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METABOLISM AND DISPOSITION OF A POTENT AND SELECTIVE GABA-A$_{\alpha 2/3}$ RECEPTOR AGONIST IN HEALTHY MALE VOLUNTEERS

Stacey L. Polsky-Fisher, Stanley Vickers, Donghui Cui [1], Raju Subramanian [2], Byron H. Arison, Nancy G. B. Agrawal, Thanh V. Goel, Laura K. Vessey, M. Gail Murphy, Kenneth C. Lasseter , Richard C. Simpson, Jose M. Vega [3], and A. David Rodrigues [4]

Drug Metabolism, Merck Research Laboratories, West Point, PA and Rahway (S.P.F., S.V., D.C., R.S., B.H.A., N.G.B.A., T.V.G., R.C.S., A.D.R.); Clinical Pharmacology, Merck Research Laboratories, Blue Bell, PA (L.K.V., M.G.M., J.M.V.); Clinical Pharmacology Associates, Miami, FL (K.C.L.)

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Abbreviations: GABA, gamma-aminobutyric acid; GAD, general anxiety disorder; HPLC, high-performance liquid chromatography; LC-MS/MS, liquid chromatography tandem mass spectrometry; CID, collision induced dissociation; UDPGA, uridine 5'-diphosphoglucuronic acid; ^{1}H-NMR, proton nuclear magnetic resonance spectroscopy; TOCSY, total correlation spectroscopy; ROESY, rotating Overhauser spectroscopy; C_{max}, maximum concentration in plasma; T_{max}, time of occurrence of maximum concentration in plasma; AUC, area under the plasma concentration *versus* time curve; %CV, coefficient of variation.

Corresponding Author: Stacey L. Polsky-Fisher, M.S.
Department of Drug Metabolism
WP75B-200
Merck Research Laboratories
West Point PA 19486
Tel: 215 652 5741
Fax: 215 993 1245
E-mail: stacey_polsky@merck.com

ABSTRACT

[^{14}C]TPA023 (99 μCi/dose) was administered to five young, healthy, fasted male subjects as a single oral dose (3.0 mg) in solution (propylene glycol/water, 10/90 v/v). The parent compound was rapidly absorbed (plasma T_{max} ~2 hr), exhibited an apparent terminal half-life of 6.7 hours, and accounted for approximately 53% of the total radioactivity in plasma. After seven days of collection, the mean total recovery of radioactivity in the excreta was 82.6%, with 53.2% and 29.4% in urine and feces, respectively. Radiochromatographic analysis of the excreta revealed that TPA023 was metabolized extensively, and only trace amounts of unchanged parent were recovered. Radiochromatograms of urine and feces showed that TPA023 underwent metabolism via three pathways (*t*-butyl hydroxylation, *N*-deethylation and direct *N*-glucuronidation). The products of *t*-butyl hydroxylation and *N*-deethylation, together with their corresponding secondary metabolites, accounted for the majority of the radioactivity in the excreta. Additionally, approximately 10.3% of the dose was recovered in urine as the triazolo-pyridazine *N*1-glucuronide of TPA023. The *t*-butyl hydroxy and *N*-desethyl metabolites of TPA023, the TPA023 *N1*-glucuronide, and the triazolo-pyridazine *N*1-glucuronide of *N*-desethyl TPA023 were present in plasma. In healthy male subjects, therefore, TPA023 is well absorbed, is metabolized extensively (*t*-butyl hydroxylation and *N*-deethylation > glucuronidation), and the metabolites are excreted in urine and feces.

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Gamma-aminobutyric acid (GABA) is the major inhibitory neurotransmitter in the brain and is essential for the overall balance between neuronal excitation and inhibition (Young and Chu, 1990; Chebib and Johnston, 2000). GABA influences neurons via three classes of receptors, $GABA_A$ and $GABA_C$, which are ligand gated ion channels, and $GABA_B$ which is a G-protein coupled receptor (Chebib and Johnston, 2000). The most widely studied of these, both in terms of its molecular biology and pharmacology, is the $GABA_A$ receptor. $GABA_A$ receptors are heteromeric pentamers of subunits expressed by a large multigene family, currently consisting of 6α, 3β, 3γ, δ, ε, θ, 3ρ and π genes (Graham et al., 1996; Johnston, 1996; Barnard et al., 1998; Chebib and Johnston, 2000; McKernan et al., 2000). Modulation of the $GABA_A$ receptor has a number of effects on behavior and is the site of action of a number of drugs, including ethanol, neurosteroids, general anesthetics, barbituates and benzodiazepines, which mediate their action through binding sites on various subunits.

Generalized Anxiety Disorder (GAD) is a disease state characterized by excessive anxiety and persistent worry for a period of six months or more (Syramek et al., 2002). The ideal agent for the treatment of GAD would have a rapid onset of action, elicit few adverse effects and show no tolerance (Syramek et al., 2002). Benzodiazepines, which modulate their activity by binding to the α1,2,3 and 5 subunits of the $GABA_A$ receptor and increase the potency of GABA by increasing the frequency of channel openings in response to GABA binding (Chebib and Johnston, 2000), are the most commonly prescribed drugs used to treat GAD. They are extremely effective anxiolytics with a rapid onset of action, although several adverse effects limit their use including sedation, impaired psychomotor performance, memory impairment and the potential for abuse and

dependency (Syramek et al., 2002). However, not all of these effects are mediated through the same α subunits and therefore novel subunit specific $GABA_A$ receptor modulators may result in compounds with an improved side effect profile (McKernan, et al., 2000; Rudolph, et al., 2001; Blackaby, et al., 2006; Goodacre et al., 2006)

TPA023 is being developed as an anxiolytic for use in the treatment of GAD. The compound behaves as a potent $GABA_A$ receptor agonist with functional selectivity towards α_2 and α_3 receptor subtypes (Atack et al., 2006; Carling et al., 2006; Goodacre et al., 2006; Jennings, et al., 2006; Russell, et al., 2006). In animal models, it is an efficacious anxiolytic that lacks the side effects experienced with more non-selective $GABA_A$ receptor agonists. The objectives of the present study were: 1) to investigate the routes of elimination of TPA023 in healthy subjects following a single oral dose; 2) to quantitate the levels of total radioactivity and TPA023 in plasma after the single oral dose; 3) to examine the metabolism of TPA023 in humans; and 4) to demonstrate mass balance for [^{14}C]TPA023 in humans.

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MATERIALS AND METHODS

Chemicals. [^{14}C]TPA023 (>99% radiochemical purity) and authentic standards of TPA023, M11 (*t*-butyl hydroxy), M15 (aldehyde), M10 (carboxylic acid), M12 (*N*-desethyl metabolite), M9 (alcohol of *N*-desethyl metabolite), M14 (*O*-dealkylation), and M5 (*N*1-glucuronide of TPA023) were synthesized at Merck Research Laboratories. All other reagents were purchased from Sigma Chemical Co. (St. Louis, MO), or Aldrich Chemical Co. (Milwaukee, WI). Pooled human liver microsomes were purchased from Xenotech LLC (Kansas City, KS).

Human Studies. The study protocol was approved by the Southern Institutional Review Board (Miami, FL). All subjects understood the procedures and agreed to participate in the study by giving written informed consent (Declaration of Helsinki). No concomitant medication was taken during the study.

[^{14}C]TPA023 (99 μCi, 3 mg; 0.05 mg/mL) was administered to five healthy fasted male subjects as a single oral dose in solution (propylene glycol/water; 10/90, v/v). The dose solution was followed by two separate rinses of water (125 mL), which were completely ingested within 2 minutes of dose consumption. Thereafter, heparinized blood was collected at intervals through 168 hours (7 days). The plasma was separated and stored at -20°C. Urine and feces were collected at various intervals for 7 days and stored at -20°C.

Measurement of Total Radioactivity. Aliquots of plasma and urine (0.2 to 1.0 mL) were transferred to polyethylene vials and 15 mL of scintillation cocktail (Ready-Safe, Beckman Instruments, Somerset, NJ) was added for counting in a Packard 2500 TR liquid scintillation counter (Packard Instrument Co., Meriden, CT). Aliquots of the fecal

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homogenate and red blood cells (2 x ~0.2 g) were combusted in a Packard robotic tissue oxidizer (Model 307). The resultant carbon dioxide was trapped in a Carbosorb/Permafluor mixture (Packard Instrument Co). Radioactivity in all samples was determined with a Packard 2500 TR liquid scintillation counter.

LC-MS/MS Analysis of Plasma for TPA023. Plasma samples were centrifuged (3000 rpm, 5 minutes) in a Beckman GS-6KR refrigerated centrifuge (Beckman Coulter Inc., Fullerton, CA) and a 1-mL aliquot of the supernatant was transferred to clean test tubes. To each sample tube was added 1:1 acetonitrile/water (50 μL), internal standard working solution (0.05 μg/mL MRK-2 in 1:1 acetonitrile /water, 50 μL), and 0.15 M phosphate solution (pH 2; 1-mL). The tubes were vortexed after each addition. An Ansys SPEC Plus® 96 well solid phase extraction plate (MP3 Mixed Phase Cation, 15 mg; Varian, Inc., Palo Alto, CA) was conditioned with methanol (800 μL), water (800 μL), and 0.1 M phosphate buffer (pH 2; 800 μL), each drawn through the plate wells with low negative pressure (< 10 inches Hg). Two 1-mL aliquots of each tube were then added to the plate wells followed by the addition of water (1-mL), 1.0 M acetic acid (800 μL), and methanol (800 μL), each drawn through with low negative pressure (< 10 inches Hg). After removing any excess wash solvent, the plate was placed onto a disposable microtiter tray and centrifuged for 5 minutes at 1000 rpm to remove any residual liquid from the plate. The plate was then placed on top of a 650 μL 96-position deep-well sample collection plate and 300 μL of 78/20/2 (v/v/v) methylene chloride/isopropanol/ammonium hydroxide was added into each well. The assembly was then centrifuged for 5 minutes at 1000 rpm to elute the analyte and internal standard into the collection plate. The eluate was evaporated to dryness under nitrogen using an

EvapArray evaporator (Jones Chromatography, Lakewood, CO) set at 40 ^{o}C. The residues were reconstituted with 100 μL of reconstitution solvent (85:15 v/v 10 mM ammonium acetate: acetonitrile, v/v), and the plate sealed, vortexed, sonicated, vortexed a second time, and placed into a Perkin Elmer series 200 autosampler tray for analysis.

The samples were run on a Perkin Elmer LC series 200 system (Norwalk, CT). TPA023 and the internal standard, MRK-2, were separated on a Restek Ultra IBD column (30 x 4.6 mm, 3 micron) with a mobile phase eluting at a flow rate of 1 mL/minute. The mobile phase consisted of 35 % acetonitrile in 10 mM ammonium acetate (pH 4) held isocratically over the 5 minute run time. Under these conditions, [^{14}C]TPA023 and [^{12}C]TPA023 co-eluted at a retention time of 2.1 minutes.

The HPLC was interfaced with a SCIEX API 3000 tandem mass spectrometer (Applied Biosystems, Foster City, CA) equipped with a heated nebulizer interface. Mass spectral analyses were carried out in the positive ion mode. The heated nebulizer probe temperature was 500 ^{o}C. Tandem mass spectrometry (MS/MS) monitoring of the precursor/product ion transitions was carried out. The precursor → product ion combinations of *m/z* 396 → 287, 398 → 289, and *m/z* 362 → 334 were used to quantify [^{12}C]TPA023, [^{14}C]TPA023, and the internal standard, respectively.

Using this procedure, the assay for the total concentration of TPA023 in human plasma was validated in the range of 0.1 to 50 ng/mL. The precision (%CV) was better than 6% at all concentrations within the standard curve range, except at the lower limit of quantitation (10.1% at 0.1 ng/mL). Accuracy was within 5% of nominal at all standard concentrations.

Preparation of Plasma, Urine, and Fecal Samples for Metabolite Profile Analysis. *Plasma:* For each subject, aliquots of plasma were pooled (based on total C-14 in sample) to produce a representative 0.5 to 8 hour sample for each subject. The plasma proteins in the samples were precipitated by the addition of four volumes of acetonitrile while vortexing vigorously, followed by centrifugation (3000 rpm, 10 minutes). The supernatants were transferred to clean tubes, and an aliquot (0.5 or 1-mL) was taken for liquid scintillation counting. Recovery of radioactivity after extraction was > 90%. The supernatants were evaporated to dryness in a centrifugal vacuum concentrator (Speed-Vac, Forma Scientific Inc., Marietta, OH). The residues were reconstituted in 300 µL of 30% acetonitrile in water, and the solutions were transferred to autosampler vials for radiochromatography. The injection volume was 60 µL for HPLC-MS/MS radiochromatography.

Urine: Representative 0- to 48 hour samples for each subject were obtained by combining aliquots from each time interval (up to and including 48 hour) in proportion to their respective volumes. A 0.1 or 0.2-mL aliquot of the pooled mixture was taken for scintillation counting. To concentrate the urine samples prior to radiochromatography, aliquots of the 0-24 hour (1 or 2-mL) and 24-48 hour (5-10-mL) urine samples were transferred to glass centrifuge tubes, and three volumes of acetonitrile was added while vortexing vigorously. The samples were then treated as the plasma samples. The reconstituted samples were transferred to autosampler vials for radiochromatography (100 µL injection volume). The remaining material was submitted for evaluation by mass spectrometry.

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Feces: To concentrate the fecal samples prior to radiochromatography, aliquots (1, 2, or 10-mL) were transferred to glass centrifuge tubes and prepared for analysis as the plasma and urine samples. One hundred μL aliquots of the resulting supernatant were taken for liquid scintillation counting. Recovery of radioactivity was acceptable (>60%).

Radiochromatographic Analysis of Plasma, Urine, and Fecal Extracts. The above samples were subjected to HPLC on a Perkin Elmer LC 235C system. The metabolites were separated on a Zorbax RX C_8 column (4.6 x 250 mm, 5 micron; Agilent Technologies, Palo Alto, CA) with mobile phase eluting at a flow rate of 1 mL/minute. A linear gradient started at 10% acetonitrile in 25 mM ammonium formate (pH 3) and increased to 50% acetonitrile over 30 minutes followed by a 10 minute isocratic run at 50% acetonitrile for a total run time of 40 minutes. A post-run time of 5 minutes under starting mobile phase conditions permitted re-equilibration of the column. The effluent was monitored using a photodiode array detector (260 and 300 nm) and by an in-line radiochemical detector (β-RAM Model 2B, INUS Systems, Inc., Tampa, FL). A 3 mL/minuet flow rate was used for the scintillation cocktail (total flow rate of 4 mL/minute through the radiochemical detector).

Incubation of Urine with β-Glucuronidase. Aliquots of the pooled urine (0-24 hour) were transferred to 13 x 100 mm test tubes and dried under nitrogen in a Pierce N-Evaporator (Rockford, IL). The tube contents were resuspended in 1 mL of 0.2 M sodium phosphate buffer (pH 7.0*)* and *E. Coli* β-glucuronidase enzyme (1000 Units Type VII-A; Aldrich, Milwaukee, WI) was added to each tube. A second set of control samples were prepared to which no enzyme was added. All the samples were incubated overnight (~17 hours) at 37°C in a shaking water bath. The reactions were stopped with

the addition of five volumes of acetonitrile and the samples were prepared for analysis as described above. The reconstituted samples were sonicated and transferred to autosampler vials for radiochromatography (100 μL injection volume). The remaining material of selected samples was submitted for evaluation by mass spectrometry.

LC-MS/MS Analysis of Plasma, Urine, and Fecal Extracts. Samples were injected onto a Hewlett-Packard HP1100 gradient high-performance liquid chromatography system (Agilent Technologies) interfaced to a Finnigan TSQ 7000 tandem mass spectrometer (Thermo, San Jose, CA) and a β-RAM radiochemical detector in parallel. Ten percent of the HPLC effluent was split into the mass spectrometer. Metabolite separation was carried out on a Phenomenex Luna C_{18} (2) column (4.6 m X 25 cm, 5 micron; Torrance, CA) at a constant flow rate of 1 mL/minute. A linear gradient starting at 10% of 0.1% formic acid in acetonitrile in 25 mM ammonium formate (pH 3) was maintained for five minutes, increased to 50% of 0.1% formic acid in acetonitrile over 30 minutes, and followed by a 10 minute isocratic run at 50% of 0.1% formic acid in acetonitrile for a total run time of 45 minutes. A post-run of 3 minutes at 90% of 0.1% formic acid in acetonitrile was run prior to the next injection.

Mass spectral analyses were carried out with electrospray ionization (ESI) in the positive ion mode. The capillary temperature was 220 ^{o}C and the ESI ionizing voltage was maintained at 5.0 kV for all analyses. Tandem mass spectrometry (MS/MS) was based on collision-induced dissociation (CID) of ions entering the rf-only octapole region where argon was used as the collision gas at a pressure of 1.7 mtorr. A combination of collision offsets, ranging from –25 eV to –45 eV, were used for all MS/MS analyses.

Incubation of *N*-Desethyl TPA023 (M12) with UDPGA-Fortified Human Liver Microsomes. Incubations were performed at 37°C in a shaking water bath, employing 12 x 75 mm borosilicate glass disposable culture tubes. Briefly, the final assay volume was 1.0 mL and consisted of 0.1 M potassium phosphate buffer (pH 7.4), magnesium chloride (10 mM), D-saccharic acid 1,4 lactone (1.25 mM), microsomal protein (1.0 mg/mL), and alamethicin (25 μg). The reactions were initiated by addition of UDPGA (4 mM) and M12 (40 μM) dissolved in DMSO (<1% v/v final volume in assay) after a 3 minute pre-incubation period (37°C, open to air) and then stopped by addition of 1 mL acetonitrile after a 6 hour incubation (37°C, open to air). In each case, the samples were centrifuged for 10 minutes at 3000 rpm and the supernatant was transferred into clean tubes. After evaporation to dryness under nitrogen, the residue was reconstituted in 400 μL of 10% acetonitrile in 25 mM ammonium formate (pH 3).

A series of injections were made onto an HPLC system (using the radiochromatography conditions described above) interfaced with a Foxy fraction collector (ISCO Inc., Lincoln, NE). The effluent was collected as 30-second fractions (1 mL/min). Fractions corresponding to the retention times of the glucuronides tubes were pooled and concentrated for NMR analysis.

NMR Analysis of Microsomal Metabolites. NMR experiments were performed on a 500 MHz spectrometer (Inova, Varian Inc., Palo Alto) fitted with a 3 mm inverse detection probe (MIDG-3 probe, Nalorac, Martinez, CA). Samples of M12 and each of the isolated metabolite fractions were dissolved in DMSO-d_6 and NMR data sets were acquired at 25 °C. All ^{1}H chemical shifts are reported on the δ scale (parts per million) downfield from tetramethyl silane using the DMSO-d_6 lock signal for reference at 2.51

ppm. One dimensional, two dimensional total correlation spectroscopy (TOCSY, to trace through bond connectivity; Summers et al, 1986), and two dimensional transverse rotating Overhauser spectroscopy (ROESY, to show through space proximity, Hwang and Shaka 1992) data sets were acquired for all of the M12 metabolites.

Identification and Quantitation of Metabolites in Plasma, Urine and Feces. Authentic standards of TPA023 and its metabolites (M5, M9, M10; M11, M12, and M14) were available to support the characterization of TPA023 metabolites in urine, feces and plasma by similarity of chromatographic retention time and their respective UV and MS spectra. The *N*-glucuronide conjugates (M2, M6, and M7) of M12, generated in human liver microsomes (structures confirmed by NMR) also served as standards. For each sample of urine and fecal homogenate, all metabolites were quantitated based on the percent of total radioactivity for each peak observed.

Pharmacokinetic Methods. Plasma concentrations of TPA023 and radioactivity were used to estimate pharmacokinetic parameters for each subject. The C_{max} and T_{max} values were obtained by inspection of the concentration-time data. The apparent terminal $t_{1/2}$ was estimated from the best-fit parameters of a single exponential to the log-linear portion of the plasma concentration-time curve. The best-fit parameters were obtained using nonlinear regression by the Simplex algorithm with weight = 1 (Yeh and Remphrey, 1990). The area under the plasma concentration-time curve (AUC_{∞}) was calculated using the linear trapezoidal method up to the last measured concentration. The additional area was estimated from the last measured concentration and the value of apparent terminal rate constant.

RESULTS

Absorption and Excretion of [^{14}C]TPA023. The mean concentration-time profiles of TPA023 and radioactivity in plasma following oral administration are shown in Fig. 2, and the geometric mean AUC ratio of TPA023-to-total radioactivity (95% CI) was 0.53 (0.47, 0.62), indicating that metabolites accounted for an appreciable amount of the radioactivity in plasma (data not shown). The single oral dose was rapidly absorbed and the mean plasma T_{max} and C_{max} for TPA023 were 2.0 hr and 28 ng/mL, respectively (Table 1). The harmonic mean apparent terminal $t_{1/2}$ was 6.7 hour. The mean plasma T_{max} and C_{max} for the total radioactivity in plasma were 2.2 hour and 42.9 ng/mL, respectively (Table 2). The harmonic mean apparent terminal $t_{1/2}$ was 7.7 hour.

The mean total recovery of total radioactivity in the excreta after 7 days was 82.6% (Table 3 and Fig. 3). Excretion was primarily via the renal route, with 53.2% of the dose eliminated in urine and 29.4% recovered in the feces. The majority of the urinary and fecal radioactivity was recovered within 48 and 96 hour, respectively.

Metabolism of [^{14}C]TPA023. Metabolites of TPA023 were identified based on protonated molecular ions and their CID fragmentation. A number of TPA023 ($[M+H]^+$ion at *m/z is* 396) metabolites were observed in plasma, urine and fecal extracts and they were identified by LC-MS/MS. The major fragmentation pathway of TPA023 occurs between the carbon and the oxygen at the ethyl triazole methoxy moiety, which gives rise to two major fragments; *m/z* 287 (from the substituted triazole pyridazine region of the molecule) and *m/z* 110 (ethyl triazole ring).

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The retention times (t_R), $[M+H]^+$ion at *m/z*, and major CID fragments are listed in Table 4. The t_R and CID fragmentations of M2, M6, and M7 were similar to those obtained with M2, M6, and M7 isolated from human liver microsomes (see below).

^{1}H NMR data of M2 determined it to be a conjugate at nitrogen *N*1 in the triazolo-pyridazine ring. The anomeric proton of the glucuronide appears as a doublet with a scalar coupling constant of 8.8 Hz at 5.95 ppm. Correlations are seen from proton 8 in the triazolo-pyridazine moiety to glucuronide anomeric protons and also to the *t*-butyl protons in the ROESY spectrum. Metabolite M6 was determined to be a conjugate at nitrogen *N4* of the triazole moiety on the bottom portion of M12 by NMR. In the ROESY spectrum, cross peaks are seen from the anomeric proton of the glucuronide (at 5.21 ppm, doublet with J = 9.5 Hz) to the methylene connected to the triazole**,** the triazole at proton 3 and the *t*-butyl group protons. *M7* was determined to be a conjugate at nitrogen *N1* of the triazole by NMR. The anomeric proton of the glucuronide (at 5.39 ppm, doublet with J=8.9 Hz) shows cross peaks to bridge methylene proton to the triazole, and the *t*-butyl group protons in the ROESY spectrum.

A representative radiochromatogram of 0 to 24 hour pooled urine is shown in Figure 4A, and mass spectral analysis revealed the presence of 12 metabolites. Only trace amounts of parent compound were detected. The triazolo-pyridazine *N*1-glucuronide (M5) and *t*-butyl hydroxy (M11) derivatives of TPA023 were identified as the most abundant metabolites, accounting for 10.3 and 14% of the dose in 0 to 48 hour urine, respectively (Table 5). Two additional metabolites (M1 and M2) were not resolved radiochromatographically and together accounted for a further 10.8% of the dose present in the urine. However, M1 and M2 were resolved mass spectrally and were

identified as the glucuronide of M14 (*O*-dealkyl TPA023) and M12 (*N*-desethyl TPA023 glucuronidated at the *N*1 of the triazolo-pyridazine moiety), respectively. In addition, glucuronides M3, M4, M5, M6, M7 and M8 wee identified in the urine. Individually, none of these metabolites accounted for more than 4% of the dose. Collectively, however, they represented about 8% of the dose. In addition to the *t*-butyl hydroxy metabolite of TPA023 (M11), three other aglycones (M9, M10, and M12) were detected in urine. M9 (1.4% of the dose) was identified as *t*-butyl hydroxy/*N*-desethyl TPA023. M10 (3.0% of the dose) was identified as the carboxylic acid product resulting from the oxidation of the *t*-butyl moiety. M12 (0.3% of the dose) was identified as *N*-desethyl TPA023. *In toto*, the data indicated that up to 30% of the dose was recovered in 0 to 48 hr urine as the glucuronide conjugates of TPA023 and its oxidative metabolites (Table 5). In agreement, only five metabolites (M9, M10, M11, M12 and M14) were detected in urine following overnight hydrolysis with β-glucuronidase (Fig. 4B). These data showed that TPA023 was largely cleared by oxidative metabolism involving *t*-butyl hydroxylation and *N*-deethylation (Fig. 5).

Fecal extracts were also subjected to radio-LC/MS analysis and only five metabolites were detected (Fig 6A). Qualitatively, the pattern of metabolism was similar to that obtained with urine after hydrolysis. This is to be expected, as the metabolites would be secreted into bile and hydrolyzed by gut bacteria on route to the lower colon. As in hydrolyzed urine, *t*-butyl hydroxy (M11) and *N*-desethyl (M12) metabolites of TPA023 were present in the feces and accounted for 7.8 and 4.8% of the dose, respectively (Table 6). The carboxylate of TPA023 (M10) and *t*-butyl hydroxy-*N*-desethyl metabolite (M9) respectively accounted for a further 8.5% and 3.6% of the dose.

The product of fluoro phenyl ring hydroxylation (M13) appeared unique to feces, but was a relatively minor metabolite (0.9% of the dose).

Plasma samples, pooled over the interval from the first time point through 8 hr, were subjected to radio-LC/MS analysis also (Fig 6B). In agreement with the data presented in Fig. 2, TPA023 was the major component and comprised the majority of the total radioactivity present therein. In addition, the triazolo-pyridazine *N*1-glucuronide of M12 (M2), the *t*-butyl hydroxy (M11), *N*-desethyl (M12) and triazolo-pyridazine *N*1-glucuronide (M5) metabolites of TPA023 were also observed in the plasma.

DISCUSSION

The purpose of this study was to investigate the absorption, disposition, and mass balance of [^{14}C]TPA023 in healthy male volunteers following a single 3 mg oral dose. Mass balance was reasonably achieved, with total recovery of radioactivity averaging 82% of the dose. Recovery of radioactivity averaged 53% of dose in urine, and 29% in feces. The results of this study also showed that radiolabeled TPA023 was well absorbed following administration of an oral solution in propylene glycol as indicated by the large fraction of the dose (53%) recovered in the urine.

The plasma concentration-time profiles following administration indicated that TPA023 accounted for the majority of the radioactivity in plasma (~53%, based upon AUCs), and qualitatively, it appeared as if the remainder of the radioactivity was roughly equally divided between four metabolites (Fig 6B). One of the metabolites (*N*-desethyl, M12) is pharmacologically active in vitro, exhibiting similar affinity and efficacy to parent compound at GABA-A $\alpha_{2/3}$ subunits. However, this metabolite along with *t*-butyl hydroxy (M11), have been shown to be poorly brain penetrant in rats. In fact, the brain-to-plasma ratios are substantially lower than those of parent (~0.4 vs. ~0.02) (B. Sohal and A. Pike, unpublished). The remaining two metabolites (M2 and M5), triazolo-pyridazine *N*1 glucuronides, also would not be expected to be brain penetrant. Therefore, it is anticipated that the metabolites of TPA023 would not contribute to the overall pharmacological activity of the compound.

Analysis of the excreta demonstrated that TPA023 is metabolized extensively, with less than 1% of the administered oral dose recovered unchanged. Therefore, renal excretion plays a minor role in the clearance of TPA023. In contrast, the primary

metabolites of TPA023 (*t*-butyl hydroxy and *N*-desethyl), and their respective metabolites, are largely excreted in the urine. The absence of parent in the feces, not only indicates good absorption, but also indicates that the triazolo-pyridazine *N*1-glucuronide (M5) of TPA023 is cleared via the urine not the bile. Only a relatively small amount of the dose (10%) is recovered in the urine as the *N*-glucuronide of parent compound, and in the absence of biliary secretion, no enterohepatic recirculation would be expected. In agreement, there was no evidence for secondary peaks in the plasma profiles (Fig. 2).

The observation that *t*-butyl hydroxylation and *N*-deethylation are major pathways is in accord with the results of in vitro metabolism studies. The in vitro data show that these two pathways are operative when TPA023 is incubated in the presence of NADPH-fortified human liver microsomes (S. L. Polsky-Fisher, unpublished). In vitro data also show that the oxidation of TPA023 is catalyzed exclusively by members of the CYP3A subfamily, and other cytochromes P450 (e.g., CYP2C9, 2C8, 2C19, 2D6, and 1A2) play a minor role. The *N*-glucuronidation of TPA023 in vitro is catalyzed by recombinant UGT1A4 only and was highly correlated with imipramine *N*-glucuronidation in a bank of human liver microsomes (S. L. Polsky-Fisher, unpublished).

Because the oxidative metabolism of TPA023 is catalyzed by members of the CYP3A subfamily only, the clearance of TPA023 can be attenuated by potent CYP3A inhibitors such as ketoconazole and itraconazole. In agreement with this hypothesis, itraconazole elicits a clinically significant effect on the oral pharmacokinetics of TPA023 (~5-fold increase in plasma AUC) (N.G.B. Agrawal, unpublished). In this regard, TPA023 is similar to agents such as zolpidem, triazolam and midazolam (Greenblatt et

al., 1998; Yasui et al., 1998; Varhe et al., 1994; Olkkola et al., 1994). Moreover, it is highly likely that the clearance of TPA023 will be increased in the presence of CYP3A inducing agents such as rifampicin and St John's Wort (Schmider et al., 1999; Backman et al., 1996; Villikka et al., 1997; Wang et al., 2001).

Based on the results of this study, it is concluded that TPA023, following a single oral dose, is absorbed rapidly and substantially and is metabolized extensively in human subjects (Fig 5). *t*-Butyl hydroxylation and *N*-deethylation represent the major routes of clearance. These metabolites are either excreted directly or are metabolized further to secondary metabolites that are also excreted (urine > feces). In comparison to oxidative metabolism, only a relatively minor fraction of the dose (~10%) is cleared via direct glucuronidation at the *N1* position of the triazolo-pyridazine moiety.

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FOOTNOTES

Address correspondence to: Stacey L. Polsky-Fisher, M.S.

Department of Drug Metabolism

Merck Research Laboratories

West Point PA 19486

E-mail: stacey_polsky@merck.com

[1] Current address: Biotransformation Department, Bristol-Myers Squibb Pharmaceutical Research Institute, Princeton, NJ 08543; [2] Current address: Department of Drug Metabolism and Pharmacokinetics, Amgen, Thousand Oaks, CA 91320; [3] Current address: Department of Global Clinical Development General Medicine, Amgen, Thousand Oaks, CA 91320; [4] Current address: Department of Drug Metabolism and Pharmacokinetics, Bristol-Myers Squibb Pharmaceutical Research Institute, Princeton, NJ 08543

FIGURE LEGENDS

Figure 1. *Structure of TPA023.*

Asterisk indicates position of carbon-14 label.

Figure 2. *Mean concentrations of TPA023 (ng/mL) and total radioactivity (ng.eq./mL) in plasma following administration of a single oral 3 mg (99 μCi) dose of [^{14}C]TPA023 to five healthy male volunteers*

Figure 3. *Recovery of radioactivity in urine and feces.*

Samples were collected for 7 days following administration of a single oral dose of [^{14}C] TPA023 to five healthy male volunteers

Figure 4. *Representative radiochromatograms of pooled urine extracts (0-24 hr) before (A) and after (B) overnight incubation with β-glucuronidase. The subject had received a single oral 3 mg (99 μCi) dose of [^{14}C]TPA023.*

See Table 4 for a detailed description of the metabolites of TPA023.

Figure 5. *Proposed metabolic pathways of TPA023 in healthy male volunteers.*

Figure 6. *Representative radiochromatograms of pooled 0-96 hr feces (A) and pooled (0.5-8 hr) plasma (B) of a human subject following a single oral 3 mg (99 μCi) dose of [^{14}C]TPA023.*

See Table 4 for a detailed description of the metabolites of TPA023.

TABLE 1

Calculated pharmacokinetic parameters of TPA023 following a single oral 3 mg (99 μCi) dose of [^{14}C]TPA023

Subject No.	C_{max}[a] *ng/mL*	t_{max}[b] *hr*	$t_{1/2}$[c] *hr*	$AUC_{0\ to\ inf}$ *ng*hr/mL*
007	32.8	2.0	5.2	238.8
008	26.5	2.0	10.4	339.1
009	29.3	2.0	6.9	277.3
010	29.1	2.0	5.3	241.8
011	24.5	2.0	5.5	187.2
Mean	28	2.0	6.7	257
SD	3.0	0.0	2.2	56
CV (%)[d]	11.0	0.0	33.2	21.8

[a] C_{max}, maximum plasma concentration

[b] t_{max}, time at maximum concentration

[c] $t_{1/2}$, terminal half-life

[d] CV, coefficient of variation

TABLE 2

Calculated pharmacokinetic parameters of plasma radioactivity following a single oral 3 mg (99 μCi) dose of [^{14}C]TPA023

Subject No.	C_{max}[a] *ng eq/mL*	t_{max}[b] *hr*	$t_{1/2}$[c] *hr*	$AUC_{0\ to\ inf}$ *ng eq*hr/mL*
007	49.8	2.0	6.0	503.5
008	37.7	3.0	10.8	593.4
009	48.0	2.0	7.9	587.1
010	41.9	2.0	7.0	392.0
011	37.2	2.0	6.9	365.6
Mean	42.9	2.2	7.7	488.3
SD	5.8	0.4	1.9	106.5
CV (%)[d]	13.5	20.3	24.0	21.8

[a] C_{max}, maximum plasma concentration

[b] t_{max}, time at maximum concentration

[c] $t_{1/2}$, terminal half-life

[d] CV, coefficient of variation

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TABLE 3

Recovery of radioactivity in urine and feces of five healthy male subjects following a single oral 3 mg (99 μCi) dose of [^{14}C]TPA023 [a]

Sample	Interval (hr)	Percent of Dose Recovered: Subject Number 007	008	009	010	011	Mean	S. D.
Urine	0-2	4.5	1.7	2.4	3.9	2.5	3.0	1.1
	2-4	5.9	3.9	6.4	8.7	7.9	6.6	1.9
	4-6	8.6	4.6	7.5	7.7	6.4	7.0	1.5
	6-12	20.2	14.0	12.4	8.1	15.6	14.1	4.4
	12-24	10.5	13.3	14.3	17.7	13.0	13.7	2.6
	24-48	5.7	10.3	7.2	7.4	4.1	6.9	2.3
	48-72	1.3	3.9	1.2	0.8	0.9	1.6	1.3
	72-96	0.2	0.8	0.2	0.1	0.1	0.3	0.3
	96-120	0	0.1	0.1	0	0	0	0
	120-144	0	0	0	0	0	0	0
	144-168	0	0	0	0	0	0	0
	Sub Total	**57.0**	**52.5**	**51.7**	**54.4**	**50.5**	**53.2**	**2.6**
Feces	0-24	0.4	n.s. [b]	n.s. [b]	3.1	12.9	5.5	6.6
	24-48	4.8	6.1	11.6	3.3	5.5	6.3	3.2
	48-72	6.5	15.2	13.3	n.s. [b]	33.4	14.6	13.5
	72-96	7.6	2.8	8.7	2.2	1.4	4.5	3.4
	96-120	1.1	2.4	2.0	0.4	0.2	1.2	1.0
	120-144	0.6	0.4	0.4	0.1	0.2	0.3	0.2
	144-168	0.1	0.1	0.2	0.1	0	0.1	0.1
	Sub Total	**21.1**	**27.0**	**36.0**	**9.1**	**53.6**	**29.4**	**16.7**
Urine and Feces	**Total**	**78.1**	**79.5**	**87.7**	**63.5**	**104.1**	**82.6**	**14.9**

[a] [^{14}C]TPA023 was administered as an oral solution in propylene glycol/ water (10/90, v/v) at a concentration of 0.05 mg/mL.

[b] n.s. = no sample collected

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TABLE 4

Chromatographic Retention Times and Mass Spectral Characteristics of TPA023 Metabolites identified in urine, feces, and plasma of five healthy male subjects following a single oral 3 mg (99 μCi) dose of [^{14}C]TPA023

Metabolites of TPA023	Description of Metabolites	Retention time (t_R) (min)	*m/z* of $[M+H]^+$ ion	*m/z* of major CID fragments
M1	Glucuronidation of M14[a]	11	463	287
M2	Glucuronidation of M12	11	544	287, 368
M3	Glucuronidation of M11[a]	11.5	588	110, 285, 382, 412
M4	Glucuronidation of M9[a]	12.5	560	82, 285, 354, 384
M5	Glucuronidation of TPA023	14.5	572	110, 287, 396
M6	Glucuronidation of M12	16	544	287, 368
M7	Glucuronidation of M12	17.2	544	287, 368
M8	Glucuronidation of M11[a]	17.2	588	110, 285, 382, 412
M9	*t*-Butyl hydroxylation and *N*-desethylation of TPA023	18.5	412	82, 285, 354, 384
M10	Carboxylic acid of TPA023	21.5	426	110, 271, 299, 382
M11	*t*-Butyl hydroxylation of TPA023	23	412	110, 275, 287, 382
M12	*N*-desethylation of TPA023	27	368	82, 273, 287
M13	Hydroxylation of TPA023	27.7	412	110, 303
M14	*O*-dealkylation of TPA023	34.5	287	228, 271

[a] Site of glucuronidation was not determined

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TABLE 5

Quantitation of TPA023 metabolites in human urine (0-48 hr) following a single oral 3 mg (99 μCi) dose of [^{14}C]TPA023

Metabolites of TPA023 [b]	Percent of Dose Recovered [a]					
	Subject 007	Subject 008	Subject 009	Subject 010	Subject 011	Mean ± SD
M1 M2	13.8	8.9	11.9	9.7	9.5	10.8 ± 2.0
M3	1.8	1.3	1.6	1.6	1.9	1.6 ± 0.2
M4	0.5	0.5	0.4	0.7	0.6	0.5 ± 0.1
M5	11.6	10.7	9.6	7.6	12.1	10.3 ± 1.8
M6	1.6	2.9	1.7	3.6	2.6	2.5 ± 0.9
M7 M8	3.4	4.2	2.9	4.0	4.6	3.8 ± 0.7
M9	1.3	2.3	1.6	1.1	0.9	1.4 ± 0.6
M10	2.4	1.8	3.4	4.5	2.8	3.0 ± 1.0
M11	14.6	14.0	14.2	15.7	11.6	14.0 ± 1.5
M12	0.2	0.3	0.4	0.3	0.3	0.3 ± 0.1
Total	**51.2**	**46.9**	**47.6**	**48.6**	**46.9**	**48.3 ± 1.8**

[a] The mean (n=5) percent of the dose in urine (0-48 hr) was 48.3 ± 1.8%. Therefore, 91% of the excreted radioactivity in urine is accounted for.

[b] See Table 4 for detailed description of metabolites of TPA023

TABLE 6

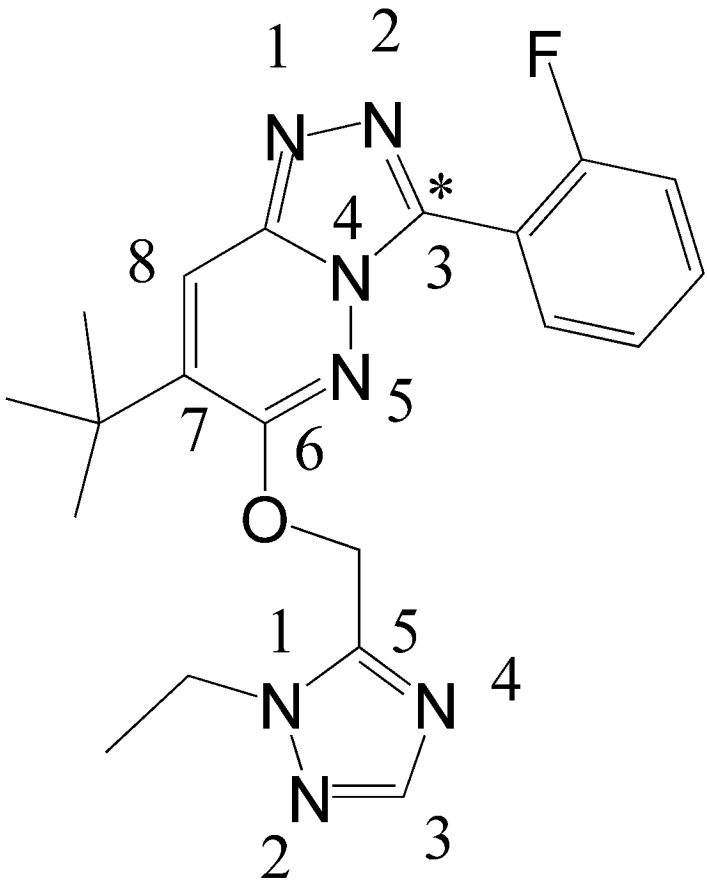
Quantitation of TPA023 metabolites in human feces (0-96 hr) following a single oral 3 mg (99 μCi) dose of [^{14}C]TPA023

Metabolites of TPA023 [b]	Percent of Dose Recovered [a]					
	Subject 007	Subject 008	Subject 009	Subject 010	Subject 011	Mean ± SD
M9	3.5	3.0	4.4	0.7	6.2	3.6 ± 2.0
M10	8.7	5.9	11.2	1.5	15.1	8.5 ± 5.2
M11	3.7	8.7	10.0	2.6	14.2	7.8 ± 4.7
M12	1.8	4.9	5.4	0.8	11.5	4.8 ± 4.2
M13	0.4	0.9	1.1	0.2	1.9	0.9 ± 0.7
Other	1.3	1.2	1.5	0.4	3.0	1.5 ± 1.0
Total	**19.4**	**24.4**	**33.5**	**6.3**	**51.9**	**27.1± 17.0**

[a] The mean (n=5) percent of the dose recovered in feces (0-96 hr) was 27.1± 17.0%. Therefore 93% of the excreted radioactivity in feces is accounted for.

[b] See Table 4 for detailed description of metabolites of TPA023

Figure 1



[^{14}C] TPA023

Figure 2

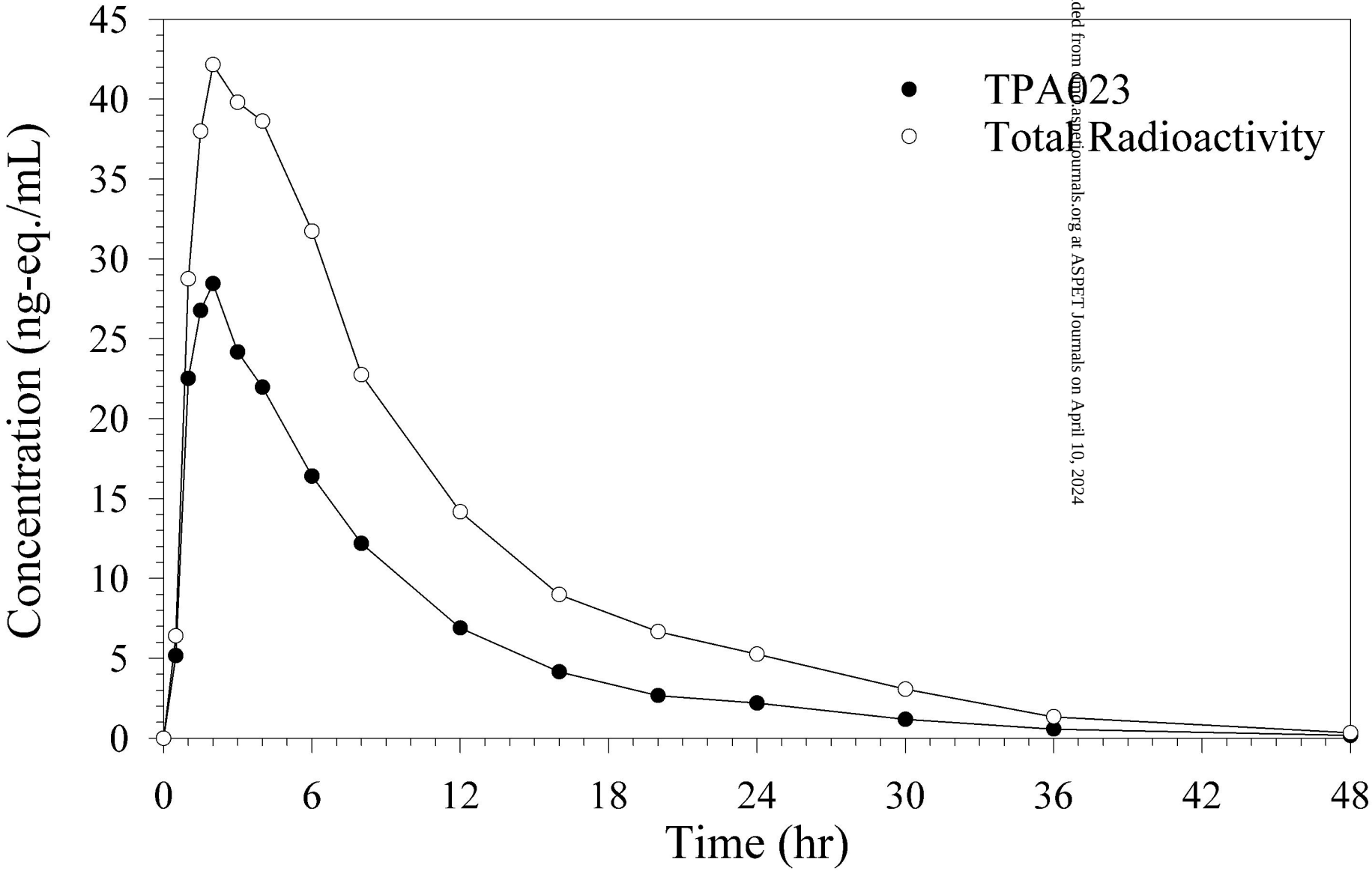


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Figure 3

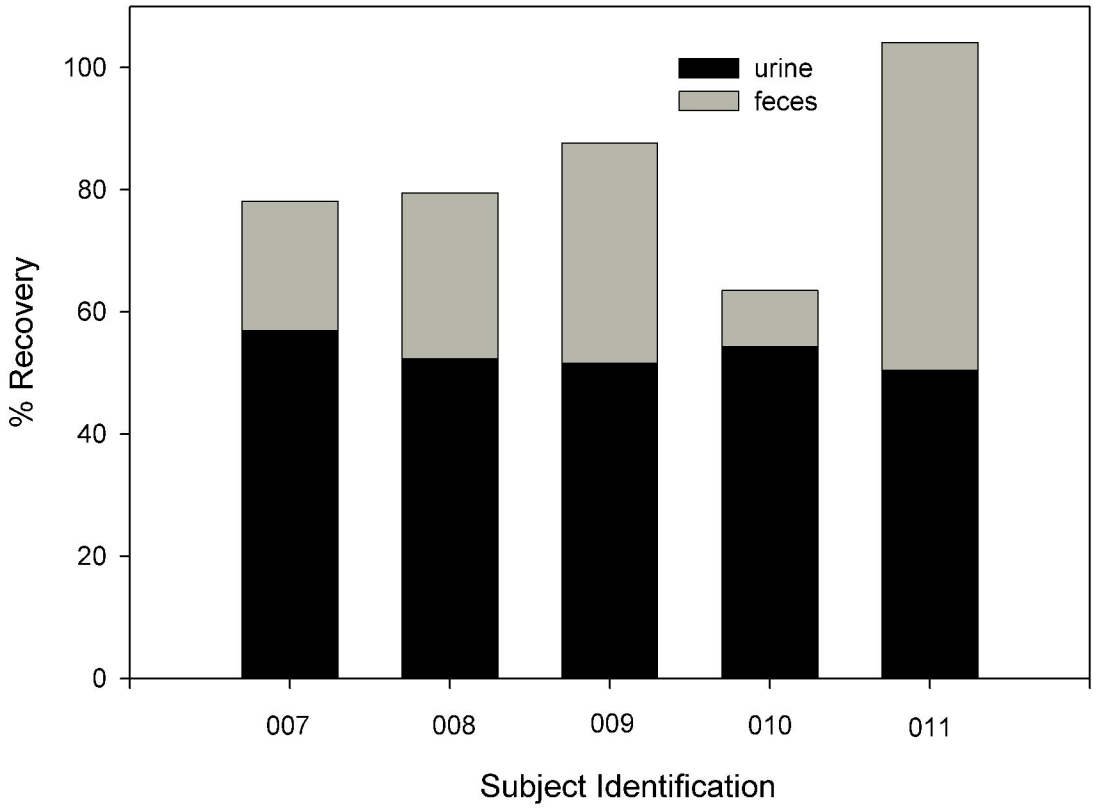

Figure 4

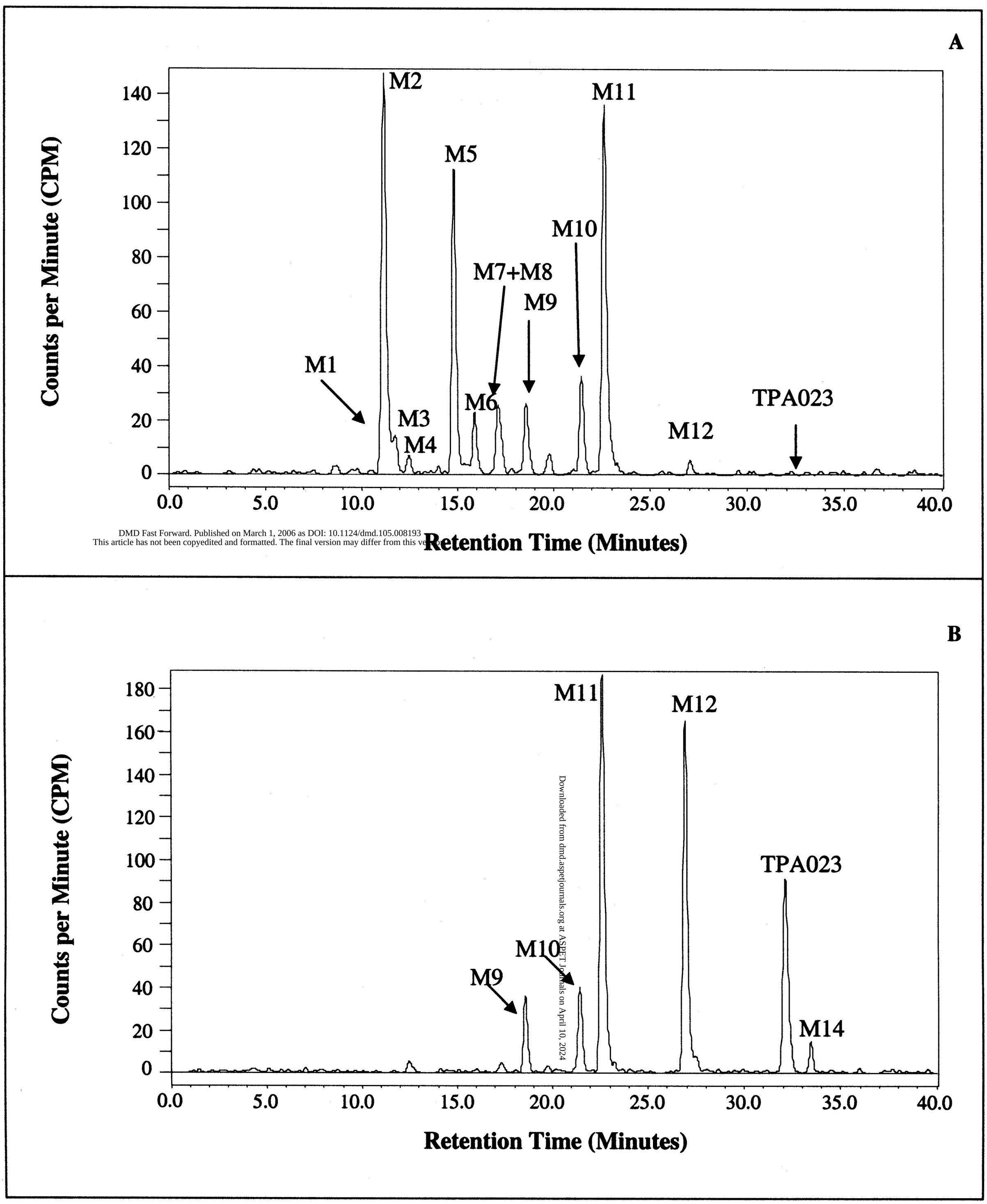

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Figure 5

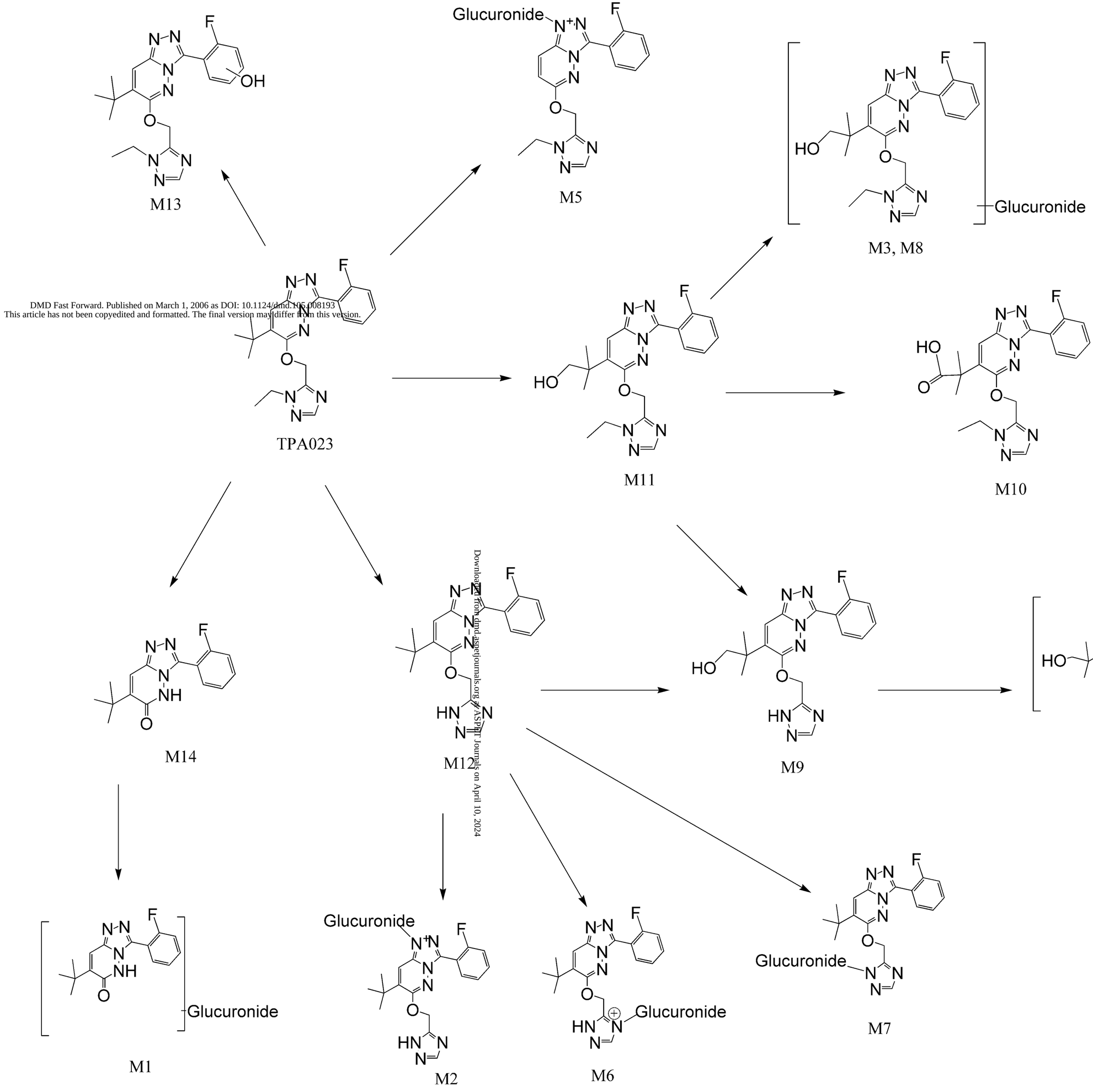

Figure 6