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## Metabolism of Fentanyl in Isolated Hepatocytes from Rat and Guinea Pig

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The metabolites of fentanyl (FT) were identified in isolated rat and guinea pig hepatocytes by gas chromatography-mass spectrum (GC-MS) combined with the stable isotope tracer technique. Isolated hepatocytes were prepared by the collagenase perfusion method and were incubated with a 0.3 mM equimolar mixture of FT and FT-*d*<sub>5</sub> citrate in a rotating round-bottomed flask at 37 °C. The metabolites were extracted with chloroform. The extracts were subjected to GC and GC-MS after trimethylsilylation.

Characteristic doublet peaks in the mass spectra indicated the presence of 6 and 7 metabolites in rat and guinea pig hepatocytes, respectively. 4-(*N*-Propionylanilino)piperidine was identified as the main metabolite in both species. Other metabolites were oxidation products of the phenethyl, piperidine and propionyl groups of FT. Oxidation of the anilino ring and hydrolysis of the amide bond were not found in hepatocytes.

**Keywords**—fentanyl; fentanyl metabolism; isolated hepatocyte; deuterium label; stable isotope tracer; GC-MS analysis

Fentanyl [1-phenethyl-4-(*N*-propionylanilino)piperidine, FT] has been widely used as an analgesic for the purpose of neuroleptanalgesia, but the metabolism of this drug has not yet been clarified. 4-(*N*-Propionylanilino)piperidine (M-1)<sup>1)</sup> and 1-phenethyl-4-anilino-piperidine (M-2)<sup>2)</sup> have been reported as urinary metabolites of FT in the rat. We have identified four additional metabolites in the urine of rats<sup>3)</sup>: 4-(*N*-2-hydroxypropionylanilino)piperidine (M-3), 4-(*N*-propionylanilino)hydroxypiperidine (M-4), 1-phenethyl-4-(*N*-2-hydroxypropionylanilino)piperidine (M-5) and 1-phenethyl-4-(*N*-propionylanilino)hydroxypiperidine (M-6).

Recently, we reported that M-1, M-3 and M-5 were detected in the urine of surgical patients who had received FT, and that the amounts of FT and M-1 excreted within 12 h after administration accounted for 0.3—4.0% and 26—55% of the dose, respectively.<sup>4)</sup> The low urinary recovery indicates the presence of unknown metabolites of FT in humans. Indeed, unknown metabolites were detected in the urine of guinea pig after administration of FT.<sup>5)</sup>

It was difficult to establish the structures of the minor metabolites of FT in the urine, because administration of a relatively high dose of FT was lethal to animals due to respiratory depression, and at a lower dose, endogenous substances in the urine interfered with the determination. In the present study, therefore, we attempted to detect and identify unknown metabolites of FT in isolated hepatocytes from rat and guinea pig by using the stable isotope tracer technique, and to determine whether or not the identified metabolites are present in patients.

### Experimental

**Chemicals**—FT, deuterium-labeled FT [1-phenethyl-4-(*N*-propionylpentadeuteroanilino)piperidine, FT-*d*<sub>5</sub>], M-1 and M-2 were prepared as described in a previous paper.<sup>3)</sup> Collagenase (from *Clostridium histolyticum*)

was purchased from Boehringer Mannheim. *N*-2-Hydroxyethylpiperadine-*N'*-2-ethanesulfonic acid (HEPES) was obtained from Nakarai Chemicals, Ltd. Bovine serum albumin (fraction V) was the product of Armour Pharmaceutical Co.

**Synthesis of Reference Compounds**—4-(*N*-2-Hydroxypropionylanilino)piperidine (Nor- $\omega$ -1-OH), 1-phenethyl-4-(*N*-2-hydroxypropionylanilino)piperidine (FT- $\omega$ -1-OH) and 1-(*p*-hydroxyphenethyl)-4-(*N*-propionylanilino)piperidine (FT-*p*-OH) were prepared as follows.

Nor- $\omega$ -1-OH: Chloroformic acid ethyl ester (6.9 g) was added dropwise to a solution of lactic acid (4.5 g) in 10% NaOH (20 ml) while the temperature was kept below 0 °C. After being stirred for 3 h at room temperature, the solution was acidified with conc. HCl and extracted with ethyl acetate. The organic layer was dried over anhydrous sodium sulfate and evaporated to yield 5.1 g of 2-ethoxycarbonyloxypropionic acid. A chloroform solution of thionyl chloride (7 g) was added to a solution of the above acid (5.1 g) in chloroform (6 ml), and the mixture was refluxed for 2 h. The resulting solution was evaporated *in vacuo* to yield 4 g of 2-ethoxycarbonyloxypropionyl chloride. A mixture of 1-benzyl-4-anilinopiperidine (2.3 g),<sup>6</sup> potassium iodide (14 mg) and sodium carbonate (2.7 g) in 4-methyl-2-pentanone (21 ml) was added dropwise to a solution of the chloride (6.2 g) in 4-methyl-2-pentanone (4 ml). The mixture was refluxed for 48 h under an atmosphere of nitrogen. The reaction mixture was filtered and the filtrate was evaporated *in vacuo*. The residue was chromatographed (silica gel C-200, with chloroform as the eluting solvent), yielding 3.5 g of 1-benzyl-4-(*N*-2-ethoxycarbonyloxypropionyl)anilino)piperidine. The amide (3.5 g) was dissolved in 20% NaOH (20 ml) and MeOH (20 ml), and refluxed for 2 h. The solution was extracted with chloroform and the organic layer was evaporated. The residue was dissolved in EtOH (100 ml) and hydrogenated in the presence of 5% Pd-C (2 g). The catalyst was removed by filtration and the filtrate was concentrated *in vacuo*. The residual oil was crystallized from ether to give 0.7 g of Nor- $\omega$ -1-OH: mp 138 °C.

FT- $\omega$ -1-OH: FT- $\omega$ -1-OH was prepared from M-2 (1 g) and 2-ethoxycarbonyloxypropionyl chloride (2.6 g) by a method similar to that used for the preparation of Nor- $\omega$ -1-OH. Recrystallization from EtOH-chloroform yielded 0.9 g of FT- $\omega$ -1-OH: mp 178 °C.

FT-*p*-OH: *p*-Hydroxyphenethylacetic acid (14 g) was dissolved in HCl-EtOH (150 ml) and the solution was refluxed for 4 h. After evaporation, the residue was mixed with benzyl chloride (14 ml), potassium carbonate (28 g) and methyl ethyl ketone (200 ml). The mixture was refluxed for 45 h and the catalyst was removed by filtration. The filtrate was evaporated, and the residual oil was dissolved in ether (200 ml). This solution was added to a suspension of LiAlH<sub>4</sub> (2.6 g) in ether (20 ml), and the mixture was refluxed for 3 h. After cooling, the mixture was poured into ice and extracted with ether. The extract was evaporated, and the residue was recrystallized from chloroform to give 9.2 g of *p*-benzyloxyphenylethanol. A solution of thionyl chloride (4.5 ml) in chloroform (20 ml) was added to a solution of the above alcohol (9.2 g) in chloroform, and the mixture was refluxed for 3 h. The solution was evaporated and a mixture of the residue with M-1 (2.5 g), sodium carbonate (10 g), potassium iodide (80 mg) and 4-methyl-2-pentanone (100 ml) was refluxed for 48 h, then filtered, and the filtrate was evaporated *in vacuo*. The residual oil was chromatographed (silica gel C-200; cyclohexane-chloroform) and the product was crystallized from benzene-*n*-hexane to yield 0.9 g 1-(*p*-benzyloxyphenethyl)-4-(*N*-propionylanilino)piperidine. The amide (0.9 g) was dissolved in EtOH (200 ml) and hydrogenated in the presence of 5% Pd-C (0.45 g). The catalyst was removed by filtration and the filtrate was concentrated *in vacuo*. The residue was crystallized from EtOH-ethyl acetate to give 0.4 g of FT-*p*-OH: mp 159 °C.

The purities of all reference compounds were checked by gas chromatography (GC) and the structures of these compounds were confirmed by mass spectra (MS).

**Preparation and Incubation of Isolated Hepatocytes**—Isolated hepatocytes were prepared from male Wistar rats (300–350 g) and male Hartley guinea pigs (350–400 g) by the collagenase perfusion method.<sup>7</sup> In the case of the guinea pig, the inserted point of the cannula at the portal vein was clamped with the fingers and the liver was supported by hand during the perfusion because of the structural weakness of the portal vein and liver capsule in the guinea pig. In the subsequent experiments we used cells with over 95% viability according to the lactic dehydrogenase latency test.<sup>7</sup> The hepatocytes were diluted to a final concentration of  $5 \times 10^6$  cells/ml in about 100 ml of suspension with Krebs-Henseleit buffer (pH 7.4) containing 2% bovine serum albumin and 12.6 mM HEPES, and were incubated with a 0.3 mM equimolar mixture of FT and FT-*d*<sub>5</sub> citrate in a rotating round-bottomed flask at 37 °C under a stream of oxygen-carbon dioxide (95 : 5).<sup>8,9</sup> The viability of the cells was over 90% after incubation for 3 h in both species.

**Sample Preparation and Extraction**—Ten ml of incubated fluid was collected after incubation for 1, 2 and 3 h, and filtered. The filtrate was extracted twice with 10 ml of chloroform. The combined extracts were dried over anhydrous sodium sulfate and evaporated to dryness. Trimethyl silyl derivatives were formed by treating the residue with 20  $\mu$ l of bis-(trimethylsilyl)acetamide in 20  $\mu$ l of pyridine at 60 °C for 30 min.

**GC and GC-MS Conditions**—GC was carried out in a Shimadzu GC-4CM equipped with a hydrogen flame ionization detector. A glass column (1 m  $\times$  3 mm i.d.) packed with 1.5% OV-1 on Chromosorb W (80–100 mesh) was used. The nitrogen gas flow rate was 40 ml/min. The column temperature was programmed from 170 to 280 °C at 10 °C/min. GC-MS was carried out in a JEOL D-300 GC-MS-computer system. A glass column (1 m  $\times$  2 mm i.d.) containing the same stationary phase as in the case of GC was used. Helium gas flow rate, 20 ml/min; column temperature, 260 °C; accelerating voltage, 3 kV; ionizing energy, 20 eV for electron impact ionization (EI) or 200 eV

for chemical ionization (CI). Isobutane was used as the reagent gas in CI measurement.

## Results and Discussion

In order to detect the metabolites of FT by the ion cluster technique, isolated rat hepatocytes were incubated with an equimolar mixture of FT and FT- $d_5$  citrate (FT:FT- $d_5$ ). Extract from the incubated fluid after osmotic shock treatment showed many interfering peaks on GC, and the detection of minor metabolites was extremely difficult. Therefore, filtrate of the incubated fluid was used for the detection of the metabolites. Gas chromatograms of the trimethylsilylated extract from the filtrate before and after 1 or 2 h of incubation are shown in Fig. 1. The peak of FT decreased with increasing incubation time, and disappeared after 3 h. EI and CI MS of the GC peaks which appeared during the incubation were measured by GC-MS. Ion clusters were observed in the mass spectra corresponding to the peaks a, b, c, d, e and f in the gas chromatogram. The  $m/z$  values of the ions observed in each peak are summarized in Table I. The presence of ion clusters separated by 5 mass units in these peaks indicated that oxidation at the anilino group did not occur. The metabolite corresponding to peak a was confirmed to be the trimethylsilyl derivative of M-1 by comparing its retention time and MS with those of authentic M-1-TMS.

FT and FT- $d_5$  did not give rise to detectable molecular ions ( $m/z$  336 and 341) in their EI MS; the base peaks of the spectra were at  $m/z$  245 and 250 (ion A). The postulated fragmentation pathway of FT is shown in Fig. 2.

In the case of peak b, the molecular weight was estimated to be 424 from the QM ion ( $m/z$  425) and M-15 ion ( $m/z$  409). The value was 88 (trimethylsilyl-ether group) higher than that of FT. This observation and the presence of ions A, B and C indicated the introduction

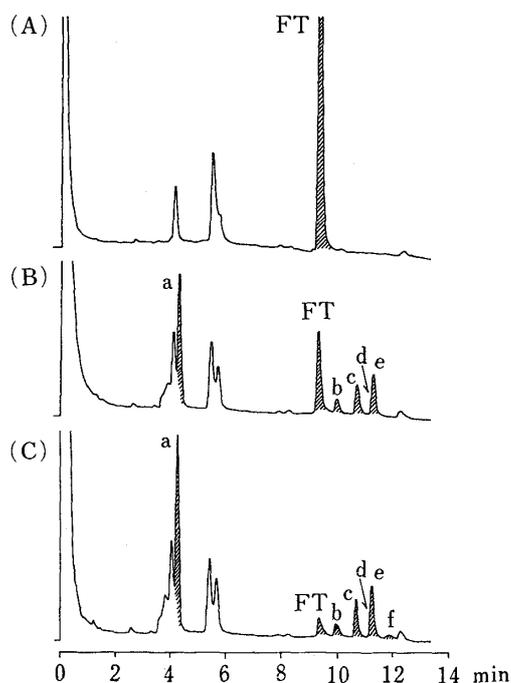


Fig. 1. Gas Chromatograms of the Trimethylsilylated Extract from Isolated Rat Hepatocytes before (A) or after Incubation of FT for 1 h (B) or 2 h (C)

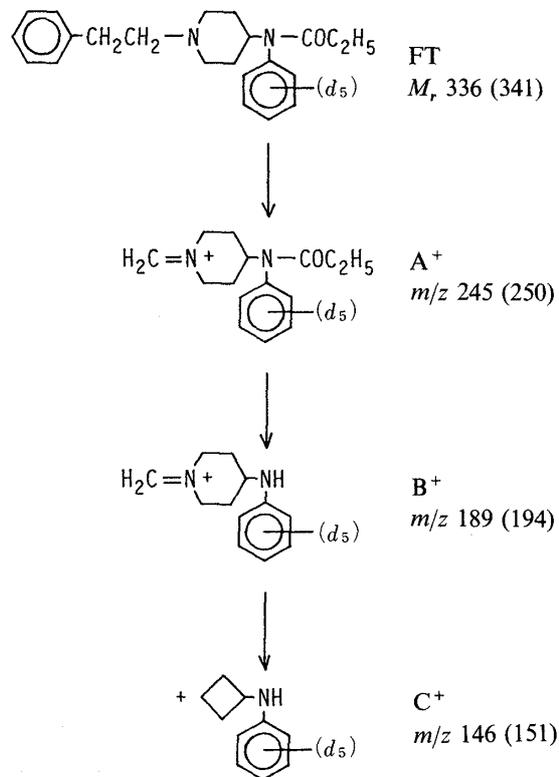


Fig. 2. Postulated Fragmentation Pathway of FT

Numbers in parentheses indicate the  $m/z$  values of the deuterium-labeled compound.

TABLE I. Mass Spectral Data for FT Metabolites in Isolated Rat Hepatocytes

Peak	QM <sup>+</sup> (CI)	<i>m/z</i> value of observed ion	
		M <sup>+</sup>	Fragment ion
a	305 (310)	304 (309)	289 (294) 247 (252) 231 (236) 206 (211) 155
b	425 (430)	—	409 (414) 245 (250) 189 (194) 146 (151)
c	425 (430)	—	409 (414) 333 (338) 261 (266) 189 (194) 146 (151) 117
d	513 (518)	—	497 (502) 333 (338) 261 (266) 189 (194) 146 (151) 117
e	425 (430)	—	409 (414) 333 (338) 261 (266) 243 (248) 189 (194) 146 (151)
f	513 (518)	—	497 (502) 421 (426) 333 (338) 261 (266) 245 (250) 189 (194) 146 (151)

—: Not observed. Numbers in parentheses indicate the *m/z* values of the deuterium-labeled compound.

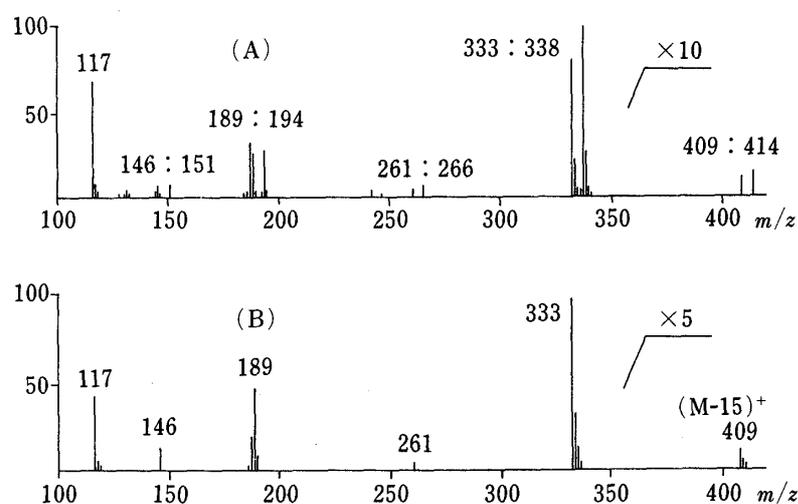


Fig. 3. Mass Spectra of the Metabolite in Peak c (A) and the Trimethylsilyl Derivative of Authentic FT- $\omega$ -1-OH (B)

of a hydroxyl group into the phenethyl group of FT. The retention time differs from that of FT-*p*-OH so the metabolized position may be the *ortho*, *meta* or  $\alpha$  position of the phenethyl group.

The MS corresponding to peak c is shown in Fig. 3A. The *m/z* values of the QM and M-15 ions in peak c indicated the introduction of a hydroxyl group. The presence of a singlet ion at *m/z* 117 ( $^+C_2H_4OTMS$ ) and cluster ions at *m/z* 333 : 338, which are 88 amu higher than ion A, indicated that peak c was the trimethylsilyl derivative of M-5. The metabolized position was determined to be the  $\omega$ -1 position of the propionyl group by comparing the retention time and MS (Fig. 3B) with those of authentic FT- $\omega$ -1-OH-TMS.

In the case of peak d, the molecular weight was estimated to be 512 from the QM (*m/z* 513) and M-15 (*m/z* 497) ions. The value was 176 higher than that of FT, and indicated the introduction of two hydroxyl groups into the FT molecule. The presence of the ions at *m/z* 333 and 117 suggested that hydroxylation had occurred on the phenethyl group and the propionyl moiety.

In the case of peak e, the presence of the ion at *m/z* 333 and the absence of the ion at *m/z* 117 were assumed to be the result of hydroxylation on the piperidine ring. The metabolite detected as peak e was assigned as M-6, as described in the previous report.<sup>3)</sup>

In the case of peak f, QM (*m/z* 513), M-15 (*m/z* 497) and M-90 (*m/z* 421) ions were observed. In addition, the presence of the ion at *m/z* 333 and the absence of the ion at *m/z* 117

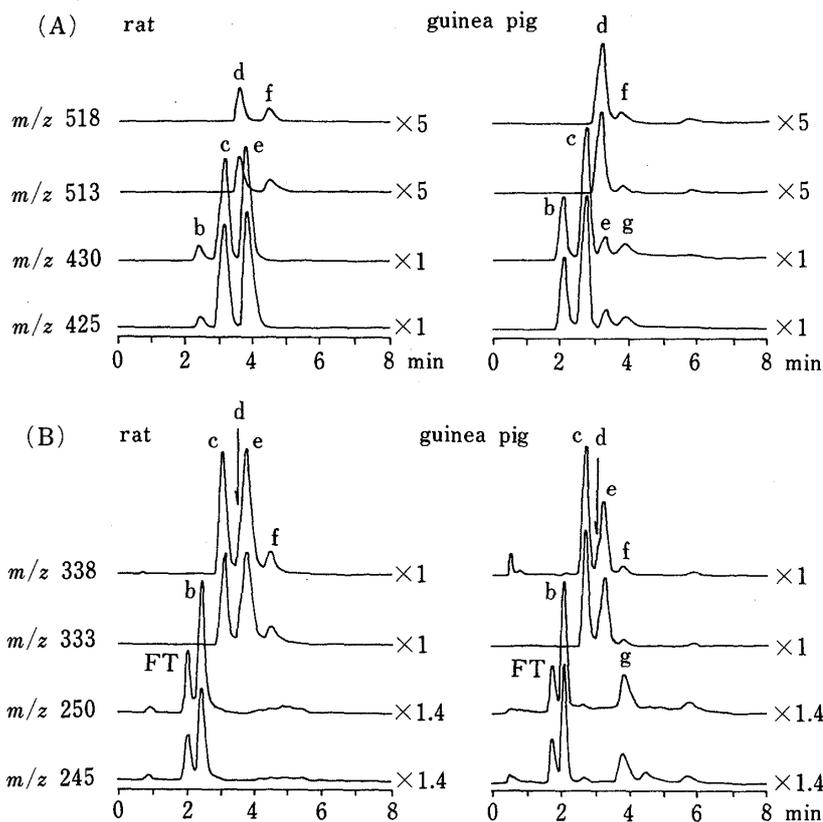


Fig. 4. CI (A) and EI (B) Mass Chromatograms of Trimethylsilylated Extract from Isolated Rat and Guinea Pig Hepatocytes after Incubation of FT:FT- $d_5$  for 3 h

suggested that two hydroxyl groups had been introduced into the phenethyl group and the piperidine ring.

In guinea pig hepatocytes, M-1 was detected as the main metabolite of FT, as in rat hepatocytes. Detection of other metabolites was performed by mass chromatography. Typical examples of mass chromatograms obtained from the hepatocytes of rat and guinea pig after incubation with FT:FT- $d_5$  are presented in Fig. 4. The QM ions in the CI spectra and the base peak ions in the EI spectra of the mono- and dihydroxy-FT detected in rat hepatocytes were selected to obtain these mass chromatograms.

Peaks b, c, d, e and f were also observed in the case of guinea pig, and a new metabolite (peak g) was also observed. The  $m/z$  value of the QM ion ( $m/z$  425:430) and the presence of the ion A ( $m/z$  245:250) indicated that hydroxylation at the phenethyl group had occurred. Peak g was identified as the trimethylsilyl derivative of FT-*p*-OH by comparing its retention time and MS with those of authentic FT-*p*-OH-TMS.

On incubation of FT- $\omega$ -1-OH and FT-*p*-OH with isolated rat hepatocytes, FT- $\omega$ -1-OH was metabolized to Nor- $\omega$ -1-OH, whereas FT-*p*-OH was metabolized to the conjugate and partly to M-1. This conjugate was expected to be the glucuronide, but could also include sulfate, since the evidence of the conjugation was simply the appearance of FT-*p*-OH after treatment of the filtrate with  $\beta$ -glucuronidase obtained from marine mollusc.

The metabolic pathways of FT in isolated hepatocytes of rat and guinea pig established by the above procedure are summarized in Fig. 5. FT was mainly metabolized to M-1 by oxidative *N*-desalkylation. Phenylacetic acid which was formed concomitantly by desalkylation, could be detected in the extract from the acidified filtrate of incubation fluid by GC-MS. Other metabolites of FT were oxidation products of the phenethyl, piperidine or propionyl group of FT.

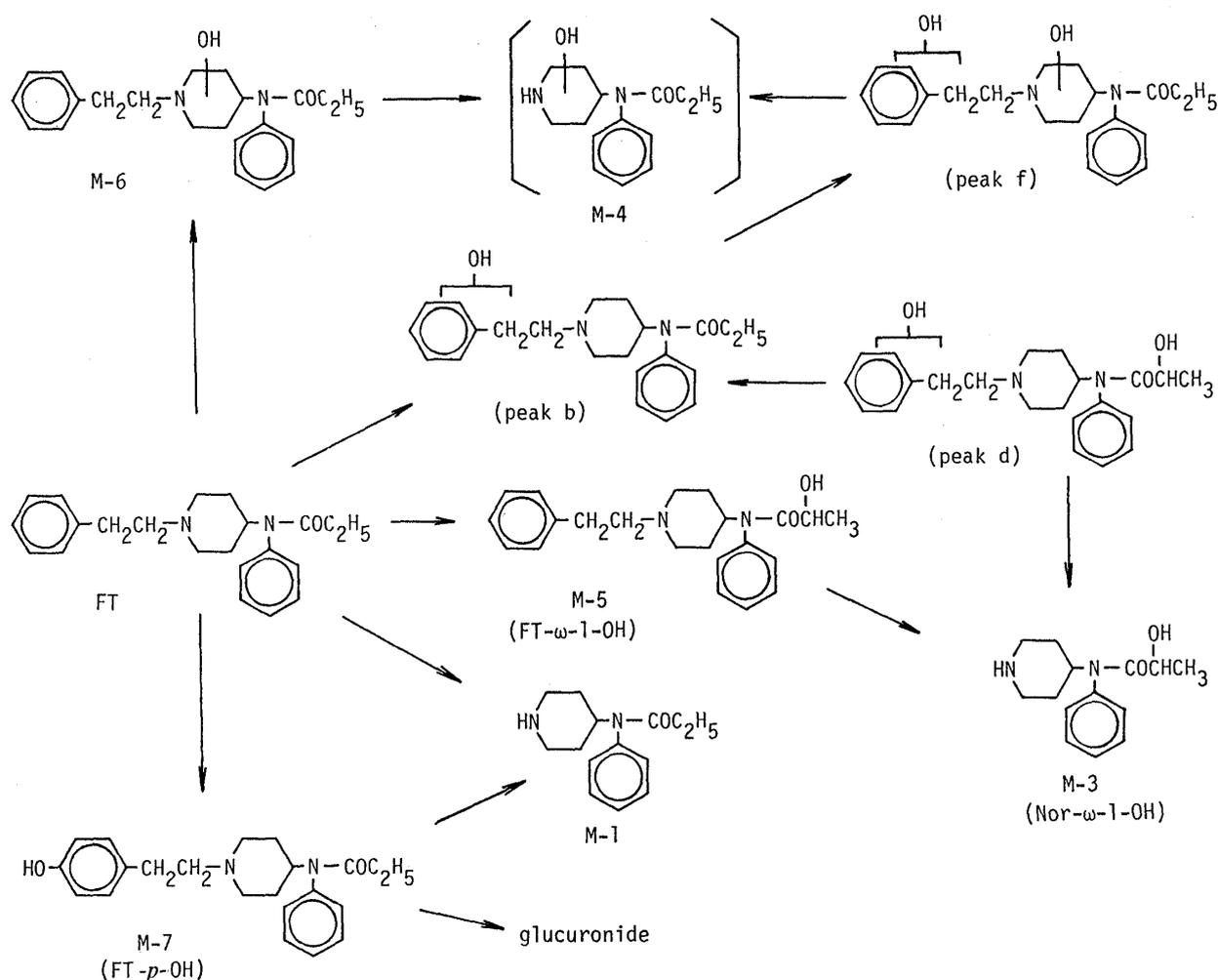


Fig. 5. Postulated Metabolic Pathways of FT in Rat and Guinea Pig

We attempted to detect these identified metabolites in the urine of rat and guinea pig after *p.o.* administration of FT : FT- $d_5$ . Peaks a, c, d, e, f and g were detected in the rat urine by mass chromatographic monitoring of the base peak ions of the identified metabolites in hepatocytes. Nor- $\omega$ -1-OH and an unknown metabolite, which might be a hydroxyl metabolite of FT, were also detected. Thus, the metabolism of FT in hepatocytes seems to be very similar to that *in vivo*.

On the other hand, only FT-*p*-OH and two unknown metabolites which might be hydroxyl and hydroxyl-methoxyl metabolites of FT were detected in guinea pig urine. It is assumed that the discrepancy between the metabolites in the urine and those in the hepatocytes of guinea pig may be due to biliary excretion or renal reabsorption of undetected metabolites.

Hug *et al.*<sup>10)</sup> detected M-2 in rat liver tissue after *i.v.* injection of tritium-labeled FT by radio chromatography, and van Rooy *et al.*<sup>11)</sup> detected M-2 as the acetyl derivative in the plasma extracts of patients receiving FT by selected ion monitoring using GC-MS. In contrast, M-2 was not found in this hepatocyte experiment or in the previous studies.<sup>3,4)</sup> When M-2 was added to control hepatocytes, M-2 clearly was detected by the procedure used in this experiment. These observations suggest that M-2 is not formed as a metabolite of FT and that metabolic hydrolysis of the amide bond does not occur.

Lehmann *et al.*<sup>12)</sup> detected FT-*p*-OH in mouse liver homogenates by the radioisotope tracer technique. We also detected FT-*p*-OH in the urine of surgical patients who had received

FT by *i.v.* infusion. It appears that hydroxylation of the phenethyl group is one of the main metabolic pathways of FT.

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