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Short communication

Analysis of urinary and biliary metabolites of (+)-4-[4-(4-methylphenyl)phenylmethoxy-1-piperidinyl]butyric acid in rats by liquid chromatography–frit-fast atom bombardment mass spectrometry

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Abstract

The urinary and biliary metabolites of (+)-4-[4-(4-methylphenyl)phenylmethoxy-1-piperidinyl]butyric acid [(+)-MPPB] were examined in rapid-metabolizing (RM) and slow-metabolizing (SM) male Sprague–Dawley rats by means of liquid chromatography–frit-fast atom bombardment mass spectrometry. In the RM-phenotyped rats, unchanged (+)-MPPB could not be detected in urine or bile, but 4-carboxyphenyl-MPPB was detected in bile. In the SM-phenotyped rats, unchanged (+)-MPPB was detected in bile and unchanged (+)-MPPB and β-oxidized MPPB in urine. Thus, the inter-individual difference in (+)-MPPB metabolism in rats was confirmed in vivo.

1. Introduction

(+)-4- [4- (4-Methylphenyl)phenylmethoxy-1-piperidinylbutyric acid hydrochloride [(+)-MPPB], a strong anti-allergic agent, showed remarkable inter-individual differences in plasma concentration and urinary excretion in rats of a single strain and sex [1]. The rats could be divided into two phenotypes, the rapid-metabolizing group (RM) and the slow-metabolizing group (SM). (−)-MPPB also showed similar inter-individual metabolic differences in rat. RM-phenotyped Sprague–Dawley rat liver microsomes metabolized (+)-MPPB to 4-hydroxymethylphenyl-MPPB (M1), and (−)-MPPB to M1 and 4′-hydroxyphenyl-MPPB (M2) [2]. The cause of these inter-individual differences was found in vitro to be a polymorphism of a cytochrome P-450 involved in the metabolism of MPPB. To confirm this phenomenon in vivo we investigated the urinary and biliary metabolites in phenotyped rats.

Fast atom bombardment (FAB) ionization gives mainly a pseudo-molecular ion, from which the molecular mass can be obtained. Thus a combination of liquid chromatography and FAB mass spectrometry has been applied to the structural analysis of various non-volatile or polar compounds, such as bile acids [3,4], oligosaccharides [5], peptides [6] and metabolites of

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drugs [7]. LC–MS is more suitable when searching for unknown metabolites than gas chromatography–mass spectrometry (GC–MS), since the latter requires clean-up and derivatization of polar compounds. GC–MS procedures may result in loss or degradation of metabolites. Therefore, we have attempted to use LC–frit-FAB-MS for the identification of the metabolites of (+)-MPPB in rat urine and bile.

First we examined the metabolites in an incubated microsomal mixture without complicated isolation and purification pretreatments in order to assess the usefulness of LC–frit-FAB-MS for the detection of MPPB metabolites. We then successfully detected and identified the urinary and biliary metabolites in rats administered (+)-MPPB.

2. Experimental

2.1. Chemicals

(+) - 4 - [4 - (4 - Methylphenyl)phenylmethoxy - 1 - piperidinyl]butyric acid hydrochloride [(+)-MPPB], (±) - 4 - [4 - (4 - hydroxymethylphenyl) phenylmethoxy - 1 - piperidinyl]butyric acid (M1) and (±) - 4 - [4 - (4 - methylphenyl)phenylmethoxy - 1 - piperidinyl]acetic acid (M4) were synthesized in the laboratory of Hokuriku Seiyaku (Fukui, Japan). NAD^+, $NADP^+$ and glucose-6-phosphate were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals and solvents were of analytical grade.

2.2. Sample preparation

Male Sprague–Dawley rats were purchased from Clea Japan (Tokyo, Japan). Rats were acclimated to the breeding environment for one week and used at seven weeks of age. Rats were divided into RM- and SM-type on the basis of the plasma concentration of unchanged (+)-MPPB after oral administration of (+)-MPPB as previously described [2].

Urine and bile

Urine and bile samples were collected from bileduct-cannulated rats for 6 h after oral administration of (+)-MPPB at 10 mg/kg. Aliquots of 20 μl of the rat urine and bile were directly injected into the LC–frit-FAB-MS system.

In vitro metabolism with liver microsomes

(+)-MPPB was incubated with liver microsomes prepared from RM-phenotyped rats as previously described [2]. In order to concentrate M1, the incubated mixture was extracted with chloroform. The chloroform layer obtained was evaporated. The residue was taken up in water and an aliquot was subjected to LC–frit-FAB-MS.

In vitro metabolism with liver S-9

Authentic M1 was incubated with the 9000 *g* supernatant fraction (S-9) of RM-phenotyped rat liver (14 mg protein/ml), NAD^+ (0.4 m*M*) and $NADP^+$ (0.4 m*M*) in 0.1 *M* phosphate buffer (pH 7.4) for 30 min at 37°C. Then 15% $ZnSO_4$ and a saturated aqueous solution of $Ba(OH)_2$ were added to the incubation mixture. The supernatant obtained after centrifugation (1500 *g*, 5 min) was freeze-dried, because M3 was not extracted by organic solvents. The residue was taken up in water and an aliquot was subjected to LC–frit-FAB-MS.

2.3. Liquid chromatography–frit-FAB-mass spectrometry

The conditions of LC–frit-FAB-MS were as follows. The HPLC system consisted of a U6K injector (Waters, Milford, MA, USA), a 600-MS pump (Waters) for the mobile phase, a PU-980 pump (Jasco, Tokyo, Japan) for the matrix and a TSKgel 80_{TM} column (150 × 4.6 mm I.D., 5 μm; Tosoh, Tokyo, Japan). The mobile phase consisted of 10 m*M* $CH_3CO_2NH_4$ (A) and CH_3CN (B). A 45-min linear gradient was applied from 100% A to 100% B at a flow-rate of 0.8 ml/min, for both bile and urine. An isocratic mobile phase of 0.8 m*M* $CH_3CO_2NH_4$–CH_3CN (6:4, v/v) was used in the case of the microsomal

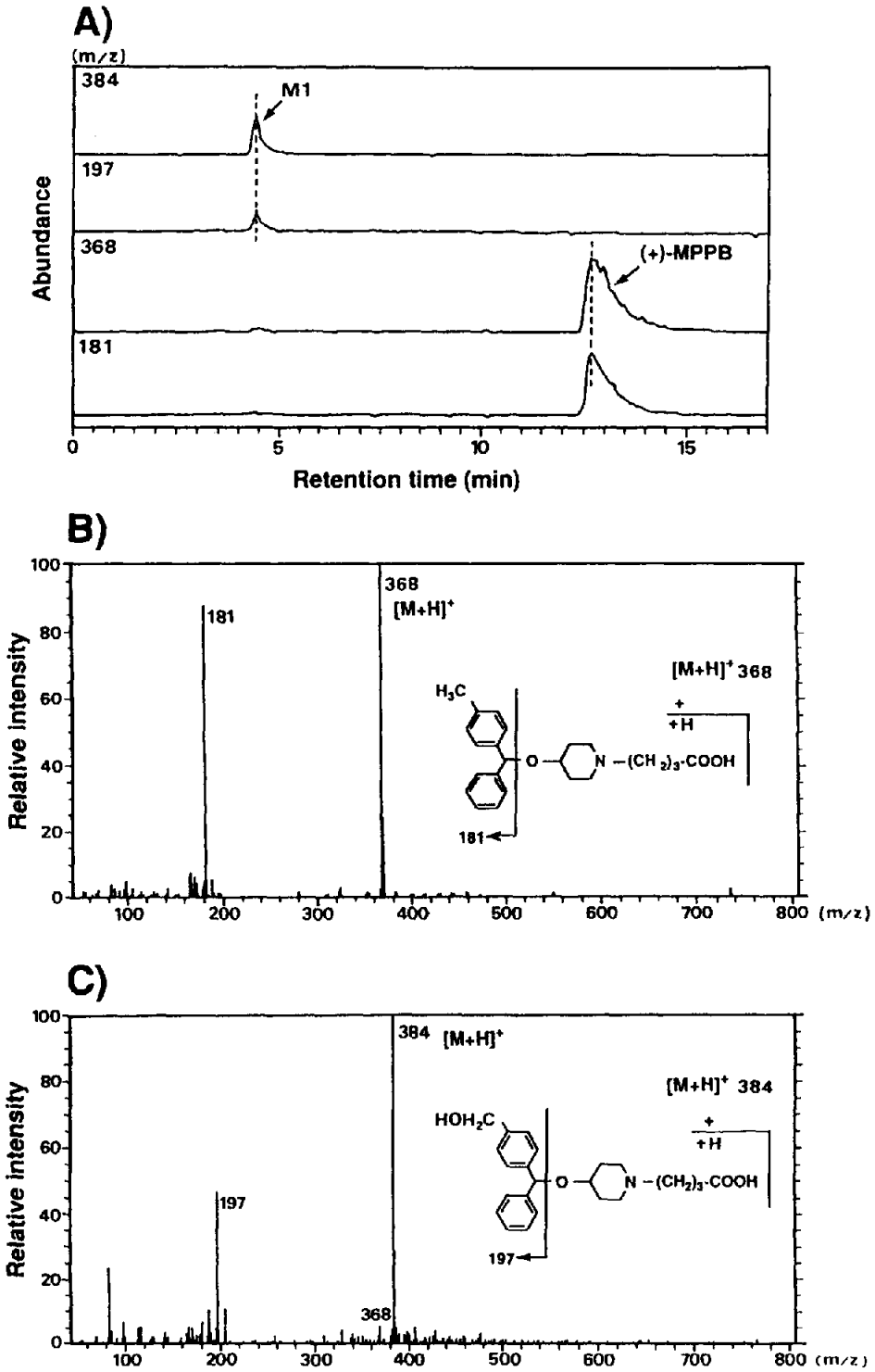

Fig. 1. LC–frit-FAB-MS chromatogram and spectra of the microsomal incubation mixture with (+)-MPPB. (A) Mass chromatogram; mass spectra of (B) (+)-MPPB and (C) M1. The incubation conditions were described in Experimental.

incubation mixture. The column effluent was mixed with 3% glycerol in methanol (0.2 ml/min) as a matrix. The mixture was split 1:100 (v/v) and directed to a DX-303 mass spectrometer (JEOL, Japan) through a FAB probe. Xenon was used as the FAB gas. The FAB gun was operated at 4 keV with a 10-mA discharge current.

3. Results

3.1. In vitro metabolism with liver microsomes

In previous papers [1,2], we showed that (+)-MPPB was metabolized to M1 by rat liver microsomes and M1 was identified as 4-hydroxymethylphenyl-MPPB by analysis of the isolated metabolite and by independent synthesis.

In the present study, we extracted the metabolites with chloroform from the microsomal mixture. A mass chromatogram of the RM-phenotyped microsomal incubation mixture with (+)-MPPB (Fig. 1) showed residual (+)-MPPB and M1 formed. In the FAB mass chromatogram, (+)-MPPB showed the pseudo-molecular ion ($[M+H]^+$) at m/z 368 and a fragment ion at m/z 181. The fragment ion at m/z 181 corresponds to the (4-methylphenyl)phenylmethyl moiety. On the other hand, M1 showed the pseudo-molecular ion at m/z 384 and the fragment ion at m/z 197. These ions are 16 mass units heavier than the corresponding ions of (+)-MPPB.

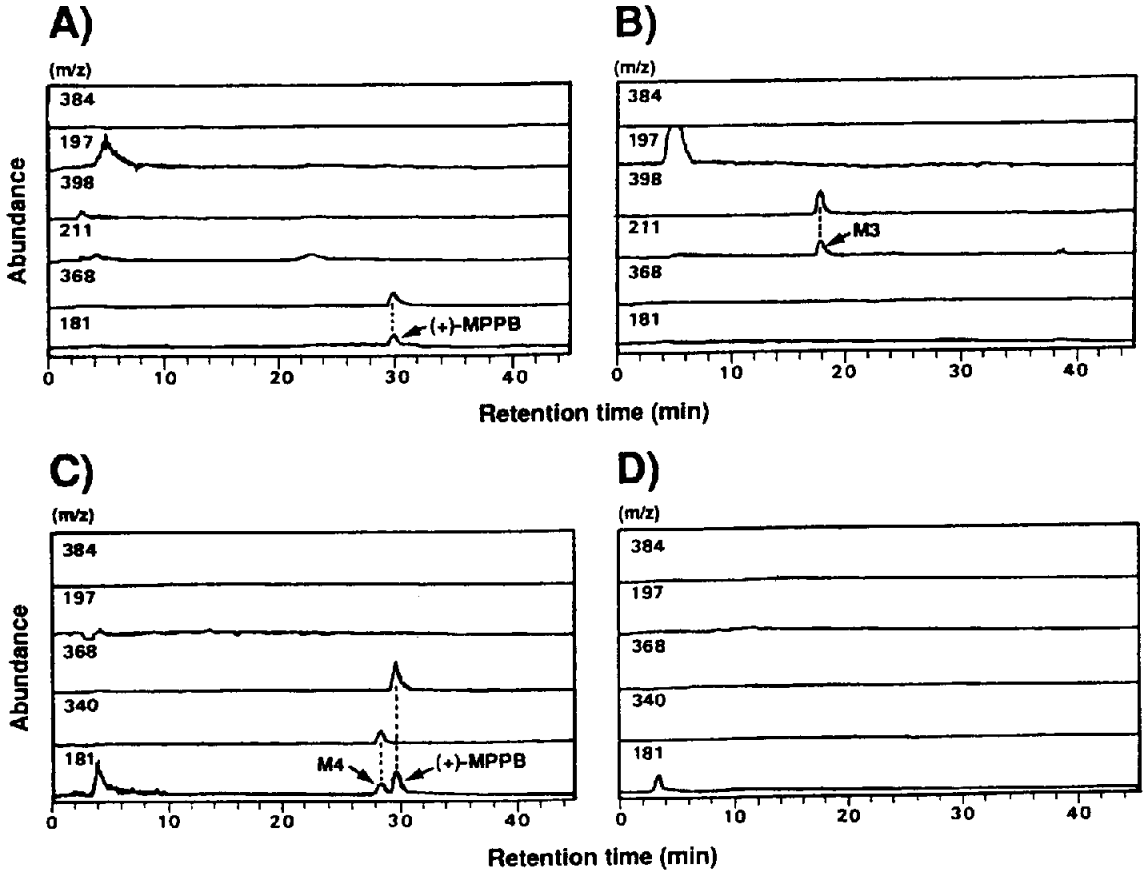

Fig. 2. LC–frit-FAB-MS chromatograms of bile and urine in the phenotyped rats administered (+)-MPPB. Bile: (A) SM- and (B) RM-phenotyped rats. Urine: (C) SM- and (D) RM-phenotyped rats.

3.2. Metabolite in the bile

Mass chromatograms for bile obtained from SM- and RM-phenotyped rats after an oral administration of (+)-MPPB are shown in Fig. 2. We searched for ions corresponding to unchanged (+)-MPPB, M1 and the other expected metabolites including conjugates. However, M1 could not be detected in SM- or RM-phenotyped rats. In SM-phenotyped rats, only unchanged (+)-MPPB was detected. On the other hand in RM-phenotyped rats, unchanged (+)-MPPB could not be detected, and an unknown metabolite (M3) was present. The mass spectra of M3 (Fig. 3) showed the pseudo-molecular ion ($[M+H]^+$) at m/z 398 and fragment ions at m/z 354, 211 and 167. The pseudo-molecular ion and the fragment ion at m/z 211 were 30 mass units heavier than the corresponding ions of (+)-MPPB. The fragment ions at m/z 354 and 167 were presumed to be formed by decarboxylation of the ions at m/z 398 and 211, respectively. Therefore, M3 was supposed to be a metabolite in which the methyl group of (+)-MPPB was oxidized to a carboxyl group.

In order to confirm the structure of M3 and

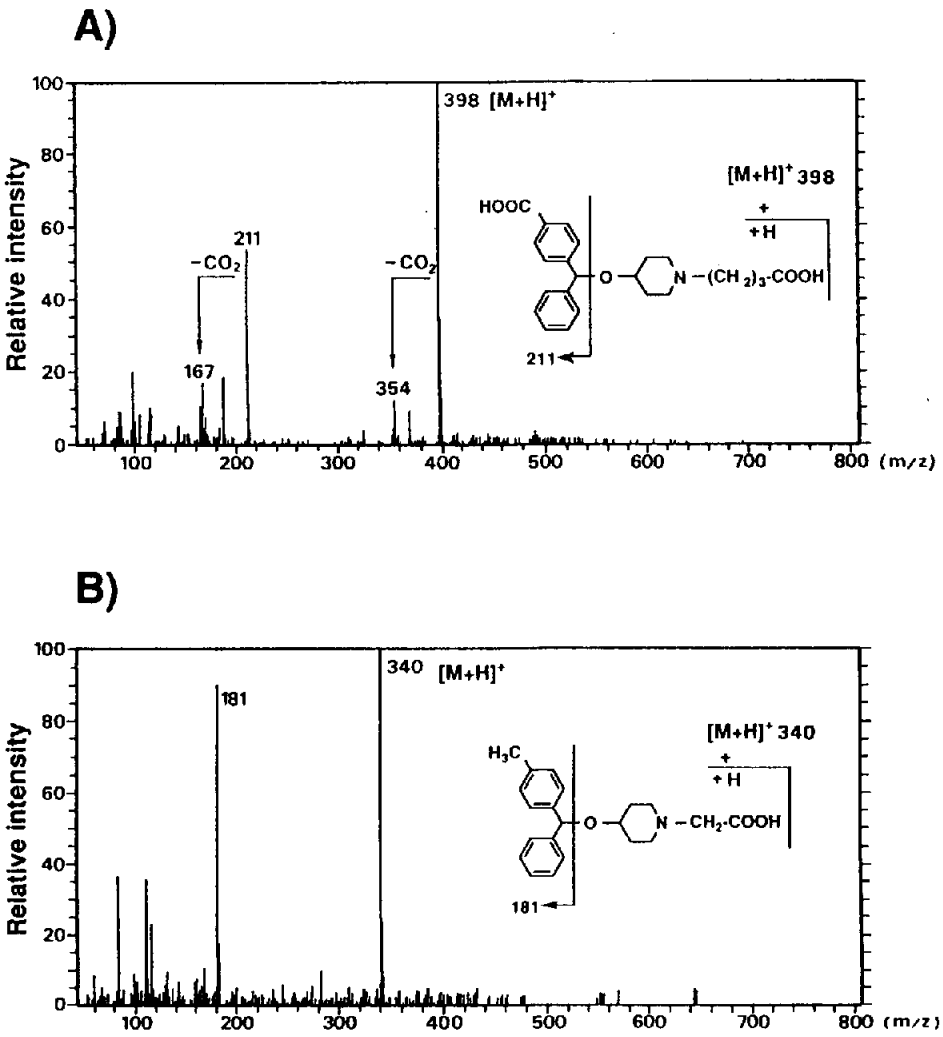

Fig. 3. FAB mass spectra of (A) M3 and (B) M4 obtained with LC–frit-FAB-MS.

the metabolic pathway leading to its formation, an in vitro study was performed with liver S-9. When authentic M1, 4-hydroxymethylphenyl-MPPB, was incubated with liver S-9, the formation of M3 was detected by of LC–frit-FAB-MS (Fig. 4). Thus, it was concluded that the biliary metabolite M3 is 4-carboxyphenyl-MPPB, formed via M1.

3.3. Metabolite in urine

Mass chromatograms of urine obtained from SM- and RM- phenotyped rats after an oral administration of (+)-MPPB are shown in Fig. 2. In SM-phenotyped rats, unchanged (+)-MPPB and an unknown metabolite, M4, were detected. The mass spectra of M4 (Fig. 3), showed the pseudo-molecular ion ($[M+H]^+$) at m/z 340 and fragment ions at m/z 181. The pseudo-molecular ion was 28 mass units less than that of (+)-MPPB. On the other hand, the fragment ion at m/z 181 was the same as the corresponding ion of (+)-MPPB, indicating that the (4-methylphenyl)phenyl moiety was not changed. Thus, M4 was supposed to be a metabolite in which the N-butyric acid group of (+)-

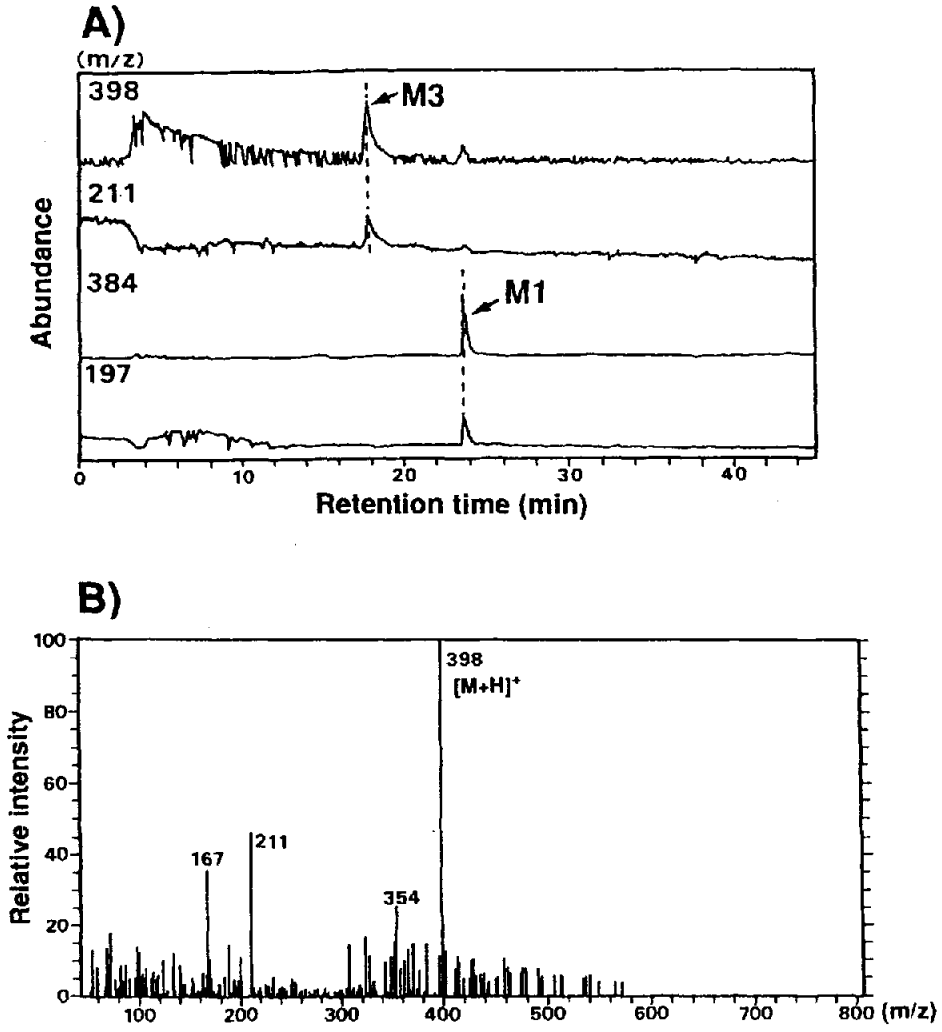

Fig. 4. LC–frit-FAB-MS chromatogram and spectra of the incubation mixture of liver S-9 and M1. (A) Mass chromatogram, (B) mass spectrum of M3. The conditions of incubation were described in Experimental.

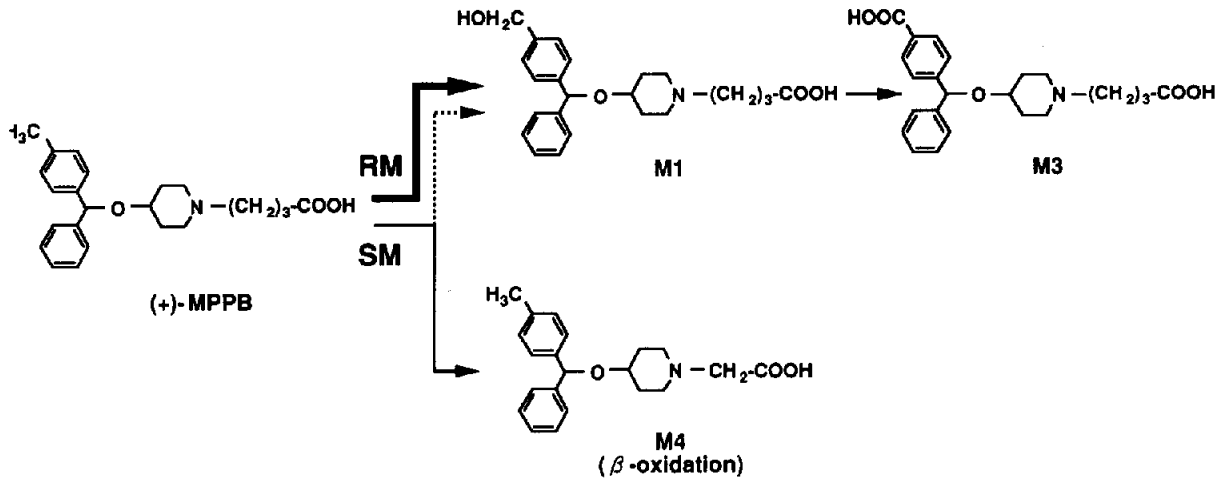

Fig. 5. Postulated metabolic pathways of (+)-MPPB in rats.

MPPB was converted to an N-acetic acid moiety by β-oxidation. The structure was confirmed by comparing the mass spectra and retention times with those of authentic compounds.

In RM-phenotyped rats, no peaks corresponding to unchanged (+)-MPPB or its metabolites were found on the mass chromatograms.

4. Discussion

In our previous study [2], M1 was identified as 4-hydroxymethylphenyl-MPPB by analysis of the isolated compound and by synthesis. In the present study, we were able to detect M1 in the microsomal incubation mixture without complicated pretreatments by using LC–frit-FAB-MS. As a next step, we attempted to detect the urinary and biliary metabolites of (+)-MPPB without pretreatment.

In RM-phenotyped rats, unchanged (+)-MPPB could not be detected in urine or bile, but the metabolite M3 was detected in bile. On the other hand, in SM-phenotyped rats, unchanged (+)-MPPB in bile, and unchanged (+)-MPPB and the metabolite M4 in urine were found. As a result of these studies, the metabolic pathways of (+)-MPPB in RM- and SM-phenotyped rats were postulated to be as shown in Fig. 5. In RM-phenotyped rats, (+)-MPPB is predominantly metabolized to M1, and M1 is further metabolized to M3. On the other hand, in SM-phenotyped rats β-oxidation proceeds instead of the metabolism to M1.

In conclusion, we have confirmed the inter-individual difference in (+)-MPPB metabolism in male Sprague–Dawley rats, not only in vitro but also in vivo, with LC–frit-FAB-MS. The LC–frit-FAB-MS method was very useful for the detection of metabolites of MPPB.

References

[1] E. Takahara, O. Nagata, H. Kato, S. Ohta and M. Hirobe, *Biol. Pharm. Bull.*, 16 (1993) 1057.
[2] E. Takahara, O. Nagata, H. Kato, S. Ohta and M. Hirobe, *Drug. Metab. Disp.*, in press.
[3] Y. Ito, T. Takeuchi and D. Ishii, *J. Chromatogr.*, 346 (1985) 161.
[4] Y. Ito, T. Takeuchi and D. Ishii, *J. Chromatogr.*, 358 (1986) 201.
[5] S. Santikarn, G.R. Her and V.N. Reinhold, *J. Carbohydr. Chem.*, 6 (1987) 141.
[6] R.M. Capriori, W.T. Moore, B. Dague and M. Martin, *J. Chromatogr.*, 443 (1988) 355.
[7] W. Luijten, G. Damien, B. Marchand and J. Capart, *Biomed. Environ. Mass Spectrom.*, 16 (1988) 93.