recovery of indomethacin. Our initial effort was directed to derivatization of indomethacin into the hexafluoroisopropyl ester. When treated with hexafluoroisopropanol in the presence of trifluoroacetic anhydride as a catalyst, indomethacin was converted into the desired ester. The structure of derivatized indomethacin was unequivocally characterized by means of GC–MS (Fig. 1). The molecular ion peak, ([M$^+$]), and a

fragment ion peak, ([M-139]$^+$), which was formed by loss of the *p*-chlorobenzoyl group, appeared at *m/z* 507 and 368, respectively. The base peak at *m/z* 139 was assignable to the *p*-chlorobenzoyl ion formed by fission of the imide linkage.

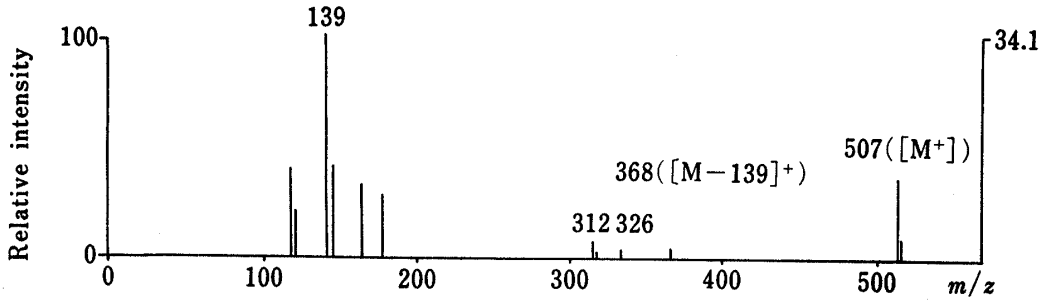

Fig. 1. Mass Spectrum of Indomethacin Hexafluoroisopropyl Ester

For the purpose of estimating the bioconversion rate of indomethacin prodrugs in living animals, the preparation of indomethacin-d_4 was undertaken. Initially, acetaldehyde *p*-methoxyphenylhydrazone was transformed into the N^1-*p*-chlorobenzoate-d_4 (I) by treatment with *p*-chlorobenzoyl chloride-d_4 in pyridine. A solution of I in methanol–toluene was bubbled through with hydrogen chloride gas, yielding the corresponding hydrochloride. When the hydrochloride salt was refluxed with levulinic acid, cyclization took place to provide the desired indomethacin-d_4. Incorporation of the heavy isotope into the benzoyl residue was confirmed by the existence of a molecular ion at *m/z* 511 and two characteristic fragment ions at *m/z* 316 and 143 in the mass spectrum of the hexafluoroisopropyl ester.

For the determination of indomethacin in canine plasma, indomethacin-d_9 was used as an internal standard. The preparation of the desired compound was carried out in the manner

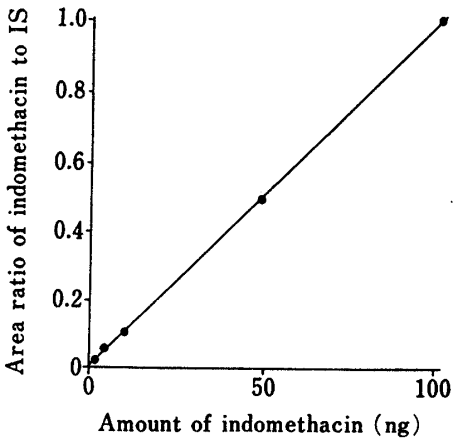

Fig. 2. Calibration Graph for Indomethacin

Indomethacin-d_9 (100 ng) was used as an internal standard (IS). The selected ions for indomethacin and IS were *m/z* 507 and 516, respectively.

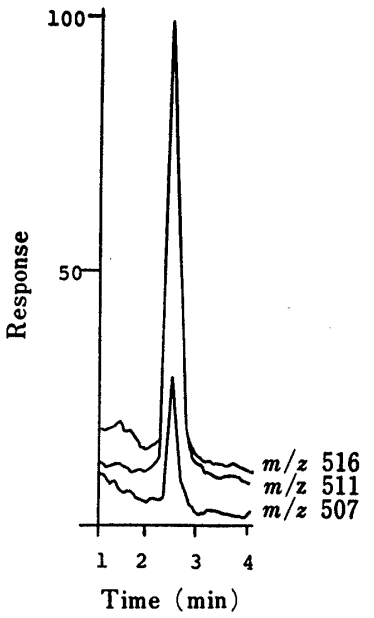

Fig. 3. Selected Ion Recording of Labeled and Non-labeled Indomethacins in Canine Plasma

1, indomethacin; 2, indomethacin-d_4; 3, indomethacin-d_9 (IS).

described for indomethacin-d_4. When treated with deuterium chloride gas, I was converted to the corresponding deuterium chloride salt. This step was necessary to obtain a high incorporation yield of deuterium. Condensation of the deuterium chloride salt with deuterated levulinic acid provided the desired indomethacin-d_9 as a major product. Incorporation of the heavy isotope was confirmed by the presence of a molecular ion at m/z 516 and two characteristic fragment ions at m/z 373 and 321 in the mass spectrum of the hexafluoroisopropyl ester.

A calibration graph was constructed by plotting the ratio of the peak area of indomethacin to that of the internal standard against the amount ratio of the two where m/z 507 and 516 were used as selected ions for indomethacin and the internal standard, respectively. Satisfactory linearity was observed in the ranger of 1—100 ng of indomethacin (Fig. 2).

Determination of plasma levels of indomethacin and indomethacin-d_4 in dogs was first undertaken. A plasma sample was extracted with ethyl acetate, and the extract was applied to PHP–LH-20[13] for elimination of interfering endogenous substances. The eluate obtained with 1% acetic acid was derivatized into the hexafluoroisopropyl ester for selected ion monitoring. A typical selected ion recording is illustrated in Fig. 3.

TABLE I. AUC, C_{max} and T_{max} Values of Indomethacin after Oral Administration of Oxametacin and Indomethacin-d_4 (each 2.8 μmol/kg) to Dogs

	AUC_0^8 (μg/ml·h)			C_{max} (μg/ml)		T_{max} (h)	
	d_0	d_4	d_0/d_4	d_0	d_4	d_0	d_4
Dog 1	1.42	8.47	0.17	0.32	2.91	3.0	1.0
Dog 2	0.71	6.35	0.11	0.23	2.29	1.5	1.5
Dog 3	2.12	14.09	0.14	0.45	3.32	5.0	1.5
Dog 4	1.03	8.34	0.12	0.25	3.24	3.0	1.5
Mean	1.32	9.31	0.14	0.31	2.94	3.1	1.4

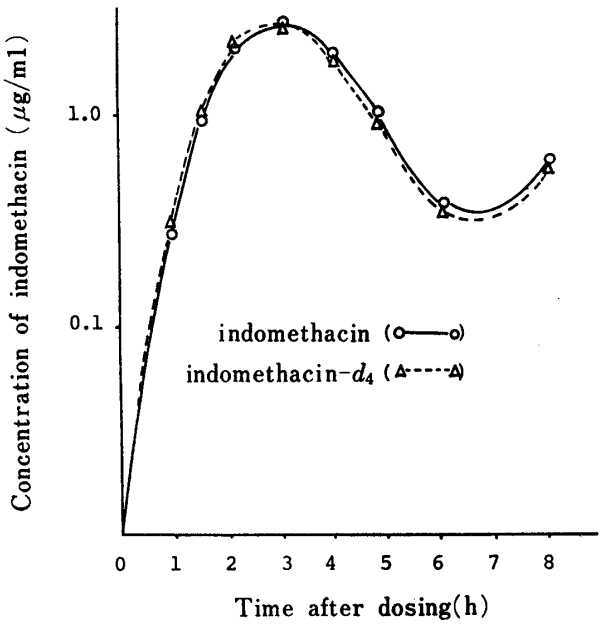

Fig. 4. Time Courses of Plasma Levels of Indomethacin and Indomethacin-d_4

A mixture of 2.8 μmol/kg each of indomethacin and indomethacin-d_4 was given orally to a dog.

The bioconversion of oxametacin into indomethacin in the dog was then investigated. When a single dose of indomethacin or oxametacin was given orally, the time courses of plasma level of indomethacin were found to vary remarkably among individual dogs. The AUC values obtained for the three dogs were significantly different from one another in both groups and therefore, the direct measurement of plasma indomethacin was inadequate for estimation of the bioconversion rate of oxametacin. The significant individual variation appeared to be associated with differences in the absorption of indomethacin from the gastrointestinal tract.

In order to overcome the difficulties due to individual variation in the absorption, an equimolar mixture of oxametacin and indomethacin-d_4 was administered to each dog, and labeled and non-labeled indomethacins in plasma were simultaneously determined by selected

ion monitoring. The results obtained are listed in Table I. The ratios of the AUC value of non-labeled indomethacin (d_0) derived from oxametacin to that of labeled indomethacin (d_4) in four dogs were found to be almost equal, although the actual AUC values obtained were remarkably different from one another.

The possibility of a biological isotope effect due to the deuterium label was ruled out on the basis of a prior experiment employing the 1:1 mixture technique. When an equimolar mixture of indomethacin and indomethacin-d_4 was administered, the time courses of plasma levels of these two were found to be almost identical, as illustrated in Fig. 4.

A mixture of oxametacin and indomethacin-d_4 in a ratio of 5 to 1 was similarly administered to dogs, and the plasma levels of non-labeled indomethacin (d_0) and labeled indomethacin (d_4) were determined. The ratios of AUC values of these two were found to be nearly constant. However, the mean value was only twice that obtained with the 1:1 mixture (Table II).

In a similar fashion, the bioconversion of acemetacin to indomethacin in the dog was also investigated by employing labeled indomethacin (d_4). The results obtained are collected in Table III. The AUC ratios of non-labeled indomethacin (d_0) to labeled indomethacin (d_4) were almost equal among four dogs.

TABLE II. AUC, C_{max} and T_{max} Values of Indomethacin after Oral Administration of Oxametacin (14.0 μmol/kg) and Indomethacin-d_4 (2.8 μmol/kg) to Dogs

	AUC_0^8 (μg/ml·h)			C_{max} (μg/ml)		T_{max} (h)	
	d_0	d_4	d_0/d_4	d_0	d_4	d_0	d_4
Dog 1	2.42	9.70	0.25	0.64	2.52	2.0	1.5
Dog 2	1.08	5.03	0.21	0.52	1.84	3.0	1.0
Dog 3	1.87	5.65	0.33	0.22	1.24	5.0	5.0
Mean	1.79	6.79	0.26	0.46	1.87	3.3	2.5

TABLE III. AUC, C_{max} and T_{max} Values of Indomethacin after Oral Administration of Acemetacin and Indomethacin-d_4 (each 2.8 μmol/kg) to Dogs

	AUC_0^8 (μg/ml·h)			C_{max} (μg/ml)		T_{max} (h)	
	d_0	d_4	d_0/d_4	d_0	d_4	d_0	d_4
Dog 1	13.75	20.70	0.66	3.34	6.86	6.0	6.0
Dog 2	4.92	7.44	0.66	0.76	1.34	5.0	4.0
Dog 3	8.68	10.28	0.84	2.39	4.22	1.5	1.5
Dog 4	4.31	6.30	0.68	1.60	3.89	3.0	3.0
Mean	7.92	11.18	0.71	2.02	4.08	3.9	3.6

The present study has demonstrated that the combined use of labeled indomethacin together with its prodrug can serve to eliminate factors due to individual variations in the absorption of indomethacin, providing a more reliable value for the conversion rate of the prodrug into a parent drug in an experiment with a small number of animals. This newly developed method for the estimation of the bioconversion rate of a prodrug by simultaneous administration of the prodrug with the labeled parent drug should be valuable for assessing the bioavailability of prodrugs.

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1) A part of this work has been presented at the 101st Annual Meeting of the Pharmaceutical Society of Japan, at Kumamoto in April, 1981.
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