METABOLISM, PHARMACOKINETICS, AND EXCRETION OF A NONPEPTIDIC SUBSTANCE P RECEPTOR ANTAGONIST, EZLOPITANT, IN NORMAL HEALTHY MALE VOLUNTEERS

Characterization of polar metabolites by chemical derivatization with dansyl chloride

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IN VIVO BIOTRANSFORMATION OF EZLOPITANT IN HUMANS

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Abbreviations: ezlopitant, (2-benzhydryl-1-aza-bicyclo[2.2.2]oct-3-yl)-(5-isopropyl-2-methoxy-benzyl)-amine; CJ-12,764, 2-[(2-benzhydryl-1-aza-bicyclo[2.2.2]oct-3-ylamino)-methyl]-4-methoxy-phenyl]-propan-2-ol; CJ-12,458, (2-benzhydryl-1-aza-bicyclo[2.2.2]oct-3-yl)-(5-isopropenyl-2-methoxy-benzyl)-amine; SP, Substance P; LSC, liquid scintillation counting; CYP450, cytochrome P450; radio-HPLC, HPLC with on-line radioactivity detector; LC-MS/MS, liquid chromatography-tandem mass spectrometry; β-RAM, radioactive monitor; BSA, bovine serum albumin; CID, collisionally induced dissociation;
Abstract

The excretion, biotransformation and pharmacokinetics of ezlopitant, a substance P receptor antagonist, were investigated in healthy male volunteers after oral administration of a single 200 mg (~93 µCi/subject) dose of [14C]ezlopitant. The total recovery of administered radioactive dose was 82.8 ± 5.1, with 32.0 ± 4.2% in the urine and 50.8 ± 1.4% in the feces. Mean observed maximal serum concentrations for ezlopitant and total radioactivity were achieved at ~2 h after oral administration, thus ezlopitant was rapidly absorbed. Ezlopitant was extensively metabolized in humans, since no unchanged drug was detected in urine and feces. The major pathway of ezlopitant in humans was due to the oxidation of the isopropyl side chain to form the ω-hydroxy and ω-1 hydroxy (M16) metabolites. M16 and ω, ω-1-dihydroxy (1, 2-dihydroxy, M12) were identified as the major circulating metabolites accounting for 64.6 and 15.4% of total circulating radioactivity, respectively. In feces, the major metabolite M14 was characterized as the propionic acid metabolite and formed by further oxidation of the ω-hydroxy metabolite. The urinary metabolites were due to cleaved metabolites resulted by oxidative dealkylation of the 2-benzhydryl-1-aza-bicyclo[2.2.2]oct-3-yl moiety. The metabolites (M1A, M1B and M4), approximately 34% of the total radioactivity in urine, were identified as benzyl amine derivatives. These were polar metabolites that were further characterized using the reaction with dansyl chloride to derivatized the primary amines and phenol moieties to less polar analytes. The other metabolites were due to O-demethylation, dehydrogenation of the isopropyl group and oxidation on the quinuclidine moiety.
Despite the significant progress made over the past decade in the development of more effective and better tolerated agents to prevent chemotherapy-induced emesis, it still remains a significant, unresolved issue in a number of clinical situations, including cisplatin-induced delayed emesis, multiple and very-high dose chemotherapy (Roila, 1996; Frakes et al., 1997). Numerous neurotransmitters have been implicated in triggering emesis; however, Substance P (SP\textsuperscript{2}), an 11-amino acid neuropeptide of the tachykinin family of peptides, by virtue of its localization in both gut and central nervous system, and its ability to produce vomiting when injected in ferrets, is thought to play a key role in emetic response (Watson et al., 1995a). SP binds to a specific neuroreceptor, neurokinin 1 (NK1) and therefore, a number of nonpeptide compounds that selectively block the NK1 receptor have been evaluated as the antiemetic agents (Watson et al., 1995b; Gardner et al., 1996; Gonsalves et al., 1996; Grelot et al., 1998; Diemunsch and Grelot, 2000).

Ezlopitant, (2-benzhydryl-1-aza-bicyclo[2.2.2]oct-3-yl)-(5-isopropyl-2-methoxy-benzyl)-amine, Fig. 1) is a highly potent and selective NK1 receptor antagonist with Ki of 0.4 nM (Evangelista, 2001). It acts on the CNS to exert the excellent anti-emetic activity for the control of both acute and delayed emesis induced by cisplatin in ferrets at a dose of 3 mg/kg (Tsuchiya et al., 2002, 2005). It also inhibited SP-induced contraction of guinea pig trachea with a pA\textsubscript{2} value of 7.8, but had no effects on the baseline tension and maximum contractile response. Recent clinical studies have shown that ezlopitant is effective for the treatment of either acute or delayed phase of emesis associated with anti-neoplastic treatment using cisplatin, suggesting a role of NK1 receptor antagonists in the clinic for the control of emesis to improve the quality of life of cancer patients undergoing chemotherapy (Hesketh et al., 1999; Lee et al., 2000).
The pharmacokinetics of ezlopitant in several animal species have been reported and characterized by high clearance values with a moderate to high volume of distribution, a low to moderate terminal half-life, and low bioavailability (Reed-Hagen et al., 1999). Preliminary in vitro studies using hepatic microsomes from rat, dog and humans and recombinant human CYP 450 suggested that ezlopitant is metabolized into two active metabolites CJ-12,458 (prop-1-ene) and CJ-12,764 (propan-2-ol), predominantly by the CYP3A4/3A5, and to a lesser extent by CYP2D6 (Obach, 2000 and 2001). The objectives of the present study were to characterize metabolism, pharmacokinetics and excretion of \[^{14}\text{C}]\text{ezlopitant}\) in humans after oral administration of a single 200 mg dose as part of the development of this NK-1 receptor antagonist. The metabolites were characterized by LC/MS/MS and the structures of polar metabolites (cleaved products) were elucidated by derivatization with dansyl chloride. The structures of metabolites, where possible, were supported by comparisons of their retention times on HPLC, and MS spectra with those of the synthetic standards.

**Materials and Methods:**

**General Chemicals.** Commercially obtained chemicals and solvents were of HPLC or analytical grade. Ultrasphere C-18 HPLC column was obtained from Beckman Instruments Inc. (Fullerton, CA). Ecolite (+) scintillation cocktail was obtained from ICN (Irvine, CA). Carbosorb and Permafluor E+ scintillation cocktails were purchased from Packard Instrument Company (Downers Grove, IL). HPLC grade acetonitrile, methanol and water were obtained from J. T. Baker (Phillipsburgh, NJ). Certified ACS grade ammonium acetate and acetic acid were obtained from Fisher Scientific Company (Springfield, NJ). Dansyl chloride and 1-methyl-3-nitro-1-nitrosoguanidine were purchased from Sigma-Aldrich Co. (Milwaukee, WI). Diazomethane was generated just before use from 1-methyl-3-nitro-1-nitrosoguanidine.
Radiolabelled Drug and Reference Compounds. $[^{14}C]$ezlopitant (HCl salt) was synthesized by the Radiosynthesis Group at Pfizer Global Research and Development (Groton, CT) as described (Zandi et al., 1999). $[^{14}C]$ezlopitant showed a specific activity of 467.6 $\mu$Ci/mmol and a radiochemical purity of $>$99.8%, as determined by HPLC using an in-line radioactivity detector. The synthetic standards, CJ-12,764, CJ-12,458 were synthesized by the Medicinal Chemistry Group at Pfizer Global Research and Development (Nagoya, Japan).

Subjects and Dose Administration. Four normal healthy male subjects between the ages of 18 and 45 years participated in the study. The study protocol was reviewed and approved by the Institutional Review Board at the Clinical Research Facility of PPD Pharmaco (Austin, TX). After being informed of the purpose, design, and potential risks of the study, the volunteers gave written consent. Subjects entered the Clinical Research Facility approximately 12 h before dosing, and remained there for 264 h (11 days) after dosing under continuous medical observation. All subjects had fasted for at least 8 h and were given a single oral 200 mg free base equivalent dose of $[^{14}C]$ezlopitant (~93 $\mu$Ci/subject). The dose was administered in an open fashion in the morning. A standard meal was provided 4 h later. The dosing formulation was prepared by dissolving the radiolabelled material in water. Total volume of water given was 240 ml. Subjects were required to refrain from lying down, eating or drinking caffeinated and carbonated beverages during the first 4 h after drug administration.

Sample Collection. After dosing, urine samples were collected for eleven days at 0 (predose), 0-12, 12-24, 24-48, 48-72, 72-96, 96-120, 120-144, 144-168, 168-192, 192-216, 216-240 and
240-264 h post-dose. One of the subjects (#1) was removed from the study after 48 h due to personal reasons. The total volumes of urine samples were recorded after each collection. Feces were collected as passed, from time of dosing until 264 h after dosing.

Blood sufficient to provide a minimum of 4 ml of serum was collected, in evacuated tubes containing no anticoagulants, phase separator or preservative, from each subject at 0 (predose), 0.5, 1, 2, 4, 8, 12, 24, 36, 48, 72, 96, 120, 144, 168, 192, 216 and 240 h after the dose. In addition, blood sufficient to yield 5 ml of serum was collected at 2, 5 and 12 h after dosing for metabolite identification. The serum was allowed to clot at room temperature and was separated within 1 h from the whole blood in a refrigerated centrifuge, transferred into labeled plastic tubes, and immediately frozen.

**Determination of Radioactivity.** Radioactivity in urine, feces, and serum was measured by liquid scintillation counting. Aliquots of serum and urine (0.2-1.0 ml, in triplicate) for each sampling time were mixed with 5 ml of Ecolite (+) scintillation cocktail and counted in a Wallac 1409 (Gaithersburg, MD) or a Packard 2500 (Downers Grove, IL) liquid scintillation counter.

Fecal samples were placed into Stomacher 400 bags and homogenized in equal amounts of water to a thick slurry, using a Stomacher homogenizer from Cooke laboratory Products (Alexandria, VA). Aliquots (30-200 mg) of the fecal homogenates were air dried prior to combustion in an automatic sample oxidizer (Packard oxidizer model 307). Radioactivity in the combustion products was determined by trapping the liberated CO₂ in Carbo-sorb followed by liquid scintillation counting using Permafluor E+ as a scintillation cocktail. Combustion efficiency of the oxidizer was determined by combustion of the [¹⁴C]methyl methacrylate standard in an
identical manner. Radioactivity less than twice the background value was considered to be below the limit of determination.

Samples collected prior to dosing were used as controls and counted to obtain a background count rate. Radioactivity in the dose was expressed as 100% and the radioactivity in urine and feces at each sampling time was defined as the percentage of dose excreted in the respective matrices at that sampling time. The amount of radioactivity in serum was expressed as nanogram-equivalents of parent drug per milliliter and was calculated by using the specific activity of the dose administered.

**Pharmacokinetic Analysis.** Serum concentrations of ezlopitant were quantitated by an LC/MS/MS assay (Kamel et al., 2000). Pharmacokinetic parameters (PK) were determined using the WinNonlin-Pro Ver. 1.5 program (Pharsight, Mountain View, CA) by non-compartmental analysis.

**Extraction of Metabolites from Biological Samples.** Urine samples obtained at 0-12, 12-24, 24-48 and 48-72 h post-dose were pooled based on collected weight for each subject, which accounts for greater than 90% of the urine radioactive excreta. The pooled urine samples (8 ml) were lyophilized overnight. The residues were reconstituted in 0.25 ml of acetonitrile:20 mM ammonium acetate (20:80) and aliquots (200 µl) were injected onto the HPLC column.

Fecal homogenates containing the highest levels of excreted radioactivity (0-96 h) were pooled, and the pooled samples (~10 g) were extracted with 2 x 20 ml of acetonitrile. The two supernatants were combined and aliquots of 0.5 ml were counted in duplicate to determine
recovery of the radioactivity. The combined supernatants were evaporated to dryness in a nitrogen Turbo Vap LV evaporator (Zymark, Hopkinton, MA). The residues were reconstituted in 250 µl of acetonitrile:20 mM ammonium acetate (20:80) and aliquots (200 µl) were injected onto the HPLC column.

Serum samples (3 ml, 1 ml from each at 2, 5 and 12 h time point) were extracted with 2 x 6 ml of acetonitrile. The mixtures were vortex mixed for 5 min and centrifuged at 3500 rpm for 5 min to remove the precipitated proteins. Supernatants were combined and a small aliquot of supernatant was counted. Approximately 90% of the radioactivity was recovered in the supernatant. The supernatants were concentrated to dryness in a nitrogen Turbo Vap LV evaporator at a room temperature. The residues were reconstituted in 150 µl of acetonitrile/20 mM ammonium acetate (20/80), centrifuged to remove insoluble matters, and 100 µl aliquots were injected onto the HPLC column.

**Reaction with Dansyl Chloride.** The metabolites M1A, M1B and M4 from human urine were isolated by HPLC. The HPLC eluent from regions for metabolites M1A, M1B and M4 were collected and evaporated to dryness. The residues were treated with 100 µl of 100 mM sodium bicarbonate solution. A 50 µl aliquot of each reconstituted fraction was reacted with 25 µl of dansyl chloride solution (1 mg/ml in acetone). The mixture was allowed to stand at room temperature for 10 min and then evaporated to dryness. The residue was reconstituted in 150 µl of acetonitrile/20 mM ammonium acetate (20/80), centrifuged to remove insoluble matters, and 100 µl aliquot was injected onto the HPLC column.
Reaction with Methanol/Sulfuric Acid. The dried fecal extract was treated with 0.25 ml of 10% sulfuric acid in methanol. The mixture was allowed to stand at room temperature for 4 h. The solvents were evaporated to dryness and the residue was reconstituted in 150 µl of acetonitrile/20 mM ammonium acetate (20/80). The mixture was centrifuged to remove insoluble matters, and 100 µl aliquot was injected onto the HPLC column.

Chromatography. The HPLC system consisted of a HP-1050 solvent delivery system, a HP-1050 membrane degasser, an HP-1050 autoinjector from Hewlett Packard (Palo Alto, CA), a Thermo Separations spectromonitor 3200 UV (San Jose, CA) and an IN/US β-RAM radioactive monitor (Tampa, FL). Chromatography was performed on a Beckman Ultrasphere C-18 HPLC column (4.6 mm x 250 mm, 5 µm) with a mobile phase containing a mixture of 20 mM ammonium acetate (solvent A) and acetonitrile (solvent B). The mobile phase was initially composed of solvent A/solvent B (90:10), it was then linearly programmed to solvent A/solvent B (30:70) over 30 min followed by a gradient to solvent A/solvent B (10:90) over 10 min. The mobile phase composition was returned to the starting solvent mixture over 5 min. The system was allowed to equilibrate for approximately 15 min before making the next injection. A flow rate of 1.0 ml/min was used for all analyses.

Quantitative Assessment of Metabolite Excretion. Quantification of metabolites in urine and feces was carried out by measuring radioactivity in the individual HPLC-separated peaks using a β-RAM. The β-RAM provided and integrated printout in CPM and the percentage of the radiolabelled material, as well as the peak representation. The β-RAM was operated in the homogeneous liquid scintillation counting mode with the addition of 3 ml/min of
Tru-Count scintillation cocktail to the effluent post-UV detection. The radiochromatograms of metabolites in serum were generated by collecting fractions at 0.33 min intervals and counting the fractions in a Packard #2500CA liquid scintillation counter. The retention times of the radioactive peaks, where possible, were compared with those of synthetic standards and/or metabolites

**Mass Spectrometry Analysis.** Identification of metabolites was performed on a PE-Sciex API 2000 LC/MS/MS using ionspray. The effluent from the HPLC column was split and ~50 µl/min was introduced into mass spectrometer. The remaining effluent was directed into the flow cell of the β-RAM. The β-RAM response was recorded in real time by the mass spectrometer that provided simultaneous detection of radioactivity (RAD) and mass spectrometry data. The interface was operated at 4500 V and the mass spectrometer was operated in the positive mode. CID studies were performed using argon gas at a collision energy of 30-40 eV.

**Results**

**Mass Balance**

After a single oral dose of [14C]ezlopitant to human subjects, a major portion of the administered radioactivity was excreted in the feces. By 264 h after the dose, the mean cumulative excretion amounted to 32.0 ± 4.2% in the urine and 50.8 ± 1.4% in the feces (Table 1). In total, 82.8% of the radioactive dose was recovered in urine and feces (Table 1). Of the entire radioactivity recovered in urine, approximately 83% was excreted in the first 48 h. Because feces were collected after natural defecation, the fecal elimination of radioactivity was delayed, compared with that in urine. The major portion of radioactivity (~88%) in feces appeared during 24-144 h
time period after the dose. While the reason for low recovery of radioactivity is not entirely clear, possible reason could be due to noncompliance of subjects in complete collection of excreta

Pharmacokinetics

Mean serum concentration-time curves for ezlopitant and total radioactivity are shown in fig. 2 and the calculated pharmacokinetic parameters are shown in Table 2.

The absorption of ezlopitant was rapid, as indicated by early appearance of radioactivity in serum after oral administration. Serum concentrations for ezlopitant and total radioactivity peaked at ~2 hr after oral administration (Table 2). \(C_{\text{max}}\) values for the parent drug ranged from 193 to 370 ng/ml with a mean value of 271 ng/ml. \(C_{\text{max}}\) values for the total radioactivity ranged from 938 to 2024 ng equiv./ml with a mean value of 1456 ng equiv/ml. These values were five times higher than those of the parent drug, suggesting that a major portion of the circulating radioactivity was attributable to metabolites. The mean terminal phase \(T_{1/2}\) values were 13.0 and 108.9 h for parent drug and total radioactivity, respectively. Mean AUC\((0-t)\) values for parent drug and total radioactivity were 3257 ng.h/ml and 34,029 ng equiv.h/ml, respectively.

Metabolic Profiles in Biological samples.

Urine. A representative HPLC radiochromatogram for metabolites in urine is shown in fig. 3. A total of 12 metabolites were detected in the radiochromatogram. The percentages of metabolites in relation to the administered dose are presented in Table 3. The major metabolites in urine were due to cleaved products, which were resulted by oxidative dealkylation of the 2-
benzhydryl-1-aza-bicyclo[2.2.2]oct-3-yl moiety. The metabolites (M1A, M1B and M4), approximately 34% of the total radioactivity in urine, were identified as benzyl amine derivatives.

Feces. On average, ~96% of the radioactivity was recovered after extraction of fecal samples. A representative HPLC radiochromatogram for fecal metabolites is shown in fig. 3. A total of 8 radioactive peaks were detected in the radiochromatogram. The percentages of fecal metabolites in relation to the administered dose are presented in Table 3. The major metabolites in feces were due to hydroxylation at the isopropyl moiety. The metabolites M12 and M14, corresponding to for 67% of the total radioactivity in feces, were identified as 1,2-propane diol and ω-carboxy metabolites.

Circulating Metabolites

A representative HPLC radiochromatogram of circulating metabolites is shown in Fig. 4. Ezlopitant and a total of 5 metabolites were detected. The relative percentages of circulating metabolites are presented in Table 4. The major circulating metabolites were due to oxidation at the isopropyl moiety. Metabolites M12 and M16 (CJ-12,764) accounting for approximately 84% of the total circulating radioactivity between 2-12 hr period, based on the pooled samples, were identified as 1,2-propane diol and Propan-2-ol (ω-1 hydroxy metabolite). Unchanged ezlopitant and its alkene metabolite accounted for only <8% of the total radioactivity.

Mass spectral fragmentation of ezlopitant

Ezlopitant had a retention time of ~38.7 min on the HPLC system. Full scan MS of ezlopitant produced a protonated molecule (MH⁺) of m/z 455. Its CID spectrum gave product ions at m/z
276, 167, 163, 133, 121 and 91 (Fig. 5, Table 5). The structure assignments of major fragment ions are proposed in fig. 5B.

Identification of Metabolites

Metabolites M1A, M1B, M4. M1A, M1B and M4 had the retention times of ~3.1, 5.7, and 8.3 min, respectively, on HPLC and they were found only in urine. The full scan MS of M1A, M1B and M4 produced protonated molecules of \( m/z \) 198, 182 and 196, respectively, suggesting that they all were cleaved products. Further the even mass of protonated molecular ions suggested that they had an odd number of nitrogen atoms.

The fragment ion at \( m/z \) 165, loss of 17 Da (NH\(_3\)), indicated the presence of a primary amine, loss of a methyl group and addition of an oxygen atom (Fig. 6A). The fragment ion at \( m/z \) 147, loss of H\(_2\)O from the ion at \( m/z \) 165, suggested the presence of an aliphatic hydroxy group. Treatment of M1B with dansyl chloride resulted in the formation of a product with a protonated molecule of \( m/z \) 648, 466 Da higher than the protonated molecule of M1B, suggesting the addition of two molecules of dansyl chloride. The CID product ion spectrum of \( m/z \) 648 showed fragment ions at \( m/z \) 398 (loss of dansylamide), 380 (loss of dansylamide and H\(_2\)O) and 171 (dimethylaminonaphthalene) (Fig. 6B). Based on these data, M1B was identified as 2-aminomethyl-4-(1-hydroxy-1-methyl-ethyl)-phenol.

The MH\(^+\) of M1A at \( m/z \) 198, 16 Da higher than the metabolite M1B, indicated that it was a hydroxylated product of M1B. The ion at \( m/z \) 181, 18 Da higher than that of the parent molecule, suggested the loss of a methyl group and addition of two oxygen atoms to the molecule (Fig. 7A). The fragment ion at \( m/z \) 163, loss of H\(_2\)O from the ion at \( m/z \) 181, suggested
the presence of an aliphatic hydroxyl group. Treatment of M1A with dansyl chloride gave a product which produced a protonated molecule of \( m/z \) 664, 466 Da higher than the protonated molecule of M1A, suggesting the addition of two molecules of dansyl chloride. The CID product ion spectrum of \( m/z \) 664 showed fragment ions at \( m/z \) 646 (loss of H\(_2\)O), 414 (loss of dansylamide), 396 (loss of dansylamide and H\(_2\)O) and 171 (dimethylaminonaphthalene) (Fig. 7B). Based on these data, M1A was identified as 2-(3-aminomethyl-4-hydroxy-phenyl)propane-1,2-diol.

The MH\(^+\) of M4 at \( m/z \) 196, 14 Da higher than the metabolite M1B, indicated that it was a methylated product of M1B. The ion at \( m/z \) 131, loss of 65 Da (NH\(_3\)+H\(_2\)O+CH\(_2\)O), indicated the presence of a primary amine and a hydroxyl group (Fig. 8A). The fragment ion at \( m/z \) 59 indicated that hydroxylation had occurred on the isopropyl moiety. Treatment of M4 with dansyl chloride gave a product which showed a protonated molecule of \( m/z \) 429, 233 Da higher than the protonated molecular ion of M4, suggesting the addition of one molecule of dansyl chloride. The CID product ion spectrum of \( m/z \) 429 showed fragment ions at \( m/z \) 179 (loss of dansylamide), 171 (dimethylaminonaphthalene), 161 (loss of dansylamide and H\(_2\)O) and 131 (Fig. 8B). Based on these data, M4 was identified as 2-(3-aminomethyl-4-methoxy-phenyl)-propan-2-ol.

**Metabolites M7A, M7B, M7C:** M7A and M7B had the retention time of ~15.4 and 18.3 min and found both in urine and feces. M7A and M7B produced protonated molecules of \( m/z \) 473, 18 Da higher than the parent compound, indicating the addition of two oxygen atoms and a loss of the methyl group. CID spectrum of M7A showed characteristic fragment ions at \( m/z \) 167, 276, and 293, similar to parent molecule suggesting that the modification had occurred remote...
from the 2-benzhydryl-1-aza-bicyclo[2.2.2]oct-3-ylamino moiety (Table 5). The fragment ion at m/z 181, 18 Da higher than the parent compound, further suggested that the modification had occurred at the isopropyl-2-methoxy-benzyl moiety. The ion at m/z 106 supported the presence of a hydroxy-phenyl moiety and supported that the addition of two oxygen atoms had occurred at the isopropyl moiety. Based on these data, M7A was tentatively identified as 2-{3-[(2-benzhydryl-1-aza-bicyclo[2.2.2]oct-3-ylamino)-methyl]-4-hydroxy-phenyl}-propane-1,2-diol.

CID spectrum of protonated M7B showed fragment ions at m/z 309 and 292, 16 Da higher than those of M7A, suggesting that an addition of one oxygen atom had occurred at the 2-benzhydryl-1-aza-bicyclo[2.2.2]oct-3-ylamino moiety (Table 5). The fragment ion at m/z 167, similar to parent compound suggested that the hydroxylation had occurred at the quinuclidine moiety and remote from benzhydryl moiety. The fragment ion at m/z 165, 2 Da higher than that of the parent molecule, suggested that an addition of one oxygen atom and a loss of the methyl group from the isopropyl-2-methoxy-benzyl moiety. The ion at m/z 147, loss of H$_2$O, suggested the presence of an aliphatic hydroxyl group. Based on these data, M7B was tentatively identified as 2-{(2-benzhydryl-1-aza-bicyclo[2.2.2]oct-3-ylamino)-methyl}-4-(1-hydroxy-1-methyl-ethyl)-phenol with hydroxylation at the quinuclidine moiety.

M7C produced a protonated molecule of m/z 457, 2 Da higher than the parent compound, suggesting the addition of one oxygen atom and a loss of the methyl group. Its CID spectrum showed fragment ions at m/z 293, 276 and 167 suggesting that the modification had occurred remote from the 2-benzhydryl-1-aza-bicyclo[2.2.2]oct-3-ylamino moiety (Table 5). The fragment ion at m/z 165, 2 Da higher than that of the parent drug, suggested that the molecule had undergone modification at the isopropyl-2-methoxy-benzyl moiety. The ions at m/z 147
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(loss of \(\text{H}_2\text{O}\)), and 59 strongly indicated the hydroxylation of the isopropyl moiety. Based on these data, M7C was tentatively identified as \(2-[\text{(2-benzhydryl-1-aza-bicyclo[2.2.2]oct-3-ylamino)-methyl}]-4-(1\text{-hydroxy-1-methyl-ethyl})\text{-phenol.}

**Metabolites M12, M15:** M12 and M15 had HPLC retention times of ~19.2 and 22.0 min, respectively and found in all matrices. Both M12 and M15 produced protonated molecules of \(m/z\) 487, 32 Da higher than the parent compound, suggesting these compounds were di-hydroxylated derivatives of ezlopitant. The fragment ion at \(m/z\) 195, 32 Da higher than that of the parent compound, further suggested that the modification had occurred at the isopropyl-2-methoxy-benzyl moiety. The ion at \(m/z\) 177, loss of \(\text{H}_2\text{O}\), suggested at least one aliphatic hydroxylation (Fig. 9A, Table 5). Based on these data, M12 was tentatively identified as \(2-[3-[(2\text{-benzhydryl-1-aza-bicyclo[2.2.2]oct-3-ylamino)-methyl}]-4\text{-methoxy-phenyl}]-propanol.

On the other hand, CID spectrum of M15 showed major fragment ions at \(m/z\) 179, 167, 161, 123 and 110 (Fig 9B, Table 5). The fragment ion at \(m/z\) 179, 16 Da higher than the fragment ion at \(m/z\) 163 of the parent compound, suggested the addition of one oxygen atom at the isopropyl-2-methoxy-benzyl moiety. The ion at \(m/z\) 161, loss of \(\text{H}_2\text{O}\) from the ion at \(m/z\) 179, suggested the presence of an aliphatic hydroxyl group. The presence of a diagnostic ion at \(m/z\) 167 suggested that the other hydroxylation had occurred remote from the benzhydryl moiety; most likely at the quinuclidine moiety. Based on these data, M15 was tentatively identified as \(2-[3-[(2\text{-benzhydryl-1-aza-bicyclo-[2.2.2]oct-3-ylamino)-methyl}]-4\text{-methoxy-phenyl}]-propan-2\text{-ol with hydroxylation at the quinuclidine moiety.}
Metabolite M14: M14 had a retention time of ~16.1 min on HPLC and found in urine and feces. It produced a protonated molecule of \( m/z \) 485, 30 Da higher than the parent compound. The 30-Da increase in molecular weight is in accordance with oxidation of the methyl group to a carboxyl group. The fragment ion at \( m/z \) 193, 30 Da higher than that of the parent compound, suggested that the modification had occurred at the isopropyl-2-methoxy-benzyl moiety. The ion at \( m/z \) 276 further indicated that the modification had occurred remote from the 2-benzhydryl-1-aza-bicyclo[2.2.2]oct-3-ylamino moiety (Fig. 10A, Table 5).

Treatment of fecal concentrate with 10% sulfuric acid in methanol resulted in the disappearance of peak corresponding to M14 and gave a product which produced a protonated molecule of \( m/z \) 499. The ion at \( m/z \) 499 was 14 Da higher than the MH\(^+\) of M14, suggesting the addition of one methyl group. The CID product ion spectrum of \( m/z \) 499 showed fragment ions at \( m/z \) 207 and 177, 14 Da higher than those of M14 suggesting the addition of a methyl group to the anisole moiety (Fig. 10B). Based on these data, M14 was identified as 2-{3-[(2-benzhydryl-1-aza-bicyclo[2.2.2]oct-3-ylamino)-methyl]-4-methoxy-phenyl}-propionic acid.

Metabolite M16 (CJ-12,764): M16 had a retention time of ~24.5 min and found in all matrices. It produced a protonated molecule of \( m/z \) 471, 16 Da higher than the parent compound, suggesting that the molecule had undergone mono-hydroxylation. Its CID spectrum showed fragment ions at \( m/z \) 276, 179, 167, 161, 131, and 121 (Table 5). The fragment ion at \( m/z \) 179, 16 Da higher than the fragment ion at \( m/z \) 163 of the parent, suggested that hydroxylation had occurred at the isopropyl-2-methoxy-benzyl moiety. The fragment ion at \( m/z \) 161, loss of \( \text{H}_2\text{O} \) from the ion at \( m/z \) 179, suggested an aliphatic hydroxylation. M16 had an identical CID spectrum to that of synthetic standard of CJ-12,764. Based on these data, M17
was identified as 2-{3-[(2-benzhydryl-1-aza-bicyclo[2.2.2]oct-3-ylamino)-methyl]-4-methoxy-phenyl}-propan-2-ol.

**Metabolite M17 (CJ-12,458):** M17 had a retention time of ~38.7 min and was detected only in serum. It produced a protonated molecule of $m/z$ 453, 2 Da lower than the parent molecule suggesting that the molecule had undergone dehydrogenation. Its CID spectrum showed fragment ions at $m/z$ 167 and 276 indicating that the modification had occurred remote from the 2-benzhydryl-1-aza-bicyclo[2.2.2]oct-3-ylamino moiety (Table 5). The fragment ion at $m/z$ 161, 2 Da lower than the fragment ion at $m/z$ 163 of the parent compound, further suggested that dehydrogenation had occurred at the isopropyl moiety. M17 had an identical CID spectrum to that of an authentic standard. Based on these data, M17 was identified as (2-benzhydryl-1-aza-bicyclo[2.2.2]oct-3-yl)-(5-isopropenyl-2-methoxy-benzyl)-amine.

**Metabolite M19:** M19 had a retention time of ~14.8 min and found only in feces. It produced a protonated molecular ion at $m/z$ 501, 46 Da higher than the parent molecule, suggesting that the M19 had the addition of three oxygen atoms and a dehydrogenation. Its CID spectrum showed fragment ions at $m/z$ 455, 439, 193, 167, 163, 147, 123 and 110 (Table 5). The fragment ion at $m/z$ 193, 30 Da higher than the fragment ion at $m/z$ 163 of the parent compound, was indicative of the presence of a carboxyl group. The ion at $m/z$ 167 suggested that the benzhydryl moiety was unchanged.

Treatment of fecal concentrate with 10% sulfuric acid in methanol resulted in the disappearance of peak corresponding to M19 and gave a product which produced a protonated molecule of $m/z$ 515. The ion at $m/z$ 515 was 14 Da higher than the protonated molecular ion of M19, suggesting
the addition of one methyl group. The CID product ion spectrum of m/z 515 showed fragment ions at m/z 207 and 177, 14 Da higher than those of M19 suggesting the addition of a methyl group to the anisole moiety. Based on these data, M19 was tentatively identified as 2-\{3-[(2-benzhydryl-1-aza-bicyclo[2.2.2]oct-3-ylamino)-methyl]-4-methoxy-phenyl\}-propionic acid with hydroxylation at the quinuclidine moiety.

**Metabolite M20:** M20 had a retention time of ~18.0 min and found only in feces. It produced a protonated molecule of m/z 503, 48 Da higher than the parent drug, suggesting that the molecule had undergone three hydroxylations. Its CID spectrum showed fragment ions at m/z 195, 177, 167, 123 and 110 (Table 5). The fragment ion at m/z 195, 32 Da higher than that of the parent compound, suggested the addition of two oxygen atoms at the isopropyl-2-methoxy-benzyl moiety. The ion at m/z 177, loss of H₂O, suggested at least one aliphatic hydroxylation. The exact site for the second hydroxylation was, however, not established by the mass spectral data. The ion at m/z 167 suggested that the benzhydryl moiety was unchanged. Based on these data, M20 was tentatively identified as 2-\{3-[(2-benzhydryl-1-aza-bicyclo[2.2.2]oct-3-yl-amino)-methyl]-4-methoxy-phenyl\}-propane-1,2-diol with hydroxylation at the quinuclidine moiety.

**Discussion**

We here report the metabolic fate and disposition of ezlopitant following oral administration of a 200 mg dose of [14C]ezlopitant, labeled at the benzylic carbon of the isopropyl anisole ring, to four healthy male volunteers. On average total radioactivity recovered from subjects was 83% not including one of the subjects that did not continue on the study after 48 h post dose. The majority of the radioactivity was excreted in the feces, suggesting that fecal/biliary excretion was
the major route of elimination of ezlopitant and its metabolites. The radioactivity recovered in urine accounts for 32% of the dose.

After oral administration, the serum concentrations of total radioactivity were greater than the parent compound at all time points. This suggested the early formation of metabolites. Unchanged ezlopitant accounted for only ~9.5% of the total radioactivity in serum pooled from 0-12 hr period. M16 and ω, ω-1-dihydroxy ((1, 2-dihydroxy, M12) were identified as the major circulating metabolites accounting for approximately 64.6 and 15.4% of total circulating radioactivity pooled from 0-12 hr period, respectively. The half-life for total radioactivity was higher than the unchanged drug suggesting that the elimination of metabolites was much slower than that of parent compound in humans.

Unchanged elimination of ezlopitant was an insignificant portion of the total elimination such that there was no unchanged drug detected in urine and feces. A total of 13 metabolites were identified. The metabolites were characterized by LC-MS/MS with simultaneous online radioactivity monitoring, which is clearly a technique of choice for the rapid structural characterization of metabolites from biological fluids (Johnson et al., 2003; Kamel and Prakash, 2006). The structures of two metabolites, M16 and M17 were confirmed by comparisons of their HPLC retention times and CID product ion spectra to those of the synthetic standards. The structures of other metabolites were proposed based on molecular ions and fragmentation patterns and further supported by derivatization with acidic methanol or dansyl chloride. All the metabolites were formed from oxidative metabolism. Based on the structures of metabolites, a plausible scheme for the biotransformation pathways of ezlopitant in humans is depicted in fig. 1.
The major metabolic pathway of ezlopitant was due to the oxidation of the isopropyl side chain to form the ω-1-hydroxy- (M16; 6% in urine and 1% in feces) and ω,ω-1-dihydroxy (M12; 8.12% in feces and 3.81% in urine) and ω-carboxy- (M14; 20.67% in feces and 1.65% in urine) metabolites. The derivatization of M14 with acidic methanol supported the presence of a carboxyl group. Although, the ω-hydroxy metabolite was not identified either in urine or feces but it could be envisioned that M14 metabolite was formed by further oxidation of the ω-hydroxy metabolite. Hydroxylation at the tert-butyl- or isopropyl-side chains to form ω-hydroxy metabolites is not uncommon and, for example, was observed for several compounds containing these side chains (Ishida et al., 1998; Johnson et al., 2003). M12 is a 1,2 dihydroxy metabolite which is most likely formed due to further oxidation of ω-1-hydroxy metabolite (M16) at the isopropyl group. Alternatively it could also be formed by further metabolism of the alkene metabolite (M17) (Obach 2000). Metabolites M12 and M15 produced the same protonated molecule of m/z 48 but the CID mass spectra of these metabolites were able to distinguish oxidative sites characterized by distinct product ions.

The major metabolites in urine were due to cleaved products resulted by oxidative dealklyation of the 2-benzhydryl-1-aza-bicyclo[2.2.2]oct-3-yl moiety. These polar metabolites (M1A, M1B and M4) had to be derivatized in order to increase the HPLC retention time away from the solvent front in order to minimize the interference from the chemical background found in the lower mass range of a conventional mass spectrum. Dansyl chloride proved to be a suitable agent for this purpose that confirmed the presence of the amine and phenol functional groups in these metabolites. Derivatization of M1A and M1B with dansyl chloride resulted in the formation of new peaks with
protonated molecules of \( m/z \) 664 and 648, respectively. These protonated molecules were 466 Da higher than the protonated molecules of underivatized M1A and M1B, respectively, suggesting the presence of both an amine and a phenolic functionality in these metabolites. On the other hand the derivatization of M4 with dansyl chloride gave a protonated molecule of \( m/z \) 429, 233 Da higher than the protonated molecule of underivatized M4, corresponding to the presence of only one amino group. Further CID spectra of dansylated metabolites supported the proposed structures.

*In vitro* studies using hepatic microsomes form humans and recombinant human CYP 450 suggested that ezlopitant is metabolized predominantly by the CYP3A4/3A5, and to a lesser extent by CYP2D6 (Obach, 2000 and 2001). (Obach 2000). This suggests that elimination of ezlopitant is primarily relying on a single enzyme, which may contribute to patient-to-patient pharmacokinetic variability. The human CYP3A family, accounting for ~50% of the total P450 in human liver, is clinically very important because it has been shown to metabolize a large number of drugs (Wrighton et al., 1996). The human CYP3A subfamily includes CYP3A4, CYP3A5, CYP3A7 (Li et al., 1995), and CYP3A43 (Domanski et.al., 2001) as sub-family members. CYP3A4 is the major human liver CYP3A enzyme, whereas CYP3A5 is present in only ~10-30% of the human liver and CYP3A7 is only present in fetal liver. Although, ezlopitant is not a potent inhibitor of CYP3A, but since it is a substrate of this enzyme attention needs to be given when it is co-administered with CYP3A inhibitors.

In conclusion, the results of this study provide the first analysis of formation and excretion of metabolites of ezlopitant in humans. Ezlopitant is rapidly absorbed in humans. The majority of the total administered ezlopitant radioactivity was excreted in the feces. Ezlopitant was
eliminated mainly by oxidative metabolism, followed by excretion of metabolites into urine and feces, as no ezlopitant excreted unchanged.

**Acknowledgments:**

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References


DMD #15362


Footnotes:

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Figure Legends

Fig. 1. Proposed routes for biotransformation of ezlopitant in humans

Fig. 2. Mean serum concentration-time curves for ezlopitant, CJ-12,764, CJ-12,458, and total radioactivity in humans following a 200 mg oral dose of [14C]ezlopitant.

Fig. 3. HPLC-radiochromatograms for metabolites of ezlopitant in human (A) urine (0-72 hr) and (B) feces (0-96 hr)

Fig. 4. HPLC-radiochromatogram for circulating metabolites of ezlopitant in human plasma (0-12 hr)

Fig. 5. CID product ion spectrum of ezlopitant; m/z 455 (A) and proposed structural assignment of major fragment ions (B)

Fig. 6. CID product ion spectra of metabolite M1B, m/z 182 (A) and its dansyl derivative, m/z 648 (B)

Fig. 7. CID product ion spectra of metabolite M1A, m/z 198 (A) and its dansyl derivative, m/z 664 (B)

Fig. 8. CID product ion spectra of metabolite M4, m/z 196 (A) and its dansyl derivative, m/z 429 (B)

Fig. 9. CID product ion spectrum of metabolites M12, m/z 487 (A) and M15; m/z 487 (B)

Fig. 10. CID product ion spectra of metabolite M14, m/z 485 (A) and its methyl ester; m/z 499 (B)
Table 1. Cumulative (mean ± SD) Percentage of Radioactivity Excreted in Urine and Feces of Humans Following Oral Administration of a Single 200 mg Dose of [14C]Ezlopitant

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Urine ± SD</th>
<th>Feces ± SD</th>
<th>Combined Total ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-12 h</td>
<td>15.1± 2.89</td>
<td>N.A.</td>
<td>15.1± 2.89</td>
</tr>
<tr>
<td>24-48 h</td>
<td>6.22 ± 1.0</td>
<td>0.80 ± 0.93</td>
<td>7.02 ± 1.38</td>
</tr>
<tr>
<td>24-48 h</td>
<td>5.21 ± 1.33</td>
<td>14.0 ± 5.94</td>
<td>19.2 ± 5.49</td>
</tr>
<tr>
<td>48-72 h</td>
<td>1.69 ± 1.02</td>
<td>18.4 ± 7.08</td>
<td>20.1 ± 7.94</td>
</tr>
<tr>
<td>72-96 h</td>
<td>1.05 ± 0.11</td>
<td>5.26 ± 5.5</td>
<td>6.31 ± 5.41</td>
</tr>
<tr>
<td>96-120 h</td>
<td>0.54 ± 0.07</td>
<td>2.66 ± 2.3</td>
<td>3.20 ± 2.24</td>
</tr>
<tr>
<td>120-144 h</td>
<td>0.3 ± 0.05</td>
<td>4.20 ± 6.63</td>
<td>4.50 ± 6.58</td>
</tr>
<tr>
<td>144-168 h</td>
<td>0.2 ± 0.02</td>
<td>1.90 ± 2.06</td>
<td>2.10 ± 2.06</td>
</tr>
<tr>
<td>168-192 h</td>
<td>0.14 ± 0.03</td>
<td>0.94 ± 0.88</td>
<td>1.09 ± 0.85</td>
</tr>
<tr>
<td>192-216 h</td>
<td>0.1 ± 0.02</td>
<td>0.49 ± 0.2</td>
<td>0.59 ± 0.2</td>
</tr>
<tr>
<td>216-264 h</td>
<td>0.09 ± 0.08</td>
<td>0.19 ± 0.13</td>
<td>0.28 ± 0.03</td>
</tr>
<tr>
<td>Total</td>
<td>32.0 ± 4.18</td>
<td>50.8 ± 1.36</td>
<td>82.8 ± 5.13</td>
</tr>
</tbody>
</table>

NA: not applicable; feces was collected over 24-h intervals
Table 2
Pharmacokinetic Parameters for Ezlopitant, CJ-12,458, CJ-12,764 and Total Radioactivity in Humans Following a Single 200 mg Oral Dose of $[^{14}\text{C}]$Ezlopitant

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Ezlopitant</th>
<th>Total Radioactivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tmax (h)</td>
<td>2.3±1.3</td>
<td>2.0±0.0</td>
</tr>
<tr>
<td>Cmax* (ng/ml)</td>
<td>271±97</td>
<td>1460±470</td>
</tr>
<tr>
<td>AUC(0-t)* (ng.h/ml)</td>
<td>3220±1310</td>
<td>34000±6070</td>
</tr>
<tr>
<td>AUC(0-∞)* (ng.h/ml)</td>
<td>3260±1310</td>
<td>NC</td>
</tr>
<tr>
<td>T1/2 (h)</td>
<td>13.0±4.7</td>
<td>109±45.8</td>
</tr>
</tbody>
</table>

*Cmax and AUC values for total radioactivity are expressed as ng equiv/ml and ng equiv.h/ml, respectively. NC=not calculated.
### Table 3
Mean Percentages of Urinary and Fecal Metabolites of Ezlopitant in Humans Following a Single 200 mg Oral Dose of $[^{14}\text{C}]$Ezlopitant

<table>
<thead>
<tr>
<th>Metabolite (#)</th>
<th>Retention Time (min)</th>
<th>Urine</th>
<th>Feces</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>M1A</td>
<td>3.1</td>
<td>3.98</td>
<td>nd</td>
<td>3.98</td>
</tr>
<tr>
<td>M1B</td>
<td>5.7</td>
<td>3.45</td>
<td>nd</td>
<td>3.45</td>
</tr>
<tr>
<td>M4</td>
<td>8.3</td>
<td>2.23</td>
<td>nd</td>
<td>2.23</td>
</tr>
<tr>
<td>M7A+M19</td>
<td>14.8</td>
<td>1.45</td>
<td>4.77</td>
<td>6.22</td>
</tr>
<tr>
<td>M14</td>
<td>16.1</td>
<td>1.65</td>
<td>20.7</td>
<td>22.3</td>
</tr>
<tr>
<td>M7B+M20</td>
<td>18.0</td>
<td>0.20</td>
<td>7.03</td>
<td>7.23</td>
</tr>
<tr>
<td>M12</td>
<td>19.2</td>
<td>3.81</td>
<td>8.17</td>
<td>12.0</td>
</tr>
<tr>
<td>M7C</td>
<td>19.8</td>
<td>2.52</td>
<td>nd</td>
<td>2.52</td>
</tr>
<tr>
<td>M15</td>
<td>22.0</td>
<td>0.91</td>
<td>1.69</td>
<td>2.60</td>
</tr>
<tr>
<td>M16 (CJ-12,764)</td>
<td>24.5</td>
<td>5.92</td>
<td>0.98</td>
<td>6.90</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td></td>
<td>26.1</td>
<td>43.3</td>
<td>69.4</td>
</tr>
</tbody>
</table>

nd = not detected
Table 4

Percentages of Circulating Metabolites of Ezlopitant in Humans Following a Single 200 mg Oral Dose of $[^{14}\text{C}]\text{Ezlopitant}$

<table>
<thead>
<tr>
<th>Metabolite*</th>
<th>Retention Time</th>
<th>Percentage of injected radioactivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>#</td>
<td>(min)</td>
<td>Subject #</td>
</tr>
<tr>
<td>M12</td>
<td>19.2</td>
<td>14.7</td>
</tr>
<tr>
<td>M7C</td>
<td>19.8</td>
<td>5.1</td>
</tr>
<tr>
<td>M15</td>
<td>22.0</td>
<td>2.2</td>
</tr>
<tr>
<td>M16 (CJ-12,764)</td>
<td>24.5</td>
<td>61.7</td>
</tr>
<tr>
<td>Parent+ M17 (CJ-12,458)</td>
<td>38.7</td>
<td>7.2</td>
</tr>
<tr>
<td>Total</td>
<td>90.9</td>
<td>98.9</td>
</tr>
</tbody>
</table>
## Table 5

CID mass spectral data of CJ-11974 metabolites in human excreta.

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Retention time (min)</th>
<th>[M+H]$^+$</th>
<th>CID Mass Spectral Data (m/z)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ezlopitant</td>
<td>38.7</td>
<td>455</td>
<td>276, 167, 163, 133, 91</td>
</tr>
<tr>
<td>M1A</td>
<td>3.1</td>
<td>198</td>
<td>181, 163, 150, 135, 107, 91, 79</td>
</tr>
<tr>
<td>M1B</td>
<td>5.7</td>
<td>182</td>
<td>165, 147, 119, 117, 107, 91, 77, 59, 43</td>
</tr>
<tr>
<td>M4</td>
<td>8.3</td>
<td>196</td>
<td>131, 116, 107, 91, 77, 59, 43</td>
</tr>
<tr>
<td>M7A</td>
<td>15.4</td>
<td>473</td>
<td>293, 276, 181, 167, 106</td>
</tr>
<tr>
<td>M7B</td>
<td>18.3</td>
<td>473</td>
<td>309, 292, 167, 165, 147</td>
</tr>
<tr>
<td>M7C</td>
<td>19.8</td>
<td>457</td>
<td>293, 276, 167, 165, 147</td>
</tr>
<tr>
<td>M12</td>
<td>19.2</td>
<td>487</td>
<td>276, 195, 177, 167</td>
</tr>
<tr>
<td>M14</td>
<td>16.1</td>
<td>485</td>
<td>276, 193, 167, 163, 147, 96</td>
</tr>
<tr>
<td>M15</td>
<td>22.0</td>
<td>487</td>
<td>179, 167, 161, 123, 110</td>
</tr>
<tr>
<td>M16 (CJ-)</td>
<td>24.5</td>
<td>471</td>
<td>276, 176, 167, 161, 131, 121</td>
</tr>
<tr>
<td>M17 (CJ-)</td>
<td>38.7</td>
<td>453</td>
<td>276, 167, 161</td>
</tr>
<tr>
<td>M19</td>
<td>14.8</td>
<td>501</td>
<td>455, 439, 193, 167, 163, 147, 123, 110</td>
</tr>
<tr>
<td>M20</td>
<td>18.0</td>
<td>503</td>
<td>195, 177, 167, 123, 110</td>
</tr>
</tbody>
</table>
Fig. 1
Fig 5B
Fig 8.