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Absorption, Metabolism and Excretion of [^{14}C]Imidafenacin, a New Compound for Treatment of Overactive Bladder, Following Oral Administration to Healthy Male Subjects

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LSC, liquid scintillation counter; HPLC, high performance liquid chromatography; RAD, radiodetector; LC/MS/MS, liquid chromatography-tandem mass spectrometry; ESI, electron spray ionization; SRM, selected reaction monitoring; Cmax, maximum concentration; Tmax, time at maximum concentration; AUC_{0-t}, area under concentration vs time curve from time zero to t hours; AUC_{0-inf}, area under concentration vs time curve from time zero to infinity; $T_{½}$el, half-life of terminal elimination phase; NMR, nuclear magnetic resonance;. NOE, nuclear overhauser effect; DEPT, distortionless enhancement by polarization transfer; ^{1}H-^{1}H COSY, proton-proton correlated spectroscopy; HMQC, heteronuclear multiple quantum coherence; HMBC, heteronuclear multiple bond coherence; CYP, cytochrome P450; UGT, uridine diphosphate glucuronosyltransferase

Abstract

The absorption, metabolism and excretion of imidafenacin (KRP-197/ONO-8025), 4-(2-Methyl-1*H*-imidazol-1-yl)-2,2-diphenylbutanamide, a new antimuscarinic drug developed for treatment of overactive bladder, were assessed in six healthy male subjects following a single oral administration of 0.25 mg [^{14}C]imidafenacin (ca. 46μCi). The highest radioactivity in the plasma was observed at 1.5 h after administration. The apparent terminal elimination half-life of the total radioactivity was 72 h. Approximately 65.6 and 29.4% of the administered radioactivity were recovered in the urine and feces, respectively, within 192 h after administration. The metabolite profiling by HPLC–radiodetector and LC/MS/MS demonstrated the main component of radioactivity was unchanged imidafenacin in the 2 h plasma. N-glucuronide conjugate (M-9) was found as the major metabolite, and the oxidized form of 2-methyl-imidazole moiety (M-2) and the ring-cleavage form (M-4) were detected as the minor metabolites in the 2 h plasma, but M-4 was found to be the main component in the 12 h plasma. Unchanged imidafenacin, M-9, M-2 and other oxidized metabolites were excreted in the urine, but the unchanged imidafenacin and M-9 were not found in the feces. Two unique metabolites were found in the urine and feces, which were identified as the interchangeable *cis*- and *trans*-isomers of 4,5-dihydro-diol forms of 2-methyl-imidazole moiety. These findings indicate that imidafenacin is rapidly and well absorbed (at least 65% of dose recovered in urine) following oral administration and circulates in human plasma as the unchanged form, its glucuronide and other metabolites,

then excreted in urine and feces as the oxidized metabolites of 2-methyl-imidazole moiety.

Introduction

Imidafenacin (KRP-197/ONO-8025), 4-(2-Methyl-1*H*-imidazol-1-yl)-2,2-diphenylbutanamide (Figure 1), is a newly synthesized antimuscarinic drug developed for treatment of overactive bladder. Acetylcholine is well known to play a major role in contracting the bladder through activation of muscarinic receptors (Braverman et al., 1998; Wang et al., 1995; Somogyi and de Groat, 1992). Compounds with high affinity for the muscarinic acetylcholine receptor, including propiverine, tolterodine, oxybutynin, darifenacin and solifenacin have been used in management of overactive bladder (Andersson, 2004; Andersson and Yoshida, 2003; Chapple et al., 2002, Robinson and Cardozo, 2005). Imidafenacin showed high *in vitro* affinity for muscarinic receptor subtypes M_1 and M_3 on the functional assay using isolated animal tissues and on the binding assay using recombinant human receptors (Kobayashi et al., 2007a; Miyachi et al., 1999). In addition, imidafenacin inhibited the carbachol-induced contraction of isolated guinea pig and human bladder mediated by M_3 receptor, and the acetylcholine release from isolated rat and human bladder mediated by prejunctional M_1 receptor (Kobayashi et al., 2007a; Murakami et al., 2003). Carbachol-induced reduction in bladder capacity and distention-induced rhythmic bladder contraction were prevented by imidafenacin dose-dependently in conscious rats (Kobayashi et al., 2007b; Miyachi et al., 1999). On the other hand, effects of imidafenacin on carbachol-induced salivary gland secretion mediated by M_3 receptor alone were less potent than those on bladder contraction in

rats (Kobayashi et al., 2007b; Miyachi et al., 1999).

In the pharmacokinetic assays in the pre-clinical toxicology studies, imidafenacin was absorbed rapidly with absolute bioavailability of 5.6% in rats and 36.1% in dogs after oral administration (unpublished observation). Orally administered [^{14}C]imidafenacin was excreted as many metabolites in the feces, and the total recovery in the urine and feces were more than 95% of the administered dose in rats and dogs (unpublished observation). In the phase I clinical trials, plasma concentration and urinary excretion of imidafenacin increased dose-dependently in the range from 0.025 to 0.5 mg of single oral dose, and apparent elimination half-life ranged from 2.6 to 3.0 h (Shimada et al., 2006a). Accumulation of imidafenacin in the plasma and urine was insignificant during multiple oral administration of 0.25 mg twice a day (Shimada et al., 2006b).

The purposes of this study were to determine the absorption and excretion kinetics of imidafenacin in humans following oral administration of [^{14}C]imidafenacin, and to investigate the metabolite profile of imidafenacin in the plasma and excreta. Imidafenacin is supposed to be transformed to many metabolites in humans; however, clinical dosage is very low. In this case, human mass-balance assay using radiolabeled drugs is a valuable measure to elucidate the fate of therapeutic drug in the body (Minematsu et al., 2005; Lantz et al., 2003; Patrick et al., 2002; Cox et al., 2000). Accordingly, ^{14}C-labeled imidafenacin ([^{14}C]imidafenacin, ^{14}C-labeled position is shown in Figure 1) was administered orally at a dose of 0.25 mg (ca. 46μCi) to six healthy male subjects, and the total radioactivity and their

metabolite profile in the plasma, urine and feces were determined by LSC or HPLC connected to RAD and MS/MS detectors. In addition, chemical structures of two unique diol-metabolites found in the excreta were identified by comparing with those of the reference product obtained from imidafenacin with the cupro-ascorbate oxidation system.

Materials and Methods

Radiolabeled Material and Other Materials [^{14}C]Imidafenacin (Figure 1) was synthesized at Amersham Biosciences Corp. (Piscataway, NJ, USA), with a certificate of analysis of the radiochemical purity (99.4%) and specific activity (178μCi/mg). [^{14}C]Imidafenacin was stored at –80°C in the dark. The radiochemical purity (more than 99.4%) and chemical authenticity of [^{14}C]imidafenacin was confirmed by HPLC before the study, following mock dose preparation and administration to subjects. Authentic standards of imidafenacin and its metabolites M-1, M-2, M-3, M-4, M-5, M-6, M-8, M-9 and M-10 (Figure 1) were prepared by chemical synthesis or isolation from biological samples in our laboratory. All other reagents were of HPLC grade or analytical grade and were obtained from commercial sources.

Dose Preparation [^{14}C]Imidafenacin was dissolved in ethanol and radioactive concentration was determined. A certain volume of the [^{14}C]imidafenacin ethanolic solution was dispensed into the hard gelatin capsules (equivalent to 0.25 mg and 46μCi per capsule). Ethanol was evaporated under steady stream of nitrogen.

The radioactive dose was set according to the human dosimetry calculations accomplished by the National Radiological Protection Board (Oxon, UK). Administration of [^{14}C]imidafenacin for the radioactive medical product was authorized by the Administration of Radioactive Substances Advisory Committee (Oxon, UK). The radiation

exposure in this study, ca 0.34 mSv, fell into Category IIa studies (0.1 to 1 mSv) in the International Commission on Radiological Protection guidelines (1992). A single oral dose of 0.25 mg imidafenacin has been known to be well tolerated in the phase I clinical trials of non-radiolabeled imidafenacin.

Study Design This study was an open-labelled and non-randomised study involving six Caucasian healthy male subjects, all of whom were to receive a single oral dose of [^{14}C]imidafenacin. Clinical phase of this study was conducted at the clinical unit of Inveresk Research (Edinburgh, UK). This study was performed in accordance with the Good Clinical Practice guidelines and the Declaration of Helsinki. Before the start of study, the study protocol and the informed consent documents were approved by an independent Ethics Review Committee. All of the subjects provided written informed consent prior to the study.

The subjects were screened within 21 days before administration of [^{14}C]imidafenacin. The mean age of the subjects was 44.0 (from 32 to 53) years, the mean height was 177.7 (from 168 to 185) cm, the mean weight was 82.43 (from 71.2 to 97.1) kg and the mean BMI was 26.08 (from 22.0 to 29.0) kg/m^2. The subjects were admitted to the clinical unit on the day before administration of [^{14}C]imidafenacin and a light supper was served about 22:00. Neither food nor beverage was permitted after the supper except for water. About 08:30 on Day 1 each subject was administered a single oral dose of 0.25 mg [^{14}C]imidafenacin with

approximately 200 mL of water. Subjects were fasted for about 4 h after administration, then a light lunch was served. Safety evaluations including physical examination, hematology, clinical chemistry, urinalysis, 12-lead electrocardiogram and vital signs were made throughout the admission period

Sample Collection Blood samples were collected from an in situ venous cannula or by venepuncture into lithium heparin tubes. From 10 to 20 mL of blood samples were taken at the following times: 0 (before administration), 0.5, 1, 1.5, 2, 3, 4, 6, 8, 12, 24, 48, 72, 96, 120, 144 and 168 h after administration. Blood samples were centrifuged for 10 min at ca 4°C and separated plasma was transferred into polypropylene tubes. The total radioactivities in the samples were analyzed as immediately as possible, then the remainders were stored at ca –80°C for metabolite profiling.

Urine samples were collected for the following intervals: 0 (before administration), 0–4, 4–8, 8–12, 12–24, 24–48, 48–72, 72–96, 96–120, 120–144 and 144–168 h after administration. For one subject, urine samples were collected until 192 h after administration because excretion of radioactivity continued in the urine. Aliquots of each sample were transferred into separate polypropylene tubes for analysis of the total radioactivity and metabolite profiling, and the samples were stored at ca –20°C and at ca –80°C, respectively.

Fecal samples were collected for the following intervals: 0 (before administration),

0–24, 24–48, 48–72, 72–96, 96–120, 120–144 and 144–168 h after administration. For one subject, fecal samples were collected until 192 h after administration because excretion of radioactivity continued in the feces. Fecal samples were homogenized in water, and then, aliquots of each homogenate were retained in separate closed containers for analysis of the total radioactivity and metabolite profiling, and the samples were stored at ca –20°C and at ca –80°C, respectively.

Analysis of Total Radioactivities in Samples Volumes or weights of all samples were measured. Duplicate portions of plasma and urine samples were diluted with water or compatible solvent and dissolved in liquid scintillation fluid, Quickszint 1 (Zinsser Analytic, Maidenhead, UK). Duplicate portions of fecal homogenate sample (ca 0.3 g) were combusted using the Tri-Carb 307 automatic sample oxidizer (Packard Biosciences Ltd., Pangbourne, UK). The $^{14}CO_2$ generated was collected in the absorbing fluid, Carbo-Sorb® CO_2 and scintillation fluid, Permafluor® E^+ (Packard Biosciences Ltd.).

All of the samples in the scintillation fluid were subjected to liquid scintillation counting for 5 min, together with representative blank samples, using the LSC, Tri-Carb 2100TR (Packard Biosciences Ltd.) with automatic quench correction by an external standard method. The limit of reliable measurement was defined as 30 dpm above the background.

Calculation of Urinary and Fecal Excretion The total radioactivities excreted in the urine and feces were calculated by the timepoints after administration of [^{14}C]imidafenacin, and expressed as percentage of the administered radioactivity (% of dose).

Estimation of Pharmacokinetic Parameters Pharmacokinetic parameters were estimated using WinNonlin® Professional version 3.1 (Pharsight Corp., Mountain View, CA, USA). A non-compartmental approach was used to generate parameter estimates, using WinNonlin® model 200 (extravascular input). All of the total radioactivities calculated from data below the limit of reliable detection (< 30 dpm above the background) were considered to be zero. The apparent terminal elimination phase was identified by regression analysis with WinNonlin®, using data points from 24 to 168 h after administration.

The observed Cmax of the total radioactivity and Tmax after administration were identified by inspection of the total radioactivity vs time data. The AUC_{0-t}, where t means the timepoint for the last sample in which the total radioactivity was reliably quantified, was calculated using the linear trapezoidal method. The AUC_{0-inf} was calculated as the sum of AUC_{0-t} and Ct/Kel, where Ct means the total radioactivity at t hours as mentioned above and Kel means the terminal elimination rate constant determined from the slope of the terminal elimination phase. The $T_{½}$el was calculated as 0.693/Kel.

Metabolite Profiling

Pretreatment of plasma Plasma samples at 2 h (around Tmax) and at 12 h (elimination phase) were selected for the samples of assessing metabolic profile of imidafenacin. The radioactivity in the latter sample stood on the brink of the detection limit. The cryopreserved plasma samples (2 and 12 h) were thawed, and designated amounts of the plasma samples obtained from six subjects after administration of [^{14}C]imidafenacin were pooled. The pooled plasma was mixed with 3-fold volume of acetonitrile containing 1% acetic acid, sonicated for 5 min, and centrifuged at 1800 × g for 15 min at 4°C to separate supernatant. The residue was re-extracted with the same solvent, followed by shaking and centrifugation. The extraction recoveries were 84.5% for 2 h plasma and 73.5% for 12 h plasma.

Entire supernatants were combined and evaporated to dryness under reduced pressure, and the residue was reconstituted in 0.3% formic acid in water / acetonitrile (90:10, v/v) and centrifuged at 1800 × g for 5 min at 4°C. The supernatant was filtered (0.45 μm), and an aliquot of the filtrate was analyzed under the conditions described below.

Pretreatment of urine and feces The cryopreserved urine samples (0-4 and 4-24 h) were thawed, and the samples obtained from six subjects after administration of [^{14}C]imidafenacin were pooled at the sample volume ratio. The cryopreserved fecal homogenates (0-48 and 48-96 h) were thawed, and the homogenates containing radioactivity of 2% or more of the administered dose, prepared from six subjects after administration of [^{14}C]imidafenacin, were

pooled at the sample weight ratio. An aliquot of the pooled sample was mixed with 3-fold volume of methanol, shaken for 10 min, and centrifuged at 1800 × g for 10 min at 4°C to separate supernatant. The residue was re-extracted with methanol, followed by shaking and centrifugation. The extraction recoveries were 93.1% for 0-4 h urine, 88.6% for 4-24 h urine, 77.2% for 0-48 feces and 69.2% for 48-96 h feces.

Entire supernatants were combined and evaporated to dryness under reduced pressure. The residue was reconstituted in 0.3% formic acid in water / acetonitrile (90:10, v/v), and an aliquot of the solution was analyzed under the conditions described below. The reconstituted fecal solution was centrifuged at 1800 × g for 5 min at 4°C. The supernatant was filtered (0.45 μm), and an aliquot of the filtrate was analyzed under the conditions described below.

Analysis of metabolites in samples Relative amounts of metabolites in the plasma, urine, and feces were determined by HPLC connected to RAD or by LSC following collection of HPLC eluates. The metabolites were identified by comparison of retention times between radioactive peaks and ion peaks on the HPLC connected to MS/MS.

TSKgel Octyl-80Ts (5 μm, 4.6 mm I.D. × 150 mm L, Tosoh Corp., Tokyo, Japan) connected to a guard column was used as the LC column. As mobile phase, the mixture of 0.3% formic acid in water / acetonitrile (90:10, v/v) (A) and 0.7% formic acid in acetonitrile (B), was flowed at 1 mL/min in the following linear gradient mode: starting with 0% of B

composition to 5 min, increasing to 10% in 5 to 10 min, increasing to 18% in 10 to 30 min, finally increasing to 100% in 30 to 35 min and maintaining 100% in 35 to 40 min. The column was maintained at room temperature. The column eluate was split in the ratio of approximately 4:1, and introduced to RAD, FLO-ONE/525TR (Packard Biosciences Ltd.) and MS/MS, TSQ7000 (Thermo Electron Corp., Waltham, MA, USA), respectively. As scintillation fluid for RAD, Flo-scint™ II (Packard Biosciences Ltd.) was delivered to the HPLC eluate at 3-fold flow rate of the mobile phase, and the radioactivity was monitored using the RAD with 6-sec integration. For the sensitive detection of radioactivity in plasma, the HPLC eluates were collected every 18 sec and dissolved in liquid scintillation fluid, Hionic-flour™ (Packard Biosciences Ltd.), and then the radioactivity in each fraction was detected for 2 min using the LSC, Tri-Carb 2500TR (Packard Biosciences Ltd.). Detection limits of radioactivity in metabolite peaks in the RAD and LSC assays were defined as 3 and 2 times of the background values, respectively.

For ionization and detection of all analytes, the conditions of TSQ7000 equipped with ESI interface were positive ionization mode, spray voltage at 4.5 kV, capillary temperature at 290°C, multiplier voltage at 1500 V, sheath gas pressure at 70 psi (N_2), auxiliary gas flow at 10 arbitrary unit (N_2), collision gas pressure at 2.0 mTorr (Ar) and collision energy at 18 to 30 eV. The precursor and product ions monitored in SRM, as shown in Figure 1, were m/z 322→240 for [^{14}C]imidafenacin, 338→240 for [^{14}C]M-1, 354→240 for [^{14}C]M-2, 356→240 for [^{14}C]M-3, 328→193 for [^{14}C]M-4, 329→193 for [^{14}C]M-5, 240→117 for [^{14}C]M-6,

344→209 for [^{14}C]M-8, 498→240 for [^{14}C]M-9, 298→240 for [^{14}C]M-10 and 514→240 for postulated metabolites.

Data processing The ratio of counts of each radioactive peak to the total radioactivity counts over run time (% on HPLC) was obtained and multiplied by the extraction recovery through the pretreatment of samples to determine the compositions of metabolites to the radioactivities in the plasma, urine and feces (% in sample). The metabolite concentration in the plasma was calculated from the mean value of the total radioactivity in six subjects and expressed as equivalents of imidafenacin (ng eq. of imidafenacin/mL). The radioactivities of the metabolites excreted in the urine and feces were calculated from the mean values of the total radioactivities excreted in six subjects and expressed as percentage of the mean radioactivity administered (% of dose).

Identification of M-11 (M-11a and M-11b)

Synthesis and purification of reference products Imidafenacin was chemically oxidized in the cupro-ascorbate system to obtain the reference product for two unknown metabolites found in the urine and feces. To 20 mL each of 100 mM imidafenacin methanolic solution, 0.5 M L(+)-ascorbate solution and 5 mM copper chloride solution was added 2 L of 0.1 M phosphate buffer solution (pH 7.2), and the mixture was stirred for 24 h at room temperature. After washing thrice with 1 L of chloroform, an aqueous layer was lyophilized and

concentrated. The reference products were semi-purified as the major-constituent-rich fraction and the minor-constituent-rich fraction by sequential column chromatography with Chromatorex ODS (Fuji Silysia Chemical Ltd., Aichi, Japan) and Wakosil 25C18 (Wako Pure Chemical Industries Ltd., Osaka, Japan), eluting with the mixture of water and acetonitrile containing 0.05 or 0.1% formic acid.

Structural elucidation of reference products The reference products were dissolved in NMR grade D_2O (Sigma-Aldrich Corp., St. Louis, MO, USA) spiked with 3-(trimethylsilyl) propionic-2,2,3,3-d_4 acid, sodium salt (Sigma-Aldrich Corp.) as an internal reference for chemical shift. The ^{1}H-NMR and ^{13}C-NMR spectra were measured on the JNM EX-400 spectrophotometer (JEOL Ltd., Tokyo, Japan) for the structural elucidation of the reference products. In addition, NOE, DEPT, ^{1}H-^{1}H COSY, HMQC and HMBC spectra were also acquired for further assignment of proton and carbon signals.

The reference products were dissolved in the mixture of water and acetonitrile or D_2O and acetonitrile. Then, the solution was injected into MS/MS system comprising API-4000 equipped with ESI interface (Applied Biosystems/MDS SCIEX, Foster City, CA, USA) at the constant rate of 3 μL/min to determine precursor and product ion spectra of the reference products.

Identification of M-11 in human urine Using HPLC connected to MS/MS system

comprising API-4000 equipped with ESI interface, the retention time and product ion spectra of reference products were compared with those of unknown metabolites in human urine collected at 2-4 h after single oral administration of non-radiolabeled imidafencin. TSKgel ODS-80Ts (5 μm, 2.0 mm I.D. × 150 mm L, Tosoh Corp.) connected to a guard column was used as the semi-micro LC column. As mobile phase, the mixture of 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B) was flowed at 0.2 mL/min in the following linear gradient mode: starting with 10% of B composition to 5 min, increasing to 19% in 5 to 10 min, increasing to 26% in 10 to 30 min, finally increasing to 91% in 30 to 35 min and maintaining 91% in 35 to 40 min. The column was maintained at room temperature.

Stability of reference products The reference products were dissolved in water or 100 mM phosphate buffer (pH 7.2), and then allowed to stand at room temperature or incubated at 50°C for 5 h. After incubation, the ratio of the major and minor constituents of the reference products or their decomposed product, amidine form M-10, was measured by HPLC. TSKgel ODS-80T_M (5 μm, 4.6 mm I.D. × 150 mm L, Tosoh Corp.) connected to a guard column was used as the LC column. As mobile phase, 0.1% formic acid in water / 0.1% formic acid in acetonitrile (73:27, v/v) was flowed at 1 mL/min. The column was maintained at room temperature. The analytes were detected by ultraviolet absorption at 210 nm.

Results

Safety Assessment Six subjects were administrated a single oral dose of 0.25 mg of [^{14}C]imidafenacin. No serious adverse events were reported and no subjects were withdrawn from the study because of adverse events.

Radioactivity in Plasma The total radioactivity in the plasma is shown in Figure 2 and the pharmacokinetic parameters are summarized in Table 1. Following oral administration of 0.25 mg [^{14}C]imidafenacin, Cmax, the mean of the maximum concentration of the total radioactivity in the plasma, was 3860 pg eq./mL (from 3230 to 5210 pg eq./mL) at 1.50 h after administration (Tmax, from 1.00 to 2.00 h). Plasma concentrations rapidly decreased to the mean of 598 pg eq./mL by 24 h and then gradually decreased to 133 pg eq./mL by 168 h after administration. The mean of $T_{½}$el, estimated from 24 to 168 h after administration, was 72.11 h. The means of $AUC_{0\text{-}t}$ and $AUC_{0\text{-}inf}$ were 69.09 ng eq. h/mL (from 60.59 to 81.11 ng eq. h/mL) and 83.06 ng eq. h/mL (from 73.45 to 101.2 ng eq. h/mL), respectively.

Urinary and Fecal Excretion of Radioactivity Urinary and fecal recovery of the total radioactivity in the urine and feces is shown in Figure 3. Following oral administration of 0.25 mg [^{14}C]imidafenacin, urinary excretion accounted for 62.5% of the administered dose by 48 h after administration and the mean of 65.6% (from 62.6 to 69.3%) of the administered dose by the end of the collection period, 192 h after administration, demonstrating

radioactivity was excreted primarily in the urine. Fecal excretion accounted for the mean of 29.4% (from 27.6 to 31.3%) of the administered dose. Recovery of the total radioactivity was quantitative with the mean of 95.0% (from 91.3 to 98.3%) of the administered dose by 192 h after administration.

Metabolite Profiles HPLC radiochromatograms of imidafenacin and its metabolites in the plasma, urine and feces are shown in Figures 4, 5 and 6. The metabolites were identified by comparison of retention times between radioactive peaks and ion peaks on LC/MS/MS chromatogram. Relative amounts of metabolites in the plasma, urine, and feces are summarized in Table 2. M-6 and M-8 were not found in all samples determined.

In the 2 and 12 h plasma (Figure 4 and Table 2), the concentrations of unchanged imidafenacin were 1.19 ng eq./mL (equivalent to 33.3% of the radioactivity in the plasma) and 0.12 ng eq./mL (10.7%), respectively. In the 2 h plasma, a major metabolite M-9 was detected, and then M-2 and M-4, and a minor metabolite M-3 and a mixture of a minor metabolite M-10 and unknown metabolite HP1 (identified as M-11b) were detected. In the 12 h plasma, a major metabolite M-4 and minor metabolites M-2 and M-9 were detected.

In the 0-4 and 4-24 h urine (Figure 5 and Table 2), unchanged imidafenacin accounted for 2.9% of the administered dose (equivalent to 14.2% of the radioactivity in the urine) and 5.1% (13.9%), respectively. In the 0-4 h urine, M-2, a mixture of M-9 and an unknown metabolite HU2 (identified as M-11a), and an unknown metabolite HU3 (identified as

M-11b) were mainly detected, accounting for 2.6 to 4.8% of the dose (equivalent to 13.0 to 23.6% of the radioactivity in urine). In the 4-24 h urine, M-2, a mixture of M-9 and HU2, and HU3 were also mainly detected, accounting for 4.6 to 9.1% of the dose (12.4 to 24.9%). Minor unknown metabolite HU1 detected by SRM would be a glucuronide of mono-oxygenated imidafenacin (m/z 514→240).

In the 0-48 and 48-96 h feces (Figure 6 and Table 2), unchanged imidafenacin was not detected. In the 0-48 h feces, M-2, M-10, an unknown metabolite HF2 (identified as M-11a), and an unknown metabolite HF3 (identified as M-11b) were mainly detected, accounting for 1.0 to 3.9% of the administered dose (equivalent to 5.7 to 21.9% of the radioactivity in the feces). In the 48-96 h feces, M-2 and HF3 accounted for 1.3 and 1.2%, respectively. There were several unknown metabolite peaks in the 0-48 and 48-96 h feces accounting for 1.2% or less of the administered dose (9.5% or less).

Identification of M-11 (M-11a and M-11b) Two major unknown metabolites at 16.8 to 17.1 min (HU2 and HF2) and 18.3 to 18.9 min (HP1, HU3 and HF3) were found in the urine and feces, and were detected by SRM as identical m/z 356→240 transition (Table 2). The product ion (m/z 240) indicated ^{14}C-labeled 2,2-diphenylbutanamide moiety, which was identical with that of [^{14}C]imidafenacin; therefore, according to the precursor ion (m/z 356), 2-methyl-imidazole moiety of [^{14}C]imidafenacin would be modified by adding 34 Da to form these unknown metabolites. However, absolute amounts of these metabolites in human

urine and feces were not enough to elucidate their chemical structures. Accordingly, imidafenacin was oxidized in the cupro-ascorbate system to obtain the reference products, its oxides of 2-methyl-imidazole moiety as reported by Ohta et al. (1998). Then, the mass spectra of the reference products were compared with those of the unknown metabolites in human urine.

Structural elucidation of the reference products was achieved by analyses of one- and two-dimensional NMR spectra including ^{1}H-^{1}H COSY, HMQC, HMBC, DEPT and NOE. ^{1}H- and ^{13}C-NMR assignments for the reference products are shown in Table 3. Because those reference products were mixtures of the major and minor constituents in each fraction, signals of H-1, H-5 and H-6 protons and C-1, C-2, C-3, C-5, C-6 and C-11 carbons in the major and minor constituents were observed in the spectra of both fractions. The oxygenated 2-methyl-imidazole moiety was analyzed as follows. The presence of two oxygenated methine carbons (87.61 and 93.10 ppm) was supported by DEPT spectra. The proton signals (5.26 and 5.33 ppm) on these oxy-methine carbons were deduced from HMQC analysis, and the neighboring connectivity of these two oxy-methines was analyzed by direct coupling between protons at 5.26 and 5.33 ppm from ^{1}H-^{1}H COSY spectra. Furthermore, the HMBC correlations between three protons (H-1, H-5 and H-6) and sp^2 carbon signal at 170.8 ppm (C-11) suggested that the presence of a 5-membered ring. These findings corroborated that the reference products were obtained by the oxidation of C-5 and C-6 carbons in imidafenacin.

Product ion spectra of reference products dissolved in water and acetonitrile or D_2O and acetonitrile are shown in Figure 7. Product ion spectra of reference products (m/z 354) and deuterium-displaced reference products (m/z 359) suggested that the reference products had two deuterium-displaceable hydrogens, such as hydroxyl groups on C-5 and C-6 carbons in 2-methyl-imidazole moiety of imidafenacin, as indicated in Figure 7.

^{1}H-NMR spectra showed vicinal proton-proton coupling (J = 8.1 Hz) on doublet signals of H-5 and H-6 protons in the minor constituent. According to the Karplus equation (Karplus, 1963), the dihedral angle between vicinal protons attached to neighboring C-5 and C-6 carbons was approximately 0 degree. In contrast, H-5 and H-6 protons in the major constituent were singlet signals, indicating orthogonal protons attached to C-5 and C-6 carbons. Consequently, structures of the major and minor constituents of the reference products were estimated to be *tarns*- and *cis*-isomers of diol forms, respectively.

The LC/MS/MS chromatogram on SRM at m/z 354→238 of human urine and the reference products are shown in Figure 8. Product ion spectra of m/z 354 derived from two unknown metabolites, HU2 and HU3, in human urine, and the major and minor constituents of reference products are shown in Figure 9. The LC/MS/MS retention time and product ion spectra of HU2 and HU3 in the urine collected at 2-4 h after single oral administration of imidafenacin corresponded to those of the minor and major constituents of reference products, respectively. From these results, HU2 and HF2 were identified as *cis*-isomer (M-11a), and HP1, HU3 and HF3 were identified as *trans*-isomer (M-11b) of the diol form.

After the reference products were allowed to stand at room temperature for 5 h in water or 100 mM phosphate buffer (pH 7.2), the ratio of the minor to major constituent remained unchanged, that is approximately 30% based on the calculation of peak areas on HPLC chromatogram. After incubation at 50°C for 5 h, however, peak areas of both constituents decreased and decomposed product, amidine form M-10, increased to more than 30% of the initial peak area of both constituents. After NMR measurements, the ratio of the major constituent increased even in the minor-constituent-rich fraction. These findings suggested that the major and minor constituents might be interchangeable and gradually decomposed to amidine form under heating condition.

Discussion

In the present study, absorption and excretion kinetics and metabolic profile of imidafenacin were investigated in six healthy male subjects following a single oral administration of 0.25 mg [^{14}C]imidafenacin. Unknown metabolites found in the excreta were identified by LC/MS/MS and NMR analyses.

The highest total radioactivity in the plasma was observed at 1.50 h after administration, which was consistent with rapid absorption of the oral dose of imidafenacin in humans (Figure 2 and Table 1). These findings are supported by the results in the pre-clinical toxicological studies in rats and dogs demonstrating that the total radioactivity in the plasma peaked within 2.0 h after oral administration of [^{14}C]imidafenacin. Furthermore, 65.6% of the administered dose of radioactivity was detected in the urine (Figure 3), suggesting no fewer than 65.6% dose of imidafenacin would be absorbed from the gut. This finding is consistent with the absolute bioavailability of 57.8% after intravenous administration of imidafenacin in humans (Ohno et al., submitted 2007). The bioavailability of humans was larger than that of rat (5.3%) and dog (36.1%) (unpublished observation).

In contrast with the rapid increase in radioactivity in the plasma, the radioactivity gradually decreased from 24 to 168 h after administration, with relatively long $T_{½}$el, 72.11 h (Figure 2 and Table 1). On the metabolite profiling, major components of radioactivity were unchanged imidafenacin and M-9 (50.9% in total) in the 2 h plasma, and M-4 (18.0%) in the 12 h plasma (Figure 4 and Table 2). M-4 and related minor metabolites might

contribute to the relatively long $T_{½}$el of radioactivity in the plasma. Approximately 65.6 and 29.4% of the administered dose of [^{14}C]imidafenacin were excreted via urine and feces, respectively, within 192 h after administration (Figure 3). The total recovery of radioactivity reached 95.0% of the administered dose. In the pre-clinical toxicological studies, urinary and fecal recoveries of orally administered [^{14}C]imidafenacin were 18.2 and 77.0%, respectively in rats, and 44.8 and 51.6%, respectively in dogs (unpublished observation). From these data, orally administered imidafenacin was thoroughly excreted via urine and feces in all of the species studied, in spite of difference in main excretion route among the species.

After oral administration to humans, imidafenacin underwent different metabolic transformations, including N-glucuronide conjugation (M-9), oxidation of the 2-methyl-imidazole moiety (M-1, M-2, M-11a and M-11b) or the ring cleavage (M-3, M-4, M-5 and M-10), the metabolic activity for N-glucuronidation was, in particular, found to be relatively high. The metabolic pathway of imidafenacin for N-glucuronidation, however, was uncommon in rats and dogs, and rats have the unique metabolic pathway for the oxidation on the phenyl moiety, generating M-8 and its related metabolites (unpublished observation). Species difference concerning N-glucuronidation was also observed in Afloqualone in common with imidafenacin, indicating high N-glucuronidation activity in humans compared with activity in the experimental animals except rabbits (Kaji and Kume 2005, Green and Tephly 1998).

Based on the metabolites found in the plasma, major metabolites of imidafenacin were M-2, M-4 and M-9, accounting for ca. 10% or more of the total radioactivity in the 2 or 12 h plasma (Figure 4 and Table 2). It has been reported that M-2, M-4 and M-9 had low affinities for muscarinic receptors, whereas unchanged imidafenacin had high affinity for muscarinic receptor subtypes M_1 and M_3 (Kobayashi et al. 2007a). These findings suggest that the *in vivo* anticholinergic effects of orally administered imidafenacin would result from unchanged form alone. In addition, toxicological symptoms attributable to M-2 and M-4 after oral administration of imidafenacin and M-9 after intravenous administration of M-9 were insignificant in the pre-clinical studies (unpublished observation), and inhibitory potentials of these metabolites on CYP isozymes were extremely low (Kanayama et al. 2007). In the meantime, because imidafenacin was metabolized by UGT1A4 to form M-9, and CYP3A4 to form M-2 and M-4, metabolism of imidafenacin might be inhibited by concomitant administration of drugs with inhibitory potential on CYP3A4 or UGT1A4 (Kanayama et al. 2007).

We identified interchangeable diol-form metabolites, M-11a and M-11b, in the urine and feces (Figure 7). Because the minor constituent of diol-form metabolite, *cis*-isomer (M-11a), was readily interchangeable to the major constituent, *trans*-isomer (M-11b), up to ca. 30% of the *trans*-isomer was found in the minor-constituent-rich fraction. Furthermore, M-11a and M-11b decomposed to amidine form M-10 under heating condition. However, M-10 was rarely detected in human plasma, urine and feces, probably because the samples

for metabolite profiling were treated under cooling condition during storage and pretreatment throughout the study. Similar metabolic pathway has been reported in a hypoglycaemic argent, midaglizole. That is, midaglizole was oxidized and hydrated at 2-substituted-imidazole moiety to form 4,5-dihydro-diol intermediate, though stereochemistry was unknown, and then amidine metabolites were obtained by ring-opening reaction (Nakaoka et al. 1987). However, this intermediate metabolite was not isolated and identified owing to its instability. In contrast, 4,5-dihydro-diol metabolites of imidafenacin, M-11a and M-11b, were relatively stable in the biological samples during storage and pretreatment, while their absolute amounts were too small to isolate for structural elucidation. Therefore, M-11a and M-11b were successfully identified by obtaining the reference product from imidafenacin with oxidation in the cupro-ascorbate system.

In conclusion, the present study clarified the absorption and excretion kinetics of imidafenacin and the characteristics of metabolites in the plasma and excreta in six healthy male subjects following a single oral administration of 0.25 mg [^{14}C]imidafenacin. The results indicate that imidafenacin is rapidly and well absorbed following oral administration and circulates in the plasma as the unchanged form, its glucuronide conjugate and other metabolites, then excreted primarily in the urine and feces as the oxidized metabolites of 2-methyl-imidazole moiety. For treatment of overactive bladder, imidafenacin is expected to provide suitable anticholinergic effects through the excellent pharmacokinetic properties in humans verified in the present study.

Acknowledgment

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Legends for Figures

Figure 1. Chemical structures of [^{14}C]imidafenacin and postulated metabolites, and their fragment ions.

Asterisk denotes ^{14}C labeled position.

Broken line denotes precursor and product ions obtained by collision–induced dissociation in LC/MS/MS.

Figure 2. Total radioactivity in plasma after a single oral administration of 0.25 mg [^{14}C]imidafenacin in six healthy male subjects.

Each point represents the mean ± standard deviation of six individuals.

Figure 3. Urinary and fecal recovery of total radioactivity in urine and feces after a single oral administration of 0.25 mg [^{14}C]imidafenacin in six healthy male subjects.

Each point represents the mean ± standard deviation of six individuals.

Figure 4. HPLC radiochromatograms of imidafenacin and its metabolites in pooled plasma after a single oral administration of 0.25 mg [^{14}C]imidafenacin in six healthy male subjects.

Figure 5. HPLC radiochromatograms of imidafenacin and its metabolites in pooled urine

after a single oral administration of 0.25 mg [^{14}C]imidafenacin in six healthy male subjects.

Figure 6. HPLC radiochromatograms of imidafenacin and its metabolites in pooled feces after a single oral administration of 0.25 mg [^{14}C]imidafenacin in six healthy male subjects.

Figure 7. Product ion spectra of reference products obtained by the cupro-ascorbate oxidation system with or without deuterium-displacement.

Figure 8. LC/MS/MS chromatograms on SRM at m/z 354→238 of human urine and reference products obtained from imidafenacin

Figure 9. Product ion spectra of m/z 354 derived from unknown metabolites, HU2 and HU3, in human urine, and major and minor constituents of reference products obtained from imidafenacin

Table 1 Pharmacokinetic parameters of total radioactivity in plasma after a single oral administration of 0.25 mg [^{14}C]imidafenacin in six healthy male subjects.

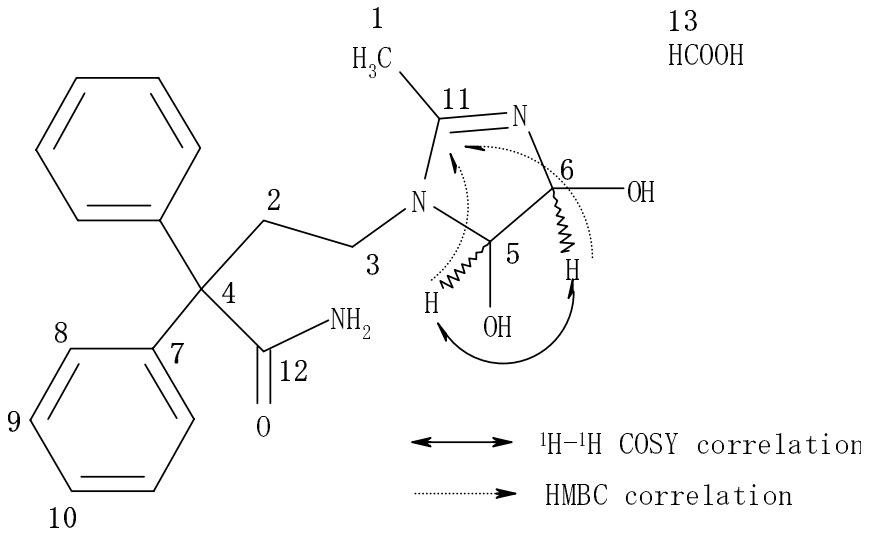
Parameters	Mean	S.D.
Cmax (pg eq./mL)	3860	723
Tmax (h)	1.50	0.45
$T_{½}$el (h)	72.11	6.54
$AUC_{0\text{-}t}$ (ng eq. h/mL)	69.09	7.63
$AUC_{0\text{-}inf}$ (ng eq. h/mL)	83.06	10.18

Table 2 Compositions of imidafenacin and its metabolites in plasma, urine and feces after a single oral administration of 0.25 mg [^{14}C]imidafenacin in healthy male subjects.

Metabolites	Mass transition (m/z)	Retention time (min)	Plasma (ng eq./mL)		Urine (% of dose)		Feces (% of dose)	
			2h	12h	0-4h	4-24h	0-48h	48-96h
Imidafenacin	322→240	21.3-21.7	1.19 (33.3)	0.12 (10.7)	2.9 (14.2)	5.1 (13.9)	–	–
M-1	338→240	23.7	–	–	0.3 (1.4)	0.2 (0.5)	–	–
M-2	354→240	26.9-27.0	0.34 (9.5)	0.09 (7.6)	2.6 (13.0)	4.6 (12.4)	1.8 (9.9)	1.3 (10.9)
M-3	356→240	22.6-22.8	0.15 (4.1)	–	0.3 (1.3)	0.7 (1.9)	–	–
M-4	328→193	28.7-28.8	0.34 (9.4)	0.21 (18.0)	0.1 (0.5)	1.3 (3.6)	–	–
M-5	329→193	24.6-24.7	–	–	0.5 (2.6)	1.8 (5.0)	–	–
M-9	498→240	16.8-17.1	0.63 (17.6)	0.11 (9.9)	4.8 [a] (23.6)	9.1 [a] (24.9)	–	–
M-10	298→240	18.7-19.4	0.28 [b] (7.9)	–	0.7 (3.7)	0.7 (1.9)	1.0 (5.7)	0.6 (4.7)
HU2, HF2 (M-11a)	356→240	16.8-17.1	–	–	4.8 [a] (23.6)	9.1 [a] (24.9)	1.1 (6.2)	0.3 (2.8)
HP1, HU3, HF3 (M-11b)	356→240	18.3-18.9	0.28 [b] (7.9)	–	4.6 (22.6)	5.5 (14.9)	3.9 (21.9)	1.2 (9.7)
HU1	514→240	15.2-15.3	–	–	1.1 (5.6)	2.0 (5.4)	–	–
HF1	–	14.7-15.0	–	–	–	–	0.6 (3.3)	0.2 (1.4)
HF4	–	20.2-20.3	–	–	–	–	0.3 (1.7)	0.8 (6.4)
HF5	–	21.5	–	–	–	–	0.6 (3.3)	0.6 (4.8)
HF6	–	22.2	–	–	–	–	0.9 (5.1)	0.2 (1.9)
HF7	–	22.7-22.9	–	–	–	–	0.2 (0.9)	0.2 (1.5)
HF8	–	34.2-34.3	–	–	–	–	0.9 (4.8)	1.2 (9.5)
Others			NC (2.7)	NC (27.3)	2.3 (11.5)	5.7 (15.6)	6.6 (22.8)	5.7 (30.8)
Total			3.57 (84.5)	1.16 (73.5)	20.2 (100)	36.7 (100)	17.9 (85.6)	12.3 (84.4)

Figures in parentheses denote % of total radioactivity in samples.
[a] Total amount of M-9 and HU2
[b] Total amount of M-10 and HP1
– : not detected; NC : not calculated

Table 3 Chemical shifts of protons and carbons in ^{1}H-NMR and ^{13}C-NMR spectra of major (M-11b) and minor (M-11a) constituents of reference products obtained from imidafenacin



a) ^{1}H-NMR data (in D_2O)

Position of proton	Major constituent (M-11b)	Minor constituent (M-11a)
1	2.18 [a] (3H [b], s [c])	2.11 [a] (3H [b], s [c])
2	2.82 (2H, m)	2.79 (2H, m)
3	3.45 (2H, m)	3.41 (2H, m)
5	5.26 (1H, s)	5.43-5.47 [d] (1H, d, J = 8.1 [e])
6	5.33 (1H, s)	5.43-5.47 [d] (1H, d, J = 8.1 [e])
8, 9 and 10	7.39-7.49 (10H, m)	7.40-7.48 (10H, m)
13	8.45 (1.3H, s)	8.45 (2H, s)

[a] Chemical shifts are reported in ppm.
[b] Intensities are represented as number of proton.
[c] Multiplicity; s: singlet; d :doublet; m: multiplet.
[d] These proton signals could not be distinguished.
[e] Coupling constants (J) are given in Hz.

b) ^{13}C-NMR data (in D_2O)

Position of carbon	Major constituent (M-11b)	Minor constituent (M-11a)
1	14.61 [a]	14.48 [a]
2	39.04	38.49
3	43.49	42.90
4	61.95-61.99 [b]	61.95-61.99 [b]
5	87.61	80.22 or 85.78 [b]
6	93.10	80.22 or 85.78 [b]
7	144.2-144.4 [b]	144.2-144.4 [b]
8, 9 and 10	130.7-131.8 [b]	130.7-131.8 [b]
11	170.8	169.5
12	181.9-182.2 [b]	181.9-182.2 [b]
13	173.8	173.8

[a] Chemical shifts are reported in ppm.
[b] These carbon signals could not be distinguished.

Figure 1

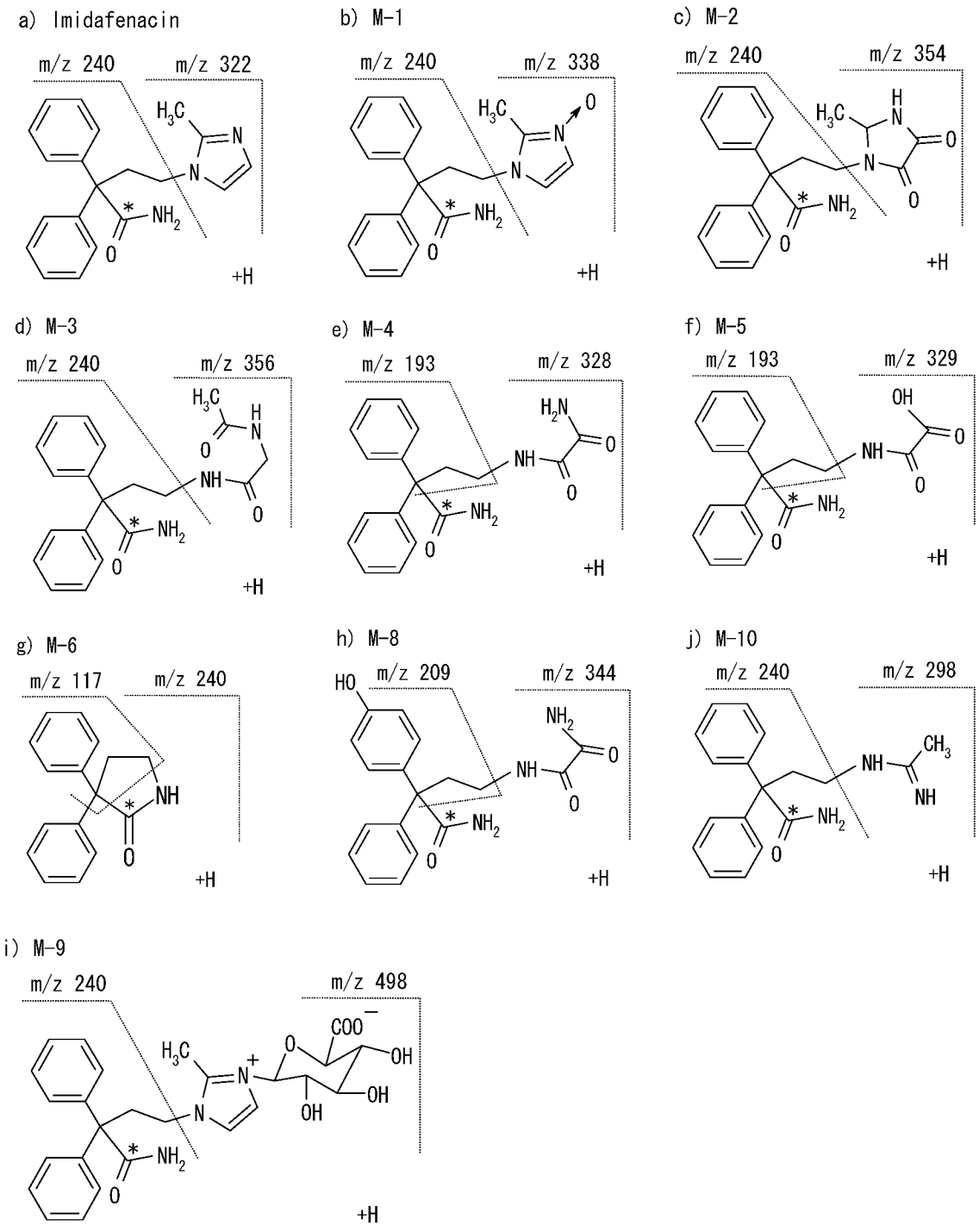

a) Imidafenacin
m/z 240
m/z 322
b) M-1
m/z 240
m/z 338
c) M-2
m/z 240
m/z 354
d) M-3
m/z 240
m/z 356
e) M-4
m/z 193
m/z 328
f) M-5
m/z 193
m/z 329
g) M-6
m/z 117
m/z 240
h) M-8
m/z 209
m/z 344
j) M-10
m/z 240
m/z 298
i) M-9
m/z 240
m/z 498
+H

Figure 2

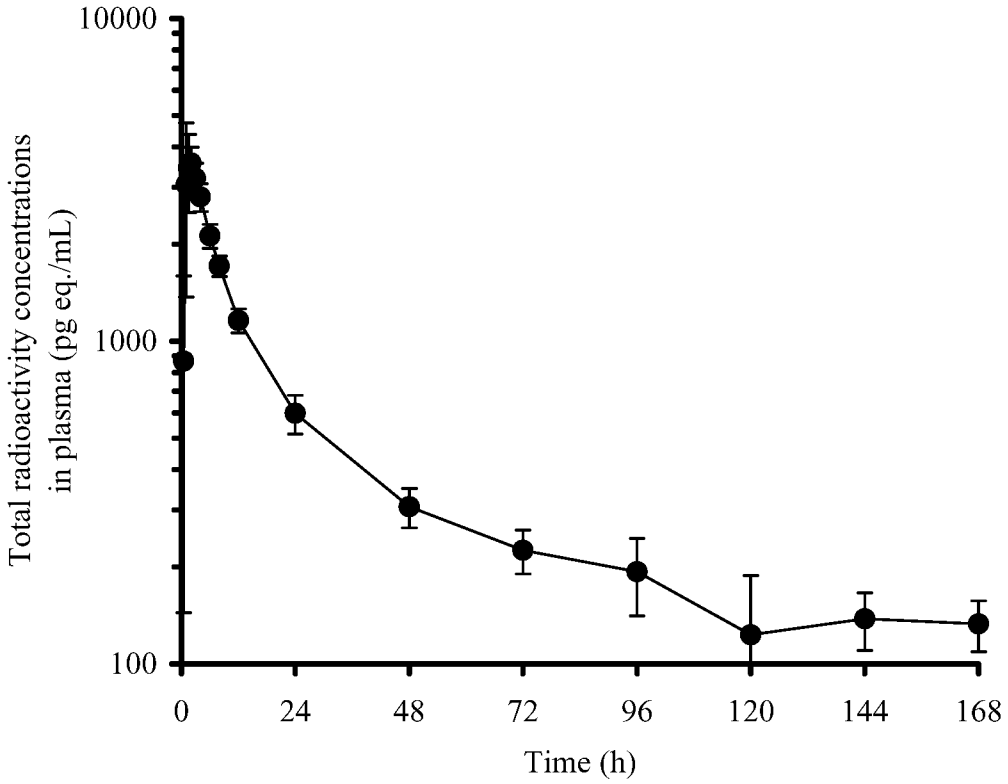

10000
1000
100
Total radioactivity concentrations in plasma (pg eq./mL)
0
24
48
72
96
120
144
168
Time (h)

Figure 3

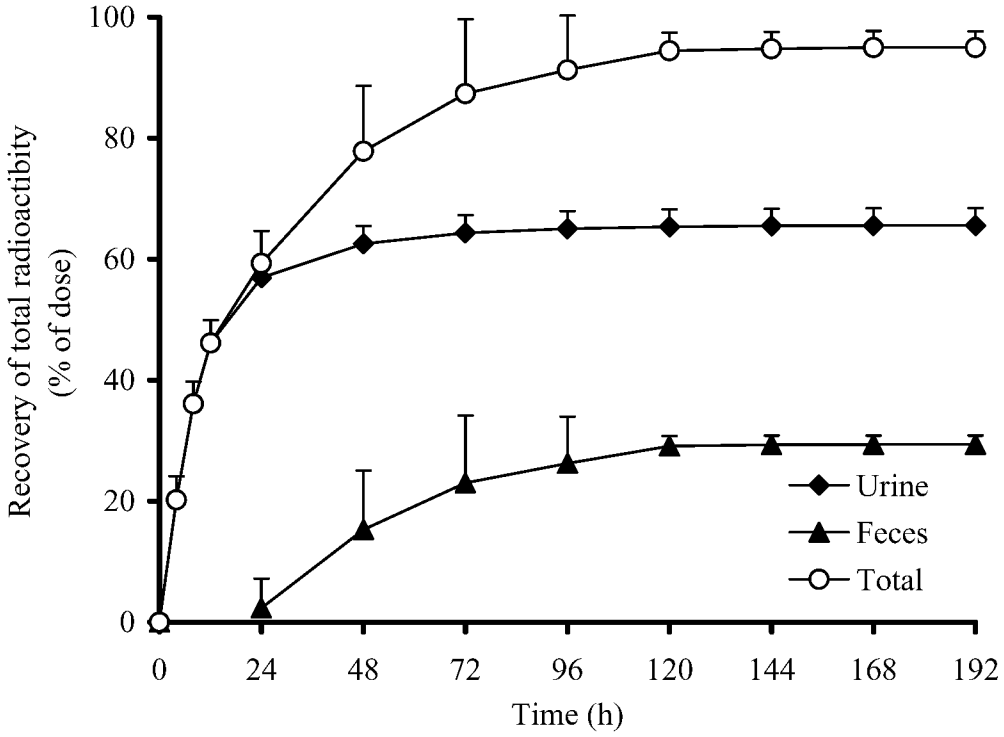

Recovery of total radioactibity
(% of dose)
100
80
60
40
20
0
0
24
48
72
96
120
144
168
192
Time (h)
Urine
Feces
Total

Figure 4

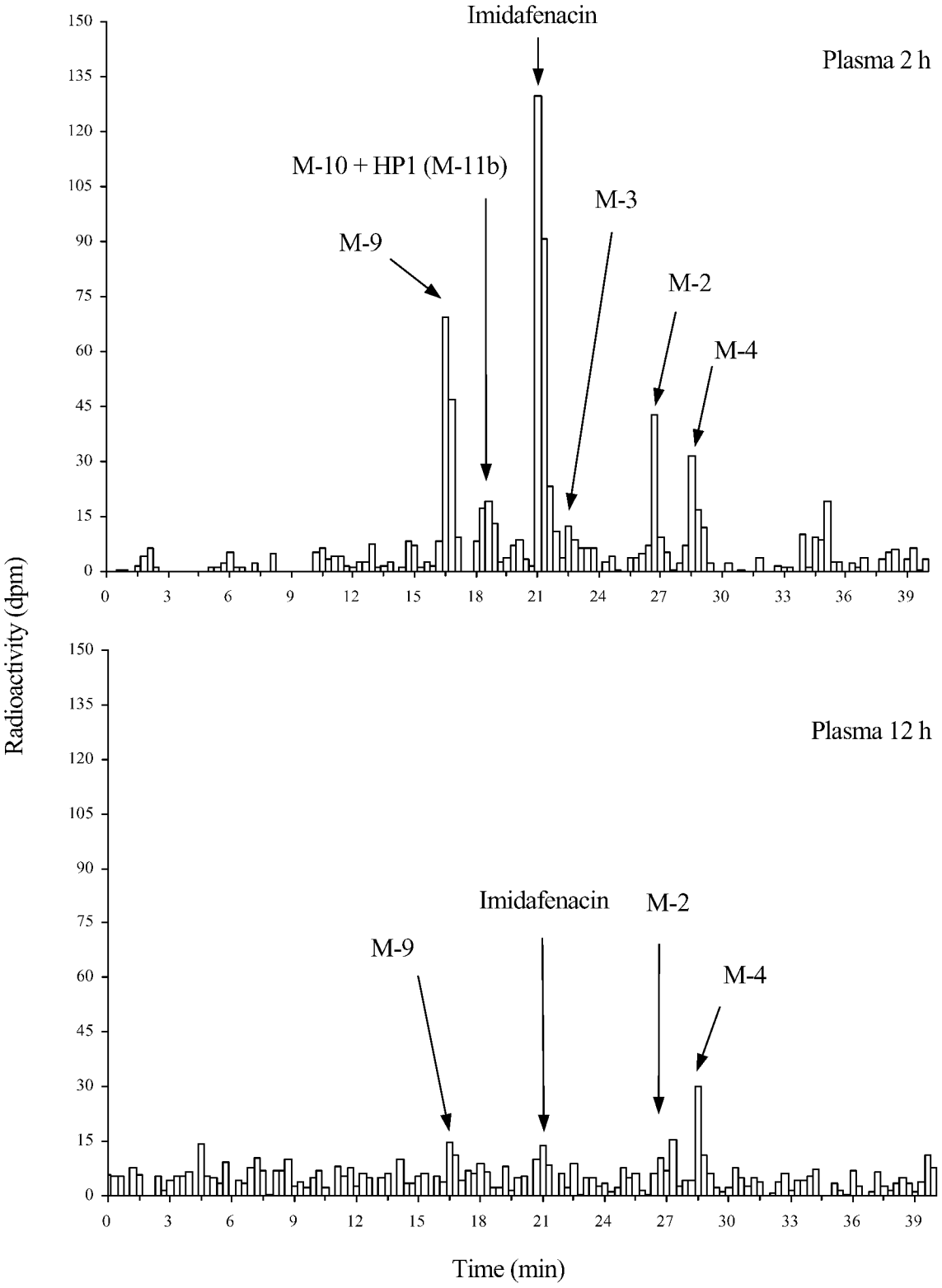

Imidafenacin
Plasma 2 h
M-10 + HP1 (M-11b)
M-3
M-9
M-2
M-4
Plasma 12 h
Imidafenacin
M-2
M-9
M-4
Radioactivity (dpm)
Time (min)
0
15
30
45
60
75
90
105
120
135
150
0
3
6
9
12
15
18
21
24
27
30
33
36
39

Figure 5

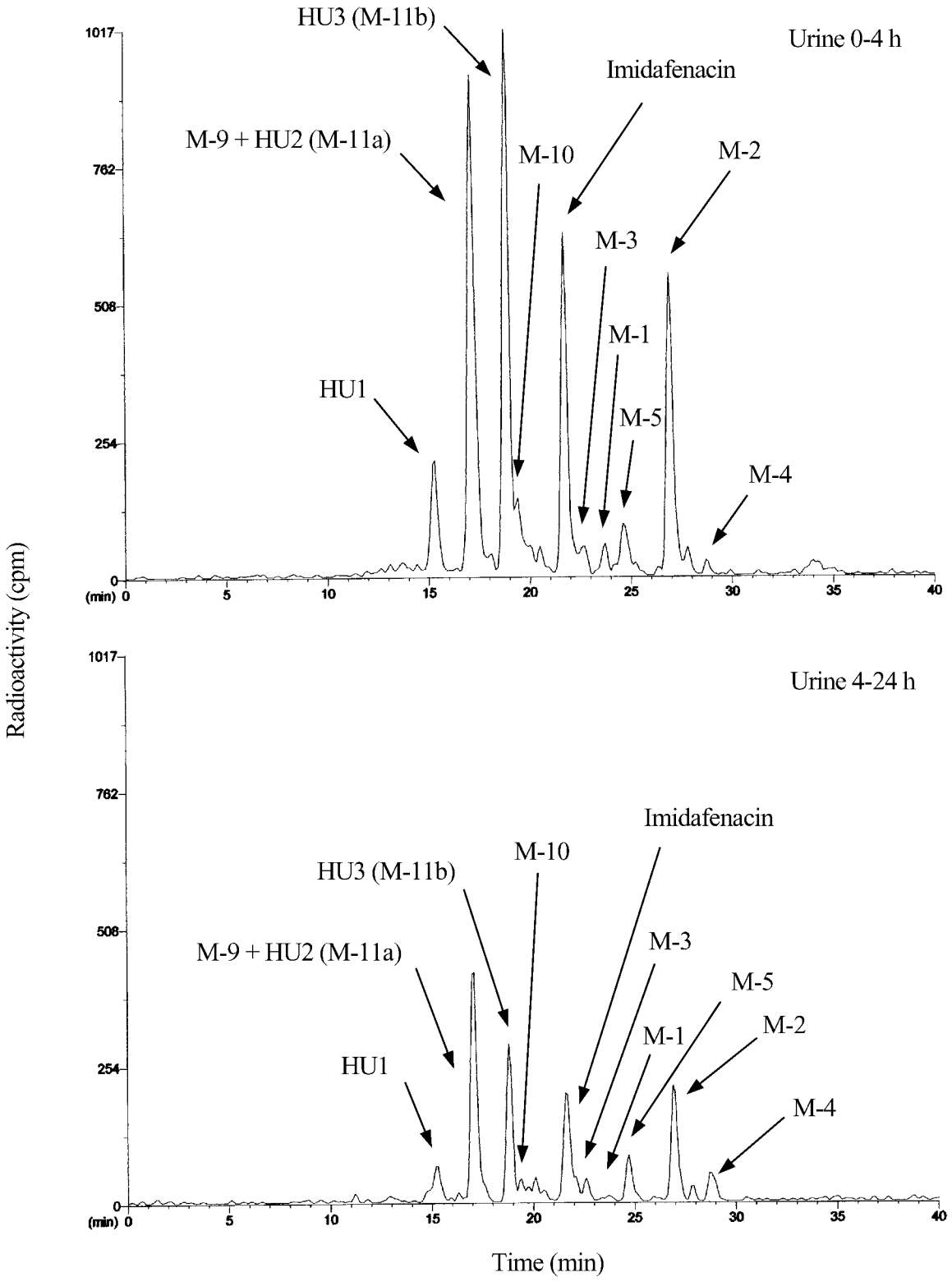

HU3 (M-11b)
Urine 0-4 h
Imidafenacin
M-9 + HU2 (M-11a)
M-10
M-2
M-3
M-1
HU1
M-5
M-4
1017
762
508
254
0
(min) 0
5
10
15
20
25
30
35
40
Urine 4-24 h
Imidafenacin
M-10
HU3 (M-11b)
M-3
M-9 + HU2 (M-11a)
M-5
M-1
M-2
HU1
M-4
Radioactivity (cpm)
Time (min)

Figure 6

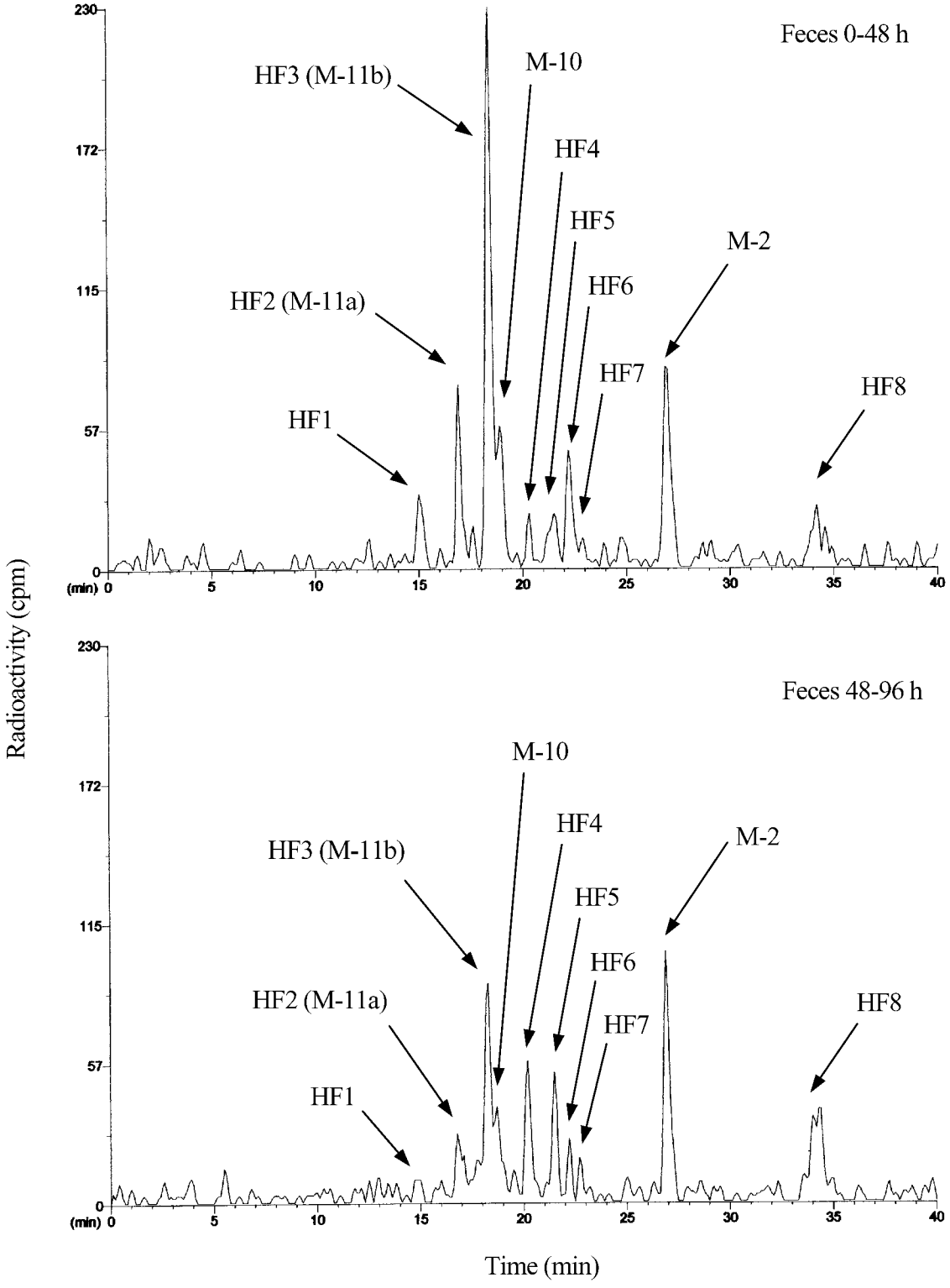
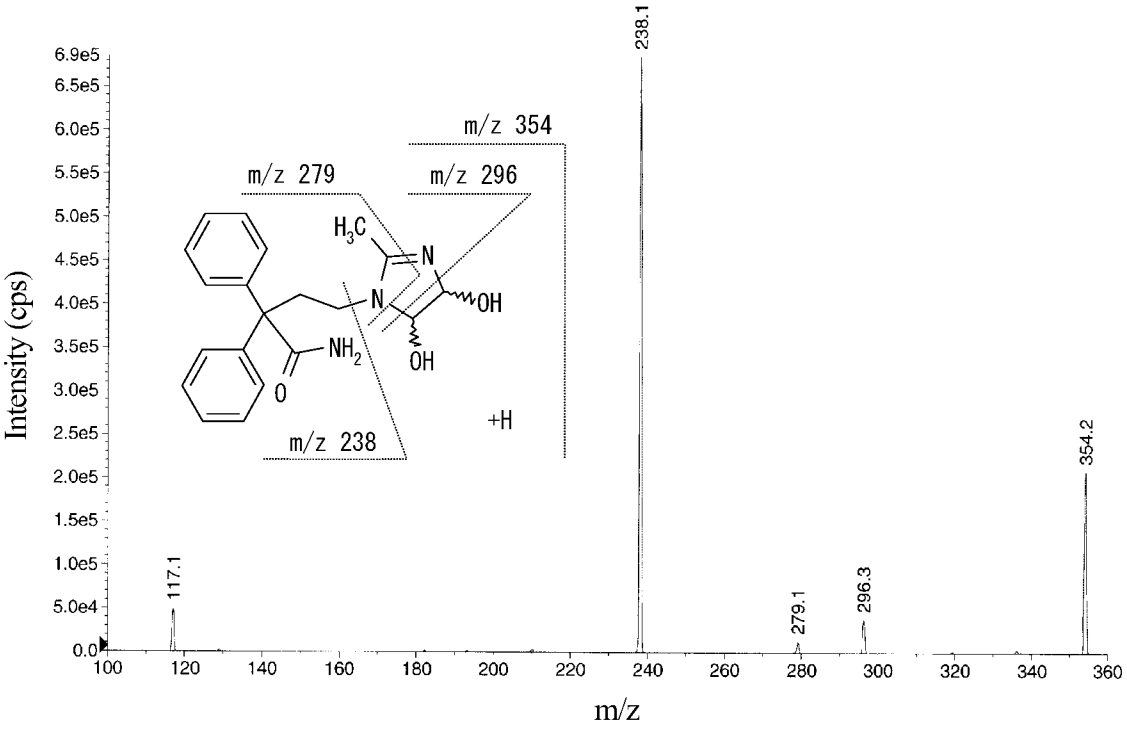

Feces 0-48 h
HF3 (M-11b)
M-10
HF4
HF5
M-2
HF6
HF2 (M-11a)
HF7
HF8
HF1
230
172
115
57
0
(min) 0
5
10
15
20
25
30
35
40
Feces 48-96 h
M-10
HF4
M-2
HF3 (M-11b)
HF5
HF6
HF2 (M-11a)
HF7
HF8
HF1
Radioactivity (cpm)
Time (min)

Figure 7

a) Reference products (m/z 354)



b) Deuterium-displaced reference products (m/z 359)

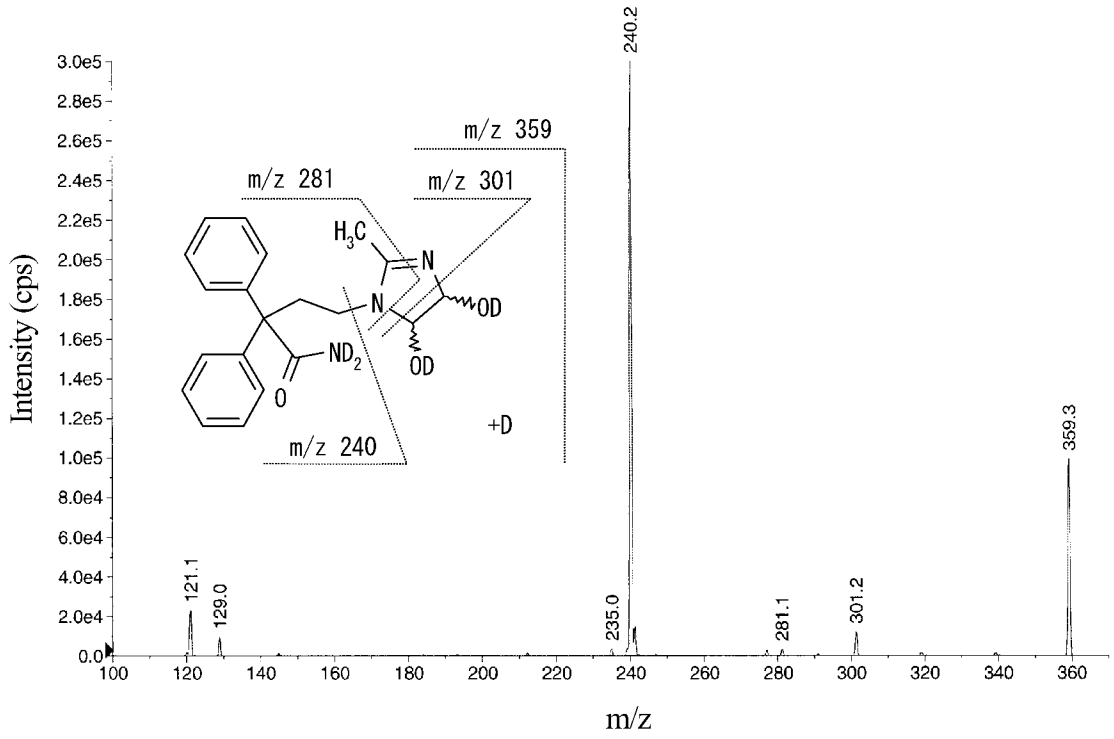


Figure 8

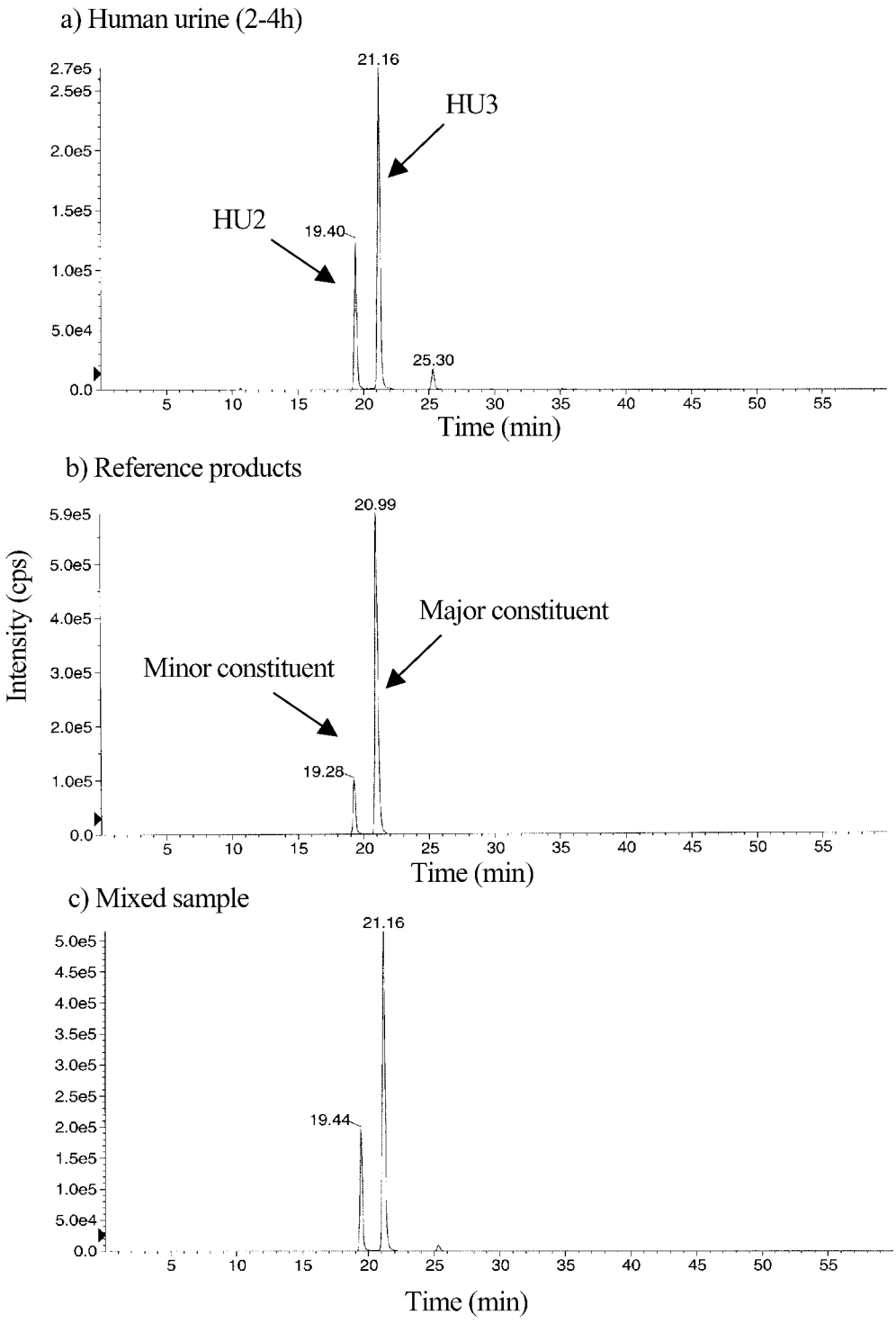
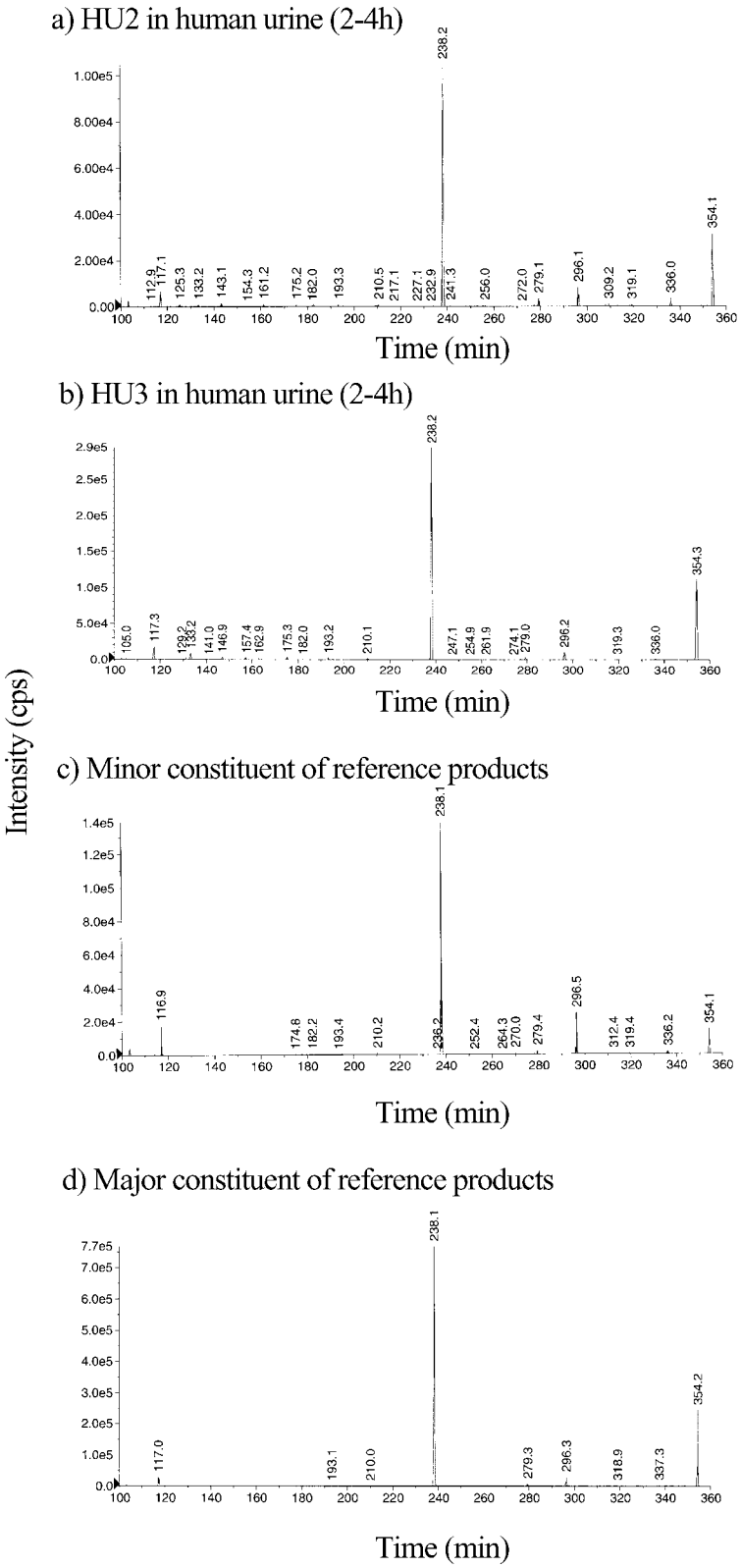

a) Human urine (2-4h)
21.16
HU3
HU2
19.40
25.30
Time (min)
b) Reference products
20.99
Major constituent
Minor constituent
19.28
Intensity (cps)
Time (min)
c) Mixed sample
21.16
19.44
Time (min)

Figure 9


a) HU2 in human urine (2-4h)
b) HU3 in human urine (2-4h)
c) Minor constituent of reference products
d) Major constituent of reference products
Intensity (cps)
Time (min)