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# Isolation and Characterization of Human Urinary Metabolites of Meclomen

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The principal metabolites of meclomen (sodium meclofenamate; sodium 2-[(2,6-dichloro-3-methylphenyl)]aminobenzoate monohydrate) were isolated from human urine and characterized by gas chromatography–mass spectrometry (GC-MS) and proton nuclear magnetic resonance ($^1$H-NMR) spectroscopy together with the Folin–Ciocalteu test. Oxidation occurred at the methyl group at C-3 and/or the aromatic ring in meclomen, yielding the methylol (M-1), dicarboxylic acid (M-2), phenols (M-3 and M-4), 3-hydroxymethylated phenol (M-7), 3-demethylated phenol (M-6) and phenolic dicarboxylic acid (M-8).

Among these metabolites, M-1, M-2, M-3 and M-4 are common to fenamate anti-inflammatory analgesics. A novel metabolite, M-6, is presumably produced from M-8 through M-7 or M-2 as an intermediate.

**Keywords**——anti-inflammatory drug; sodium meclofenamate; meclomen; metabolism; human urinary metabolite; trace characterization; GC-MS; ion cluster

Meclomen (MCM)[1] is one of the nonsteroidal anti-inflammatory analgesic fenamates, which include mefenamic acid and flufenamic acid. Glazko *et al.* disclosed the occurrence of oxygenated metabolites (M-1, M-2, M-3, M-4 and M-5) and their glucuronides in human urine after oral administration of MCM[2] by using thin-layer and gas liquid chromatography (TLC and GLC). No further investigation, however, has been done on the structural elucidation of other metabolites.[3,4]

This paper describes the isolation and characterization of urinary metabolites of MCM in human subjects administered a single dose of the drug. The appearance of cluster ions due to chlorine isotopes in the mass spectrum served as a guide for separation of the metabolites derived from MCM. These metabolites were unequivocally characterized on the basis of usual criteria.

## Experimental

**Chemicals**——Authentic MCM, M-1, M-2 and M-4 were kindly donated by Warner Lambert/Parke-Davis Co., Ltd. (Michigan, MI, U.S.A.). For enzymatic hydrolysis of glucuronides, *Helix pomatia* $\beta$-glucuronidase, Type H-2 (Sigma Chemical Co., St. Louis, MO, U.S.A.) was used. 1,1,1,3,3,3-Hexafluoro-2-propanol (HFIP) was supplied by E. Merck AG (Darmstadt, FRG), *N,O*-bis(trimethylsilyl)acetamide (BSA) by Wako Pure Chemical Industries (Tokyo), and trifluoroacetic anhydride (TFAA) and pentafluorobenzyl bromide (PFBB) by Tokyo Kasei Co. (Tokyo). All other chemicals were of analytical-reagent grade.

**Instruments**——For TLC, Kieselgel 60 $F_{254}$ plates (2 and 0.25 mm thick) were used. The lower layer of a chloroform–methanol–water (3 : 2 : 1) mixture was employed as a developing solvent. High performance liquid chromatography (HPLC) was run on a Shimadzu LC3A liquid chromatograph equipped with a UVD-2 detector (254 nm) and an ERC-ODS 1722 (250 × 8 mm i.d.) column (Ellmer Optics) using 2% acetic acid -acetonitrile (5 : 6) as a mobile phase at a flow rate of 0.7 ml/min. Gas chromatography–mass spectrometry (GC-MS) was carried out on a Shimadzu QP-1000 gas chromatograph-mass spectrometer. The column used was 2% OV-1 (1 m × 3 mm i.d.) or 1%

OV-101 (0.5 m × 3 mm i.d.). Temperatures of the injection port, separator and ion source were kept at 275, 280 and 290 °C, respectively. The carrier gas was He at a flow rate of 30 ml/min and the electron impact ionization energy was 70 eV. $^1$H-Nuclear magnetic resonance ($^1$H-NMR) spectra were recorded on a Hitachi R-20A (60 MHz) or R-90H (90 MHz) spectrometer in methanol-$d_4$ using tetramethylsilane as an internal standard. Melting points were determined on a micro hot-stage apparatus and are uncorrected.

**Administration of MCM and Collection of Urine Specimens**——Six healthy male volunteers were orally given single doses of MCM (3 capsules, each containing 100 mg). The urine was collected for 8 h and diluted to 1500 ml prior to use.

**Derivatization for GC-MS**——HFIP–TFA Derivative: HFIP (0.3 ml) and TFAA (0.05 ml) were added to a sample (100 $\mu$g), and the whole was heated at 60 °C for 1 h. After removal of the solvent in an $N_2$ gas stream, the residue was dissolved in 20% TFAA in ethyl acetate (100 $\mu$l) and subjected to GC-MS analysis.

PFB–TMS Derivative: Solution of 10% PFBB in acetone (0.3 ml) and 0.2 M KOH in methanol (0.05 ml) were added to a sample (100 $\mu$g), and the whole was heated at 40 °C for 30 min or at 80 °C for 16 h. After removal of the solvent in an $N_2$ gas stream, the residue was mixed with 20% BSA in ethyl acetate (0.3 ml) and heated at 40 °C for 30 min. After removal of the solvent in an $N_2$ gas stream, the residue was dissolved in 20% BSA in ethyl acetate (100 $\mu$l) and subjected to GC-MS analysis.

**Isolation of Metabolites**——The procedure used for separation of urinary metabolites is shown in Chart 1. The diluted urine specimen (1450 ml) from volunteers administered the drug was applied to a column of Amberlite XAD-2 resin (300 g), and the metabolites were eluted successively with water (3 l) and methanol (2 l). The eluate obtained with water was adjusted at pH 11 with 10 M NaOH and allowed to stand at room temperature for 2 h. The solution was adjusted to pH 1 with concentrated HCl and extracted with benzene (500 and 200 ml). After removal of the solvent, the residue was subjected to TLC. The adsorbent corresponding to *Rf* 0—0.1 was scraped off and eluted with methanol. The dried eluate was redissolved in dilute HCl and extracted with ethyl acetate, and the extract was in turn subjected to HPLC. M-8 (mixture, 7.1 mg) was obtained as the fraction of $t_R$ 8—21 min and pure M-2 (7.0 mg) as that of $t_R$ 23 min on the chromatogram. On the other hand, the eluate with methanol in Amberlite XAD-2 column chromatography was evaporated and the residue was redissolved in 0.1 M acetate buffer (pH 5.0, 2.5 ml), then incubated with $\beta$-glucuronidase (95400 unit/ml, 5 ml) at 37 °C for 17 h. The incubation mixture was adjusted to pH 11 with 10 M NaOH and allowed to stand at room temperature for 1 h. The resulting solution was adjusted to pH 1 with concentrated HCl and then extracted with benzene (80 ml × 5). The product was subjected to TLC and the adsorbents corresponding to *Rf* 0.43—0.52 and 0.52—0.62 were scraped off and extracted with ethyl acetate. These eluates were then subjected to HPLC. From the former band, M-4 (1.6 mg) was obtained as the fraction of $t_R$ 34 min while from the latter band, M-7 (mixture, 2.2 mg), M-1 (14.8 mg), M-6 (3.7 mg) and M-3 (16.1 mg) were obtained as those of $t_R$ 12—20, 26, 30 and 39 min, respectively.

**Properties of Urinary Metabolites of MCM**——MCM: Folin–Ciocalteu test: negative. MS *m/z* (HFIP derivative): 445 ($M^+$), 277, 242 (base); *m/z* (PFB derivative): 475 ($M^+$), 277, 242 (base). $^1$H-NMR ($CD_3OD$) $\delta$: 7.96 (1H, dd, $J=2$ and 8 Hz, 3′-H), 7.30 (1H, d, $J=8$ Hz, 5-H), 7.09 (2H, d, $J=8$ Hz, 4-H and m, 5′-H), 6.65 (1H, dt, $J=1$ and 8 Hz, 4′-H), 6.18 (1H, dd, $J=1$ and 8 Hz, 6′-H), 2.39 (s, 3-$CH_3$).

M-1: mp 190—195 °C. Folin–Ciocalteu test: negative. MS *m/z* (HFIP–TFA derivative): 557 ($M^+$), 444, 354 (base); *m/z* (PFB–TMS derivative): 563 ($M^+$, base), 438, 330. $^1$H-NMR ($CD_3OD$) $\delta$: 7.95 (1H, dd, $J=2$ and 8 Hz, 3′-H), 7.48 (2H, s, 4-H and 5-H), 7.24 (1H, dt, $J=2$ and 8 Hz, 5′-H), 6.72 (1H, br t, $J=8$ Hz, 4′-H), 6.22 (1H, br d, $J=8$ Hz, 6′-H), 4.68 (2H, s, 3-$CH_2OH$).

M-2: mp 253—255 °C. Folin–Ciocalteu test: negative. MS *m/z* (HFIP derivative): 625 ($M^+$), 457, 422 (base); *m/z* (PFB derivative): 685 ($M^+$), 487, 452, 256. $^1$H-NMR ($CD_3OD$) $\delta$: 7.98 (1H, dd, $J=2$ and 8 Hz, 3′-H), 7.64 (1H, d, $J=8$ Hz, 4-H), 7.55 (1H, d, $J=8$ Hz, 5-H), 7.30 (1H, dt, $J=2$ and 8 Hz, 5′-H), 6.76 (1H, dt, $J=1$ and 8 Hz, 4′-H), 6.27 (1H, dd, $J=1$ and 8 Hz, 6′-H).

M-3: mp 222—227 °C. Folin–Ciocalteu test: positive. MS *m/z* (HFIP-TFA derivative): 557 ($M^+$) 461, 354 (base), 292; *m/z* (PFB derivative): 671 ($M^+$), 490, 292 (base). $^1$H-NMR ($CD_3OD$) $\delta$: 7.92 (1H, d, $J=8$ Hz, 3′-H), 7.19 (1H, t, $J=8$ Hz, 5′-H), 6.90 (1H, s, 5-H), 6.65 (1H, t, $J=8$ Hz, 4′-H), 6.19 (1H, d, $J=8$ Hz, 6′-H), 2.25 (3H, s, 3-$CH_3$).

M-4: Folin–Ciocalteu test: positive. MS *m/z* (HFIP–TFA derivative): 557 ($M^+$), 460, 354 (base); *m/z* (PFB derivative): 671 ($M^+$), 490, 292. $^1$H-NMR ($CD_3OD$) $\delta$: 7.44 (1H, d, $J=3$ Hz, 3′-H), 7.35 (1H, d, $J=9$ Hz, 5-H), 7.16 (1H, d, $J=9$ Hz, 4-H), 6.80 (1H, dd, $J=3$ and 9 Hz, 5′-H), 6.16 (1H, d, $J=9$ Hz, 6′-H), 2.40 (3H, s, 3-$CH_3$).

M-6: mp 164—166 °C. Folin-Ciocalteu test: positive. MS *m/z* (HFIP–TFA derivative): 543 ($M^+$), 447, 340 (base), 278; *m/z* (PFB derivative): 657 ($M^+$), 476, 278 (base). $^1$H-NMR ($CD_3OD$) $\delta$: 7.98 (1H, br d, $J=8$ Hz, 3′-H), 7.27 (1H, br t, $J=8$ Hz, 5′-H), 6.97 (2H, s, 3-H and 5-H), 6.69 (1H, br t, $J=8$ Hz, 4′-H), 6.20 (1H, br d, $J=8$ Hz, 6′-H).

M-7: MS *m/z* (HFIP–TFA derivative): 669 ($M^+$), 466, 404; *m/z* (PFB–TMS derivative): 759 ($M^+$), 578, 380.

M-8: MS *m/z* (HFIP–TFA derivative): 737 ($M^+$), 534; *m/z* (PFB derivative): 881 ($M^+$), 700, 502.

## Results and Discussion

The chemical structures of the metabolites isolated from the urine of human subjects

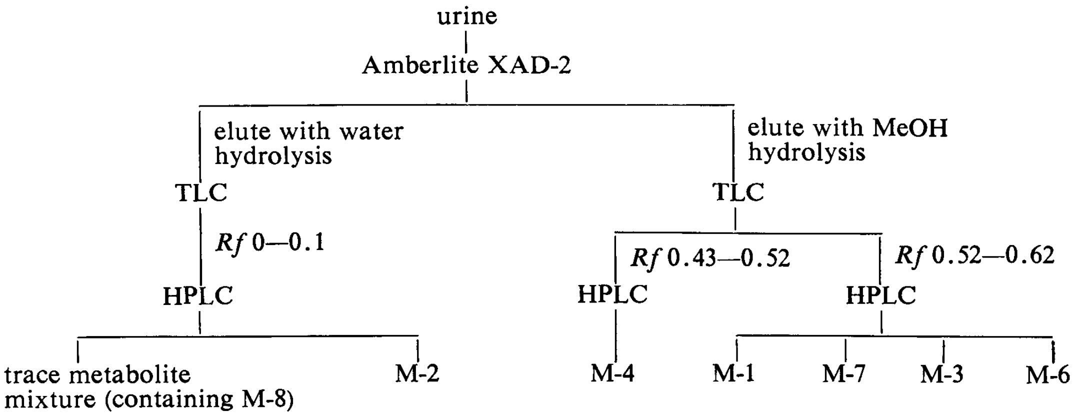

Chart 1. Procedure for Separation of Urinary Metabolites

administered MCM have been investigated. The metabolites eluted with water and methanol in Amberlite XAD-2 column chromatography were hydrolyzed with sodium hydroxide and then separated into two fractions containing M-2 and M-8 and five fractions containing M-1, M-3, M-4, M-6 and M-7, respectively, by means of HPLC (Chart 1). The M-1 HFIP–TFA derivative formed with HFIP and TFAA showed a molecular ion peak at *m/z* 557 and fragment ion peaks at *m/z* 444 and 354 in the mass spectrum, while the M-1 PFB–TMS derivative produced by pentafluorobenzylation at 40 °C and subsequent trimethylsilylation exhibited a molecular ion peak at *m/z* 563 and prominent fragmentation peaks, indicating the introduction of a PFB group into the carboxylic acid moiety and a TMS group into the alcohol. These results suggested that M-1 would have a meclofenamate skeleton with a hydroxyl group. Furthermore, on the basis of the negative Folin–Ciocalteu test on TLC and the NMR spectrum exhibiting a methylene proton signal at $\delta$ 4.68 (2H, s), the structure having the 3-hydroxymethyl group was assigned to M-1.

The mass spectra of the HFIP and PFB derivatives of M-2 showed corresponding molecular ion peaks at *m/z* 625 and 685, implying the presence of two HFIP groups and a PFB group, respectively. In the NMR spectra M-2 gave no signals assignable to the 3-methyl and 3-hydroxymethyl protons but showed C-4 and C-5 proton signals at $\delta$ 7.64 and 7.55 (2H, ABq), exhibiting a downfield shift as compared with MCM. This metabolite was thus deduced to be the structure possessing a carboxyl group at C-3.

The mass spectra of HFIP–TFA and PFB derivatives of M-3 provided molecular ion peaks at *m/z* 557 and 671 together with fragment ion peaks due to the TFA and PFB moieties, respectively, indicating the presence of an aromatic hydroxyl function. Moreover, the positive Folin–Ciocalteu test and the presence of an aromatic proton signal at $\delta$ 6.90 (1H, s) were indicative of the hydroxylated position of M-3 being C-4 rather than C-5.

Similarly, M-4 appeared to possess a phenolic hydroxyl group as judged from the mass spectra of its HFIP–TFA and PFB derivatives and positive Folin–Ciocalteu test. Inspection of the NMR spectrum indicated that the hydroxylated position would be C-4 in ring B.

The structures of M-1, M-2 and M-4 were unambiguously established by direct comparison with authentic specimens. In spite of considerable effort, M-5, reported by Glazko *et al.*,[2] could not be detected in the present study. In contrast, additional new metabolites were separated and characterized. M-6 seemed to have a phenolic hydroxyl group as judged from the mass spectra of the HFIP–TFA and PFB derivatives (Fig. 1) and positive Folin–Ciocalteu test. The mass number of each peak in the mass spectrum was 14 units smaller than that in M-3 or M-4. The absence of signals assignable to the 3-methyl or 3-hydroxymethyl group in the NMR spectrum indicated that the C-3 methyl group had been lost in M-6. In addition, the NMR proton signals assignable to the C-3 and C-5 positions

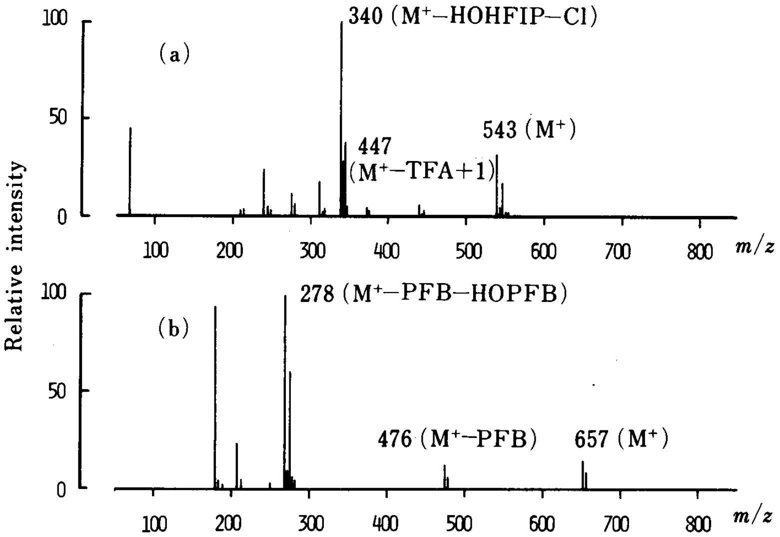

Fig. 1. Mass Spectra of M-6 HFIP–TFA (a) and PFB (b) Derivatives

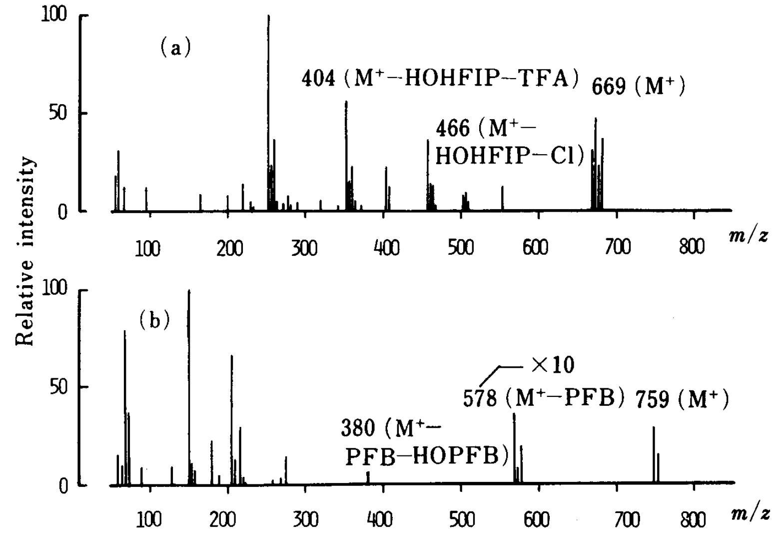

Fig. 2. Mass Spectra of M-7 HFIP–TFA (a) and PFB–TMS (b) Derivatives

appeared at $\delta$ 6.97 (2H, s), suggesting the hydroxylated position to be C-4.

The occurrence of the demethylated metabolite, M-6, prompted us to clarify the metabolites of MCM in more detail. Identification of M-7 and M-8 in the eluates with methanol and water in Amberlite XAD-2 chromatography was carried out by means of mass chromatography because only trace amounts were available.

The HFIP–TFA derivative of M-7 exhibited a molecular ion peak at $m/z$ 670 and prominent fragment ion peaks, indicating the presence of an HFIP residue and two TFA groups (Fig. 2a). On the other hand, when converted into the PFB–TMS derivative, M-7 showed a fragment ion at $m/z$ 759 and prominent fragment ions in the mass spectrum (Fig. 2b). This result implied that two PFB groups and a TMS group were introduced into M-7. Inspection of the mass spectra of the two derivatives revealed that M-7 is a 3-hydroxymethylated phenol possessing both phenolic and alcoholic hydroxyl groups beside the aromatic carboxyl group.

As for the mass spectrum of M-8, the HFIP–TFA derivative exhibited a molecular ion peak at $m/z$ 737 and fragment ion peaks due to the loss of HFIP and TFA groups, indicating

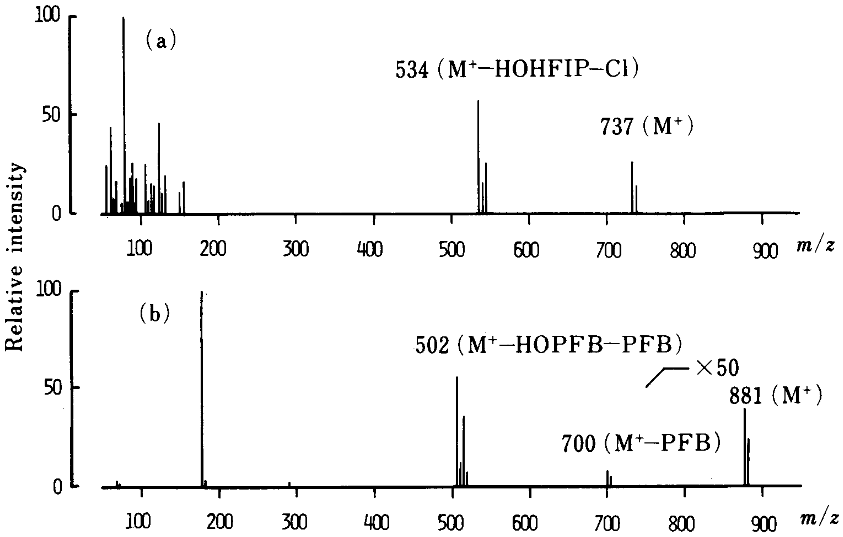

Fig. 3. Mass Spectra of M-8 HFIP–TFA (a) and PFB (b) Derivatives

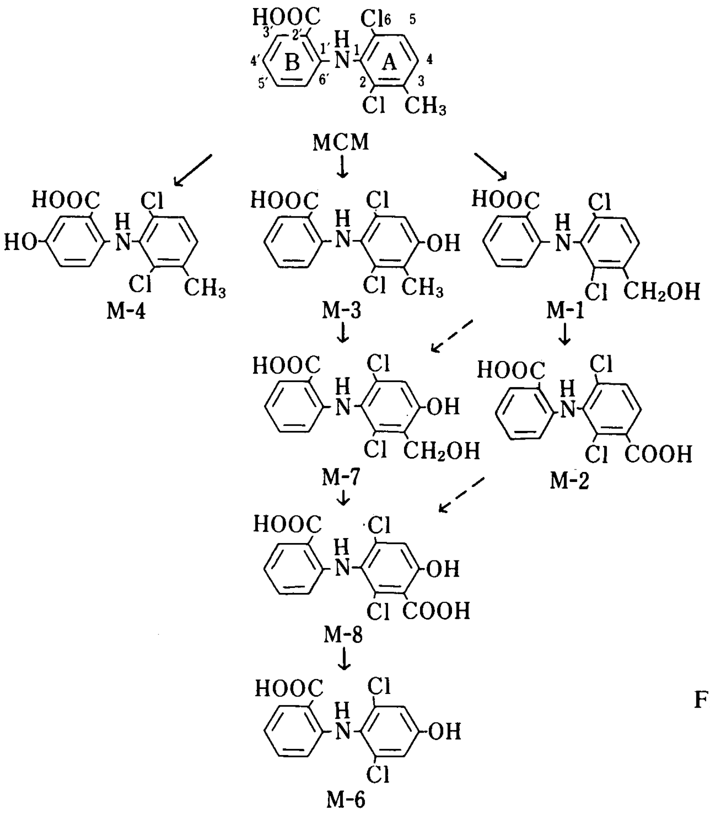

Fig. 4. Postulated Metabolic Pathway of MCM in Man

the presence of a TFA group and two HFIP groups (Fig. 3a). On the other hand, when M-8 was subjected to pentafluorobenzylation as described for M-1 at 40 or 80 °C, the bis or tris PFB derivative was mainly produced, respectively (Fig. 3b). These results suggested that M-8 has two carboxyl groups and a phenolic hydroxyl group on the aromatic ring.

The position of the phenolic hydroxyl group in M-8 was confirmed by model experiments using salicylic acid and 3-hydroxybenzoic acid. On being treated with PFBB at 40 °C, the former provided a very small amount of the bis PFB derivative besides the mono PFB one, while the latter afforded a large amount of the bis PFB derivative. Both compounds yielded almost exclusively the bis PFB derivatives at 80 °C. These data lent support to the assumption that the phenolic function in M-8 would be at the *ortho* position to the carboxyl group. It seems likely that M-8 would be an intermediate in the bioconversion of M-2 or M-7 to M-6. Therefore, the occurrence of M-8 and M-6 in urine suggests that M-7 is a metabolic precursor of M-8.

In the present study, the *in vivo* formation of a novel demethylated metabolite, M-6, following oral administration of MCM has been demonstrated. To the best of our knowledge this is the first report of the demethylated metabolite in man.[5-7] The metabolic pathway of MCM is postulated to be as shown in Fig. 4.

Unfortunately metabolite M-5 reported by Glazko *et al.*[2] could not be detected. However, the formation of two new dechlorinated phenolic metabolites was demonstrated by means of mass chromatography, although the complete structures of these compounds remain to be established.

Further studies on the pharmacokinetics of meclomen and its metabolites are being conducted in these laboratories, and the details will be reported elsewhere.

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