Metabolism and excretion of asenapine in healthy male subjects

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Abbreviations:
a.o. amongst others
ES electrospray
HPLC high-performance liquid chromatography
LC liquid chromatography
LSC liquid scintillation counting
MIM mono isotopic molecular masses
MS mass spectrometry
NMR nuclear magnetic resonance
SPE solid-phase extraction
TOCSY total correlation spectroscopy
Abstract

Metabolism and excretion of asenapine [(3aRS,12bRS)-5-Chloro-2-methyl-2,3,3a,12b-tetrahydro-1H-dibenzo[2,3:6,7]-oxepino[4,5-c]pyrrole (2Z)-2-butenedioate (1:1)] were studied after sublingual administration of [14C]-asenapine to healthy male volunteers. Mean total excretion based on the percent recovery of the total radioactive dose was ~90%, with ~50% appearing in urine and ~40% excreted in feces; asenapine itself was only detected in feces. Metabolic profiles were determined in plasma, urine and feces using HPLC with radioactivity detection. Approximately 50% of drug-related material in human plasma was identified or quantified. The remaining circulating radioactivity corresponded to at least 15 very polar, minor peaks (mostly phase II products). Overall, >70% of circulating radioactivity was associated with conjugated metabolites. Major metabolic routes were direct glucuronidation and N-demethylation. The principal circulating metabolite was asenapine N+-glucuronide; other circulating metabolites were N-desmethylasenapine-N-carbamoyl-glucuronide, N-desmethylasenapine, and asenapine 11-O-sulphate. In addition to the parent compound, asenapine, the principal excretory metabolite was asenapine N+-glucuronide. Other excretory metabolites were N-desmethylasenapine-N-carbamoylglucuronide, 11-hydroxy-asenapine followed by conjugation, 10,11-dihydroxy-N-desmethylasenapine, 10,11-dihydroxy-asenapine followed by conjugation (several combinations of these routes were found), N-formylasenapine in combination with several hydroxylations and most probably asenapine N-oxide in combination with 10,11-hydroxylations followed by conjugations. In conclusion, asenapine was extensively and rapidly metabolized, resulting in several region-isomeric hydroxylated and conjugated metabolites.
Introduction

Asenapine [SAPHRIS®; (3aRS,12bRS)-5-Chloro-2-methyl-2,3,3a,12b-tetrahydro-1H dibenzo[2,3:6,7]-oxepino[4,5-c]pyrrole (2Z)-2-butenedioate (1:1)] (Figure 1) belongs to the group of dibenzoxepinopyrrolidine compounds and is available as a fast-dissolving tablet for sublingual administration.

Asenapine has been approved for use in the United States for treatment of schizophrenia and acute treatment, as monotherapy or as an adjunct to lithium or valproate, of manic or mixed episodes associated with bipolar I disorder in adults (2010). Asenapine is also approved in Europe for the treatment of manic episodes in bipolar disorder (Reuters, 2010).

Asenapine has a unique receptor-binding profile, with potent multi-receptor antagonism for a combination of serotonin, dopamine, noradrenaline, and histamine receptors, with a higher absolute affinity than currently available antipsychotics for a subset of therapeutically relevant serotonergic (5-HT2A,2B,2C,6,7), noradrenergic (α1,2), and dopaminergic (D3,4) receptors and no appreciable activity at muscarinic cholinergic receptors (Shahid et al., 2009).

Studies to investigate the excretion balance and metabolism in preclinical species have been performed previously to support the safety assessment of asenapine (van den Wildenberg et al., 1990). The present study was performed to investigate the excretion balance and metabolism routes of asenapine in humans and to compare with the preclinical species which were used in safety assessment. Therefore, a clinical trial was performed in which sublingual asenapine was administered twice daily to healthy male volunteers as multiple doses of unlabeled asenapine followed by a final single dose of [14C]-asenapine. A 10-mg dose of asenapine was selected because clinical trials in schizophrenia (Potkin et al., 2007; Kane et al., 2010; Schoemaker et al., 2010) and bipolar disorder (McIntyre et al.,
2009a; McIntyre et al., 2009b; McIntyre et al., 2010) have indicated that the therapeutic dose range of asenapine is 5 or 10 mg twice daily (BID).

The excretion balance (urinary and fecal) and the radioactivity concentrations in plasma were determined by LSC. Metabolite profiling was performed by HPLC with radioactivity detection. Isolation and purification of the metabolites was done by HPLC or SPE or liquid-liquid extraction. Identification of the metabolites, where feasible, was performed by comparison of retention time of authentic synthesized reference compounds, LC-MS-MS, and in some cases by NMR spectrometry or enzymatic deconjugation.
Methods

Study execution and dose preparation and administration

The clinical part of this study was performed in the clinical research center of PRA (formerly Pharma Bio-Research), Zuidlaren, The Netherlands. Six healthy male volunteers (ages 21–54 years; body mass index, 20.5–28.1 kg/m²) received sublingual unlabeled asenapine according to the schedule in Table 1, which has previously been demonstrated to be well tolerated (Dogterom et al., 2009). It was anticipated that healthy subjects would not tolerate an immediate single dose of 10 mg and therefore it was decided to gradually increase the dose, titrate to 10 mg and dose until steady state, which was expected after 6 days of dosing, before administration the radioactive dose. All doses were given BID except for the radioactive dose, which was administered as a single sublingual dose on the morning of day 10. Subjects were hospitalized from day -2 until 10 days after the final radioactive dose. Four subjects completed the study; two subjects withdrew before receiving the radioactive dose.

The study was approved by the appropriate medical ethics committee and conducted in full compliance with the declaration of Helsinki and the principles of Good Clinical Practice. Laboratory assessments were performed in full compliance with Good Laboratory Practice. All study participants signed an informed consent form before screening evaluations.

The radiolabeled asenapine sublingual tablets were prepared by dispensing [14C]-labeled asenapine in ethanol on the tablets. Ethanol was removed under reduced pressure at room temperature. The average radioactivity content of the tablets as determined in 6 tablets was 2.07 MBq (RSD = 2.1%) and no radiochemical degradation was found; the radiochemical purity was >97%.

Chemicals and reference compounds
[\textsuperscript{14}C]-asenapine (label at C12b, radiochemical purity \(\geq 97\%\)) was prepared by MSD, Oss, The Netherlands.

Unlabeled asenapine and the reference compounds shown in Table 2 were synthesized by MSD (Oss, The Netherlands). N-desmethylasenapine-N-carbamoylglucuronide (Org 191475-0) (also shown in Table 2) was previously isolated from rat bile and identified by \textsuperscript{1}H-NMR, \textsuperscript{13}C-NMR, MS, and infrared spectroscopy (van den Wildenberg et al., 1990). All other chemicals were obtained from commercial sources and were of analytical grade.

\textit{Sample Collection}

Blood samples for determination of pharmacokinetics of asenapine and its N-desmethyl and N-oxide metabolite and of the concentration of total radioactivity in plasma were collected from day 10 onward at 0 (pre-dose) and 0.5, 1, 1.5, 2, 3, 4, 6, 8, 12, 24, 36, 48, 60, and 72 hours post-dosing using heparin as an anticoagulant. In addition, blood samples for metabolite profiling were collected on day 1 at 0 (pre-dose; i.e., just before the first dose of asenapine) and on day 10 at 1, 1.5, 2, 4, 8, 12, and 24 hours post-dosing, using EDTA as an anticoagulant. Plasma was prepared by centrifugation. Urine and feces were collected up to 264 hours post-dosing. Plasma, urine, and fecal samples were stored at \(-20^\circ\text{C}\) until analysis.

\textit{Determination of radioactivity in plasma, urine, and feces}

The amount of radioactivity in plasma and urine was determined by LSC. Feces were homogenized with approximately 2 volumes of milli-Q water, after which the amount of radioactivity in feces homogenates was determined by combustion in a sample oxidizer followed by LSC.
Bioanalysis

Bioanalysis of asenapine, N-desmethylasenapine, and asenapine-N-oxide in human plasma samples was performed by a validated assay using an internal standard method after SPE at the Department of Bioanalytics, Essex Pharma Development GmbH, Waltrop, Germany. For the main metabolite, asenapine N-glucuronide, no standard was available at the time of bioanalysis.

The extracts were quantified by LC/MS using ES ionization in multireaction monitoring mode (data on file, 2008). Watson Drug Metabolism Laboratory Information Management System (version 6.3.0.03) was used for all calculations. Calibration curves for asenapine, N-desmethylasenapine, and asenapine-N-oxide were constructed using linear weighted (1/x²) regression.

Pharmacokinetics

All calculations were performed using SAS system for Windows™, version 8.2 running under Windows XP at the Department of Clinical Pharmacology and Kinetics, MSD, Oss, The Netherlands. Plasma concentration-time curves of asenapine, asenapine-N-oxide, N-desmethylasenapine, and total radioactivity (^14C; expressed in mass equivalents of asenapine) were constructed for each of the 4 individuals completing the study. Pharmacokinetic parameters were calculated from the plasma concentration-time data, where feasible.

Metabolite profiling, isolation and identification

Individual and pooled plasma samples were used for metabolite profiling and identification. Plasma samples containing sufficient radioactivity were analyzed individually
per subject and per time point. In addition, plasma samples were pooled across subjects, per
time point or per time interval. Identification of the peaks was done by retention time
comparison with authentic reference compounds and LC/MS analysis, where feasible.

Individual urine and fecal samples containing more than 5% of the administered
radioactivity were selected for metabolite profiling. These urine and fecal samples were
pooled per matrix and per individual in such way that quantification of metabolites reflects
the situation in the excreta.

Sample treatment

Acidified (0.2% HCl, v/v %) plasma and urine samples were applied to pretreated
Oasis HLB SPE columns (Waters Corp, Milford, MA). The columns were washed with
milli-Q water and eluted with acetonitrile/0.01 mol⋅L⁻¹ NH₄OAc pH 4.2 (7/3 v/v %). The
eluate samples were concentrated under a gentle stream of nitrogen, centrifuged (for urine
samples), and analyzed by HPLC. The recovery of SPE was determined by LSC and was
≥81% for plasma samples. For urine samples, the mean recovery was approximately 89%.

Fecal samples were extracted with 2 volumes of acetonitrile/methanol (3/1 v/v %) +
1% acetic acid (ratio feces homogenate/extraction solution [w/v, 1/2 %]). Additionally, the
fecal samples were extracted with acetonitrile/methanol (3/1 v/v %), (ratio feces
homogenate/extraction solution [w/v, 1/2 %]). The extraction recovery was determined per
extraction by LSC. The extracts containing >5% of the radioactivity were pooled and
concentrated under a gentle stream of nitrogen. The concentrated extracts were analyzed by
HPLC. The mean extraction recovery for feces was 74%.

Metabolite profiling
The metabolic profiling of asenapine in pooled plasma, urine, and fecal samples was performed on an HP1100 liquid chromatograph (Agilent Technologies Deutschland GmbH, Waldbronn, Germany) equipped with a diode array detector using a µ-Bondapak C_{18} column (internal diameter: 7.8 mm; internal length: 30 cm; particle size: 10 µm) and a gradient of ammonium acetate buffer (0.1 M, pH 4.2) (solvent A) and methanol/acetonitrile (1/3 v/v %) (solvent B) at 50°C. Elution started with 3 minutes isocratic at 10% solvent B followed by a linear gradient of 10%–40% solvent B in 17 minutes, 40%–90% solvent B in 30 minutes, and 90%–95% solvent B in 1 minute. This solvent composition was held during at least 3 minutes and maximally 8 minutes before returning to starting conditions. The flow rate was 2.0 mL/min.

Several reference compounds, monitored by UV detection (270 nm), were analyzed by HPLC, before or after analysis of the study samples. Radioactivity in the HPLC effluent was determined online using a flow-through detector (Flo-one\Beta model A500TR, PerkinElmer Life and Analytical Science, Zaventem, Belgium) or off-line by the collection of fractions followed by solid scintillation counting.

**Isolation of metabolites**

Pooled urine samples were filtered, acidified, and extracted by SPE. The eluate samples were concentrated and injected on HPLC, after which fractions were collected. Fractions constituting a peak of radioactivity (U1-U10) were pooled and concentrated under a gentle stream of nitrogen gas. The isolated metabolite fractions were purified by SPE using Oasis HLB columns with acetonitrile/0.01 mol/L NH_{4}OAc pH 4.2 (7/3 v/v %) and methanol/0.01% trifluoroacetic acid, respectively.

Fecal samples (homogenates) were pooled and extracted. The extracts were pooled, concentrated, and injected on HPLC, after which fractions were collected. Fractions
constituting a peak were pooled (F1-F11), concentrated, and further purified by acetonitrile/methanol/acetic acid extraction followed by extraction with diethylether.

On analysis, some of the fractions consisted of more metabolites, and attempts were made to purify these fractions using a variety of SPEs, liquid-liquid extractions, HPLC columns, elution profiles, and flow rates.

**Hydrolysis of urinary metabolites**

Metabolites U1 and U2/3, isolated from urine, were incubated in a solution containing sulphatase and β-glucuronidase to identify the phase II (conjugated) metabolites. These metabolites were dried under a stream of nitrogen, dissolved in a drop of methanol/0.01% trifluoroacetic acid, and diluted with 0.1 mol·L⁻¹ ammonium acetate buffer (pH 6). Subsequently, these metabolites were incubated for 18 hours at 37 °C in the presence of sulphatase and β-glucuronidase. In addition, incubation without sulphatase and β-glucuronidase was performed as a control. Incubation mixtures were pretreated by SPE and subjected to HPLC analysis.

**Identification of metabolites**

A number of metabolites found in plasma, urine, and feces were tentatively identified by HPLC retention time comparison with authentic reference compounds and a previously isolated and identified metabolite (N-desmethyl-asenapine-N-carbamoylglucuronide) (van den Wildenberg et al., 1990) Confirmation of identities of the metabolites was obtained by LC-MS-MS and, where feasible, with NMR or enzymatic deconjugation.

**NMR spectroscopy**
$^1$H-spectra were recorded at 600 MHz on a Bruker Avance 600 (Bruker Biospin GmbH, Rheinstetten, Germany) under standard conditions. The samples were dissolved in deuteromethanol (CD$_3$OD). The CD$_3$OD was used as a reference and set to 3.30 ppm. The chemical shifts are given in parts per million.

**Mass spectrometry**

ES mass spectra and product ion scans were recorded in positive and negative ion mode using the ionspray interface on the ABI MDS Sciex QStar Pulsar hybrid QqTOF mass spectrometer (MDS Sciex, Concord, Ontario, Canada) or the ES interface on the Bruker Esquire 3000 plus quadrupole ion trap mass spectrometer (Bruker Daltonik GmbH, Bremen, Germany). Sample introduction was performed by HPLC, using different HPLC methods. The flow was split before entering the mass spectrometer. The PE Sciex QStar Pulsar mass spectrometer was operated at a positive ionspray voltage of about 5500 V. Although the mass resolution of the QStar in MS mode was >6500, only nominal masses are reported. Mass spectra were recorded from about m/z 150 to m/z 1000 or 1200. The Bruker Esquire 3000 plus was operated in positive and negative ion mode. The mass spectra were recorded from m/z 150 to 750.
Results

**Plasma concentration of asenapine and total radioactivity**

Mean plasma concentrations of asenapine plus metabolites expressed in ng equivalents/mL plasma were calculated from the total radioactivity concentrations in plasma samples and the specific activity of the $^{14}$C-labeled formulated sublingual dose. The asenapine concentration in plasma was determined by bioanalysis.

Plasma concentrations of total radioactivity greatly exceeded those of asenapine from the first time point (0.5 h post-radioactive dosing) onward (Figure 2). The peak concentration of $[^{14}$C]-radioactivity was reached 4 hours after dosing, later than for asenapine (0.75 h). Half-lives (mean ± SD) of total radioactivity (39.3±7.6 h) and asenapine (27.5±5.0 h) in plasma were in the same range or slightly longer for the total radioactivity, suggesting that the metabolites of asenapine have a comparable or slightly longer half-life. The metabolites analyzed (N-desmethylasenapine and asenapine-N-oxide) constituted only a small fraction of the total of asenapine metabolites in plasma.

**Excretion of radioactivity in urine and feces**

After sublingual administration of $[^{14}$C]-asenapine to healthy male volunteers under steady-state conditions, radioactivity was excreted via urine and feces as shown in Figure 3. The cumulative excretion of radioactivity via urine was ~50%, whereas the cumulative excretion in feces was ~40%. The total recovery of radioactivity was ~90%; More than 80% of the radioactive dose was excreted within 96 hours.

**Metabolite profiling and characterization**

Radioactive peaks in the HPLC profiles were numbered per matrix on the basis of retention time. Asenapine was extensively and rapidly metabolized and resulted in several
regio-isomeric hydroxylated and conjugated metabolites. Nearly 50% of drug-related material in human plasma was identified or quantified. The remaining radioactivity (~50%) corresponds to different very polar peaks, none of which represent >6% of the plasma radiocarbon profile. Metabolites eluting in this region have been tentatively identified and correspond mostly to phase II (sulfate, glucuronide, and methylated) products. Overall, >70% of circulating radioactivity was associated with conjugated metabolites. In addition, a significant percentage (~71%) of the excreted radioactivity was characterized. The most important MS-MS fragments and related metabolite structures of the identified metabolites in plasma, urine, and feces are given in Table 3. The metabolic routes of asenapine are shown in Figure 4.

**Plasma**

The metabolite profiles of pooled plasma samples (1 h and 1.5–12 h) consisted of at least 6 peaks (P1-P6). Peak P2/3 was the major metabolite in both pooled plasma samples. Additionally, peak P6 was a major metabolite in the 1-hour pooled plasma sample. The metabolite profile of the 1.5–12 hour pooled plasma sample is given in Figure 5.

**P1: asenapine 11-O-sulphate**

The HPLC retention time and LC-ES-MS spectra (m/z 382/384) of metabolite P1 eluting at 22.4 min were identical to the reference compound Org 214025-0 (asenapine 11-O-sulphate). Based on LC/MS data, this peak also contained some other hydroxylated and conjugated metabolites.

**P2/3: asenapine N+-glucuronide**
Peak **P2/3** eluting at 25 minutes was a major metabolite in the pooled plasma samples and co-eluted with reference compound Org 216761-0 (asenapine N⁺-glucuronide). The fragmentation patterns of the reference compound and **P2/3** resulted both in the main ions m/z 462/464, which corresponded to the cation of asenapine N⁺-glucuronide. In addition, the product ion spectrum (MS-MS) of m/z 462 (286, 159, and 131, a.o. loss of 176 Da, characteristic for the glucuronide structure) was in agreement with the asenapine N⁺-glucuronide. The fraction consisted of diastereoisomeric forms that were partly separated under the HPLC conditions applied. For these reasons, peak **P2/3** was identified as the asenapine N⁺-glucuronide.

**P4: N-desmethyласenapine-N-carbamoyl-glucuronide**

Peak **P4** eluting at 28.7 minutes co-eluted with reference compound Org 191475-0 (N-desmethyласenapine-N-carbamoylglucuronide). LC-ES-MS analyses of this fraction resulted in the main ions m/z 492/494, which corresponded to the [M+H]⁺ of N-desmethyласenapine-N-carbamoylglucuronide. In addition, the product ion spectrum (MS-MS) of m/z 492 (316 and 272, a.o. loss of 176 Da, glucuronide) was in agreement with this reference.

**P5: N-desmethyласenapine**

LC-ES-MS analyses of peak **P5** eluting at 29.7 minutes resulted in the main ions m/z 272/274, which corresponded to the [M+H]⁺ of reference compound Org 30526 (N-desmethyласenapine). In addition, the product ion spectrum (MS-MS) of m/z 272 (a.o. loss of 43 Da, C₂H₄N, characteristic for the N-desmethyl structure) and the retention time of this compound were in agreement with this reference.
P6: asenapine

Peak P6 eluting at 30.2 minutes was a major peak of moiety in the 1-hour pooled plasma sample and co-eluted with reference compound Org 5222 (asenapine). The fragmentation patterns of P6 resulted in the main ions m/z 286/288, which corresponded to asenapine. In addition, the product ion spectrum (MS-MS) of m/z 286 (a.o. loss of 57 Da, C₃H₇N, characteristic for the N-methyl structure) and the retention time of this compound were in agreement with the reference. In addition, m/z 302/304 was observed. Additional peaks found in the pooled plasma samples could not be identified, with no chlorine pattern observed in the MS analyses.

Urine

The metabolite profiles of urine samples consisted of at least 10 different peaks (U1-U10). Peak U8/9 was the major metabolite, representing 10% to 21% of the radioactive dose. In addition, peaks U2/3, U4/5, U6/7, and U10 represented >5% of the administered radioactive dose at least in one of the pooled urine samples. The other peaks (including peak U1) were minor peaks (<3% of the administered radioactive dose) in all urine samples. A representative metabolite profile is shown in Figure 6.

U1: mixture of the methoxy, glucuronide of 10,11-dihydroxy-N-desmethyl-asenapine and the glucuronide of 10,11-dihydroxy-N-desmethyasenapine

LC-ES-MS analyses of peak U1 resulted in two fractions (U1A and U1B) in which the MIM were 493 and 479. The product ion spectrum (MS-MS) (Table 3) of MIM 493 in sample U1A was slightly different from the other sample U1B (most probably the position of the methoxy and glucuronide interchanged). Both samples showed a loss of 176 Da, characteristic for a glucuronide, followed by a.o. a loss of 43 Da (C₂H₃N) as in the
N-desmethyl. This fraction was identified as the methoxy and glucuronide conjugate of 10, 11-dihydroxy-N-desmethylasenapine, in which the position of the methoxy and glucuronide is 10,11 and the reverse. Analog to the fraction with MIM 493 and based on the mass difference of 14 Da between the fractions with a MIM of 479 and 493, the fraction with a MIM of 479 is tentatively identified as the glucuronide of the 10,11-dihydroxy-N-desmethylasenapine.

Enzymatic hydrolysis of U1A+B released ES-MS spectra containing MIM 317, corresponding with the O-methylated-10,11-dihydroxy-N-desmethylasenapine. The product ion spectrum (MS-MS) of the protonated molecule showed a.o. the loss of 43 Da (C₂H₅N) toward m/z 275; the MS-MS-MS product ion spectrum of m/z 275 showed losses of both 15 Da (CH₃) and 32 Da (CH₃OH). Furthermore, after H-D exchange in D₂O/CH₃COOD, two exchangeable protons were found (MIM 319), in agreement with the mono-hydroxy, mono-methoxy, N-desmethyl structure proposed.

For these reasons, peak U1 was identified as a mixture of trans-5-chloro-10-methoxy-2,3,3a,12b-tetrahydro-1H-dibenz[2,3:6,7]-oxepino[4,5-c]pyrrole-11-yl β-D-Glucopyranosiduronic acid and trans-5-chloro-11-methoxy-2,3,3a,12b-tetrahydro-1H-dibenz[2,3:6,7]-oxepino[4,5-c]pyrrole-10-yl β-D-Glucopyranosiduronic acid.

U2/3: mixture of methoxy and sulphate of 10,11-di-hydroxy-N-desmethylasenapine, asenapine 11-O-glucuronide and some other undefined conjugated metabolites.

LC-ES-MS analyses of peak U2/3 resulted in two fractions (U2/3A and U2/3B) containing at least six different MIM values, namely 397, 383, 493, 477, 463, and 507. In the product ion spectra of MIM 397, a loss of 80 Da (SO₃) and 43 Da (C₂H₅N) was observed. For the fractions with MIM 477 and 463, a loss of 176 Da (glucuronide) was observed. The U2/3 fractions were identified as the methoxy and sulphate of
10,11-dihydroxy-N-desmethylasenapine, the glucuronide of 11-hydroxy-N-desmethylasenapine and asenapine 11-O-glucuronide. Further evidence for the proposed structures was obtained from the ES-MS spectra of the hydrolyzed U2/3A fraction containing MIM 317, MIM 287, and MIM 301, which were in agreement with the mono-hydroxy, mono-methoxy, N-desmethyl structure in peak U1, 11-hydroxy-N-desmethylasenapine, and 11-hydroxyasenapine, respectively.

Based on these results peak U2/3 was identified as a mixture of trans-5-chloro-10-methoxy-11-sulfooxy-2,3,3a,12b-tetrahydro-1H-dibenz[2,3:6,7]-oxepino[4,5-c]pyrrole and trans-5-chloro-11-methoxy-10-sulfooxy-2,3,3a,12b-tetrahydro-1H-dibenz[2,3:6,7]-oxepino[4,5-c]pyrrole, and the trans-5-chloro-2-methyl-2,3,3a,12b-tetrahydro-1H-dibenz-[2,3:6,7]-oxepino[4,5-c]pyrrole-11-yl β-D-Glucopyranosiduronic acid plus some other unidentified conjugated metabolites (fractions with MIM 383, 493 and 507).

U4/5: mixture of conjugated metabolites

LC-ES-MS analyses of peak U4/5 resulted in two fractions (U4/5A and U4/5B) containing at least five MIM values, namely 397, 411, 507, 477, and 427. No structure proposals could be given for these fractions, but based on fragmentation patterns, it can be concluded that peak U4/5 consisted of several conjugated metabolites (sulphates and glucuronides).

U6/7: mixture of asenapine 11-O-sulphate, the mono-hydroxylated, mono-O-sulphated asenapine-N-oxide, and the mono-O-sulphated asenapine-N-oxide

LC-ES-MS analyses of peak U6/7 resulted in five fractions coded U6/7A up to U6/7E. The major signals in the 1H-NMR spectrum of fraction U6/7A were comparable to the signals for the reference compound asenapine 11-O-sulphate. U6/7A resulted in two MIM
values, namely 381 and 517 (very small peak). Based on the obtained NMR, MS, MS-MS
data and retention time comparison, the fraction with MIM 381 was identified as the
asenapine 11-O-sulphate. U6/7B showed a MIM value of 413. Based on the loss of 80 Da and
the presence of m/z 57 in the product ion spectrum of the protonated molecule, it can be
concluded that MIM 413 contained a sulphate and is most probably an N-oxide of mono-
hydroxylated, mono-O-sulphated asenapine. U6/7C showed two MIM values, namely 397
and 412. Based on the loss of 80 Da and presence of m/z 57, it can be concluded that
MIM 397 contained a sulphate and is most probably an N-oxide of mono-O-sulphated
asenapine. No structure proposal could be given for MIM 412, but based on the
fragmentation (a loss of 176 Da) pattern, it can be concluded that this fraction contained a
glucuronide. U6/7D showed three MIM values, namely 301, 491, and 499. MS and MS-MS
data showed that MIM 301 was in agreement with 11-hydroxyasenapine. Since the retention
time of this compound (based on comparison with the 11-hydroxyasenapine reference) differs
from the retention time of U6/7, this mono-hydroxylated metabolite probably is an isomer.
Based on the loss of 176 Da in the product ion spectrum of the protonated molecules, it can
be concluded that MIM 491 and 499 both contained a glucuronide. For MIM 499, three times
the H2O loss was observed in the MS-MS analysis. For MIM 491, no further structural
evidence was obtained. U6/7E resulted in three MIM values, namely 491, 477, and 301. The
signal-noise ratio for MIM 301 was very low. No structure proposal could be given for these
fractions, but based on fragmentation (loss of 176 Da) for MIM 491 and 477, it can be
concluded that these fractions both contained a glucuronide.

U8/9: asenapine N\textsuperscript{+}-glucuronide

Peak U8/9 was the major metabolite in urine, coeluted with the reference compound
Org 216761-0 (asenapine N\textsuperscript{+}-glucuronide). The fragmentation patterns of the reference
compound and U8/9 resulted in the main ions m/z 462/464, which corresponded to the cation of the asenapine N\(^+\)-glucuronide. In addition, the product ion spectrum (MS-MS) of m/z 462 (a.o. loss of 176 Da, characteristic for the glucuronide structure) and the \(^1\)H-NMR spectra were in agreement with the asenapine N\(^+\)-glucuronide. The fraction consisted of diastereoisomeric forms that were partly separated under the HPLC conditions applied.

**U10: N-desmethylasenapine-N-carbamoyl-glucuronide**

The \(^1\)H-NMR spectrum of peak U10 was in agreement with NMR data of the previously isolated and identified metabolite Org 191475-0 (N-desmethylasenapine-N-carbamoylglucuronide) (van den Wildenberg et al., 1990). In addition, the MS, MS-MS, and retention time were in agreement with this reference compound.

**Feces**

The peaks present in fecal samples consisted of at least 11 different peaks (F1-F11), of which asenapine (F9, representing 5%-16% of the radioactive dose) was the major peak. Additionally peak F1/2 (representing 6%-10% of the radioactive dose) and two minor peaks (F10 and F11) were isolated and identified. Peaks F3-F7 could not be further characterized. Peak F8 coeluted with N-desmethylasenapine but was not further characterized. A representative metabolite profile is shown in Figure 7 (feces, 48–72 h, Subject 1).

**F1/2: 10,11-dihydroxy-N-desmethylasenapine and 10, 11-dihydroxyasenapine**

LC analyses of peak F1/2 resulted in two fractions coded F1/2A and F1/2B. The \(^1\)H-NMR and TOCSY (2D-NMR) spectra of peak F1/2A showed many unknown, unrelated aliphatic and some small broader aromatic impurities. Some sharper aromatic signals were present (two singlets at 6.43 and 6.59 ppm H9 and H12, doublet at 7.08 ppm H7, doublet at 7.11 ppm.
H4, and double doublet at 7.17 ppm H6). LC-ES-MS analyses of this fraction resulted in the main ions m/z 304/306 ([M+H]+). Based on MS-MS results (a.o. observed loss of 43 Da, C2H5N, in the product ion spectrum of the protonated molecule), the peak F1/2A is most probably the N-desmethylasenapine with two hydroxyl groups. Based on the chemical shift and the pattern of the sharp aromatic protons and the absence of the signal at 2.95 ppm (as compared to F1/2B) in the NMR spectra and MS results, the structure of F1/2A is most probably the trans-5-chloro-10,11-dihydroxy-2,3,3a,12b-tetrahydro-1H-dibenz[2,3:6,7]-oxepino[4,5-c]pyrrole.

The 1H-NMR and TOCSY (2D-NMR) spectra of fraction F1/2B showed many unknown, unrelated aliphatic and some small broader aromatic impurities. Some sharper aromatic signals (two singlets at 6.41 and 6.57 ppm H9 and H12, doublet at 7.06 ppm H7, doublet at 7.08 ppm H4, and double doublet at 7.15 ppm H6) and a singlet at 2.95 ppm were present. LC-ES-MS analyses of this fraction resulted in the main ions m/z 318/320 ([M+H]+). Based on MS-MS results (a.o. an observed loss of 57 Da, C3H7N, in the product ion scan of the protonated molecule), the compound is most probably asenapine with two hydroxyl groups. Based on the chemical shift and the pattern of the sharp aromatic protons and the chemical shift of the signal at 2.95 ppm (N-CH3) in the NMR spectra and MS results, the structure of F1/2B is most probably trans-5-chloro-10,11-dihydroxy-2-methyl-2,3,3a,12b-tetrahydro-1H-dibenz[2,3:6,7]-oxepino[4,5-c]pyrrole.

F9: asenapine

The 1H-NMR and TOCSY (2D-NMR) spectra of peak F9 were compared with a primary reference standard of asenapine. The asenapine-related entity in the F9 fraction was in agreement with asenapine. In addition, some unknown, unrelated aromatic and many
unknown, unrelated aliphatic compounds were present. The LC-ES-MS spectrum of fraction F9 resulted in the main ions m/z 286/288, which corresponded to the [M+H]+ of asenapine. The product ion spectrum (MS-MS) of m/z 286 was also in agreement with asenapine.

F10: 11-hydroxy-N-formylasenapine

The 1H-NMR spectra of peak F10 were in agreement with Org 213979-0 (11-hydroxy-N-formylasenapine). LC-ES-MS analyses of this fraction resulted in the main ions m/z 316/318 and 314/316 (positive and negative mode), which corresponded to the [M+H]+ and [M-H]−, respectively, of the 11-hydroxy-N-formylasenapine. In addition, the product ion spectrum (MS-MS) of m/z 316 was in agreement with Org 213979-0 and showed a.o. the loss of 28 Da (CO) and 71 Da (C₂H₅NCO).

F11: 6-hydroxy-N-formylasenapine

LC-ES-MS analyses of this fraction resulted in the main ions m/z 316/318 and 314/316 (positive and negative mode). The product ion spectrum (MS-MS) of m/z 316 also showed the loss of 28 (CO) and 71 Da (C₂H₅NCO) but was not identical to that of Org 213979-0 (11-hydroxy-N-formylasenapine). This compound also eluted at a different retention time and probably has the same structure as Org 213979-0 (F10), with only the hydroxyl group at a different position. The 1H-NMR spectra of peak F11 were compared with the NMR spectra of N-formylasenapine, 11-hydroxy-N-formylasenapine, and 7-hydroxyasenapine. For the aromatic protons, no coupling appeared to be present between H4 and H6 at 6.71 and 6.73 ppm, as found for the 7-hydroxyasenapine reference. Therefore, this metabolite is tentatively identified as trans-5-chloro-2-formyl-6-hydroxy-2,3,3a,12b-tetrahydro-1H-dibenzo[2,3;6,7]-oxepino[4,5-c]pyrrole.
Discussion

The objective of this study was to investigate the excretion mass balance and metabolic profiling after sublingual administration of a single 10-mg dose of \([^{14}\text{C}]\)-labeled asenapine under steady-state conditions in healthy male volunteers and to compare with preclinical species used in safety assessment.

Mass balance resulted in a mean overall recovery of 93% of the administered radioactivity, of which >80% was excreted within 96 hours; excretion in the urine and feces accounted for 53% and 39%, respectively.

Plasma concentrations of total radioactivity greatly exceeded those of asenapine at any time point, indicating that asenapine is metabolized extensively and rapidly. Our results showed that the asenapine-\(N^+\)-glucuronide accounted for most of the radioactivity in plasma, followed by the N-desmethyl carbamoyl glucuronide. The three other radioactive detectable circulating compounds were asenapine, N-desmethylasenapine, and asenapine 11-hydroxysulphate. All other metabolites, as identified in urine and feces, were either not present or below the limit of radioactivity detection and/or identification. Asenapine-\(N\)-oxide was detected bioanalytically in some plasma samples (0.5 hours up to 1.5 hours post dosing) at very low levels (maximally, 0.2 ng·mL\(^{-1}\)) but was below the limit of radioactivity detection in all plasma samples analyzed.

Pre-clinical data on file showed that the basic amine functionality is essential for the pharmacological activity of asenapine. Asenapine \(N^+\)-glucuronide and N-desmethyl-asenapine have a diminished potency toward the receptors to which asenapine binds. While N-desmethyl-N-carbamoyl-asenapine glucuronide was not tested for activity, the fact that the nitrogen is no longer basic would lead to loss of binding activity. 11-hydroxy-asenapine sulphate was also tested and showed a similar receptor-binding profile as asenapine. However, it was shown in the rat that this metabolite does not penetrate the brain. Other
putative metabolites that were tested for receptor activity included asenapine N-oxide, 11-hydroxyasenapine, and 7-hydroxy asenapine (data on file, 2008). These metabolites were below the limit of radioactivity detection in human circulation and thus it is unlikely that these metabolites contribute towards the pharmacological effects of asenapine. These data suggest that circulating human metabolites of asenapine do not contribute to the pharmacologic effects of asenapine.

Metabolites observed were mainly derived from reactions at the nitrogen in the five-membered ring of asenapine. The primary route is direct glucuronidation. The main enzyme responsible for the direct glucuronidation of asenapine is UGT1A4 (data on file, 2008). The biotransformation of quaternary ammonium $N^+$-glucuronidation has been characterized in humans for more than 30 drugs and xenobiotics, including antihistamines, antidepressants, and antipsychotics (Hawes, 1998).

Glucuronidation is generally considered to be a pathway of detoxification, which commonly transforms lipophilic compounds into hydrophilic metabolites. Quaternary ammonium $N^+$-glucuronides are not associated with toxicity. Asenapine $N^+$-glucuronide is not suspected to cause any adverse effects because: (1) it would not be expected to be chemically reactive and is a stable compound; (2) it is not pharmacologically active and would not contribute to suprapharmacologic effects; (3) it is present to some extent in animal species used in safety assessments after administration of asenapine; and (4) asenapine is dosed maximally at 20 mg/day, resulting in low levels of circulating $N^+$ glucuronide (<1 μM). Moreover, asenapine $N^+$-glucuronide is of a class of metabolites that have been shown to be present in humans for other drugs at much greater levels than for asenapine (Hawes, 1998).

Also important but apparently less pronounced is the demethylation pathway, followed by association with carbamic acid and conjugation with glucuronic acid, resulting in the N-desmethyl carbamoyl glucuronide. It is not unreasonable to propose that the carbamic
acid of asenapine serves as the transiently formed intermediate for glucuronidation. For several drugs containing a primary, secondary, or tertiary amine, the formation of a carbamic acid followed by a glucuronidation has been observed (Ronfeld et al., 1982; Straub et al., 1988; Brown et al., 1990; Delbressine et al., 1990; Obach et al., 2005; Obach et al., 2006; Schaeffer, 2006). An unprotonated primary or secondary amine and CO₂ are required to form a carbamic acid (Delbressine et al., 1990), which was subsequently conjugated with the glucuronic acid (Schaeffer, 2006). The formation of a carbamate glucuronide has been observed in excreta or the circulating system of several species, including humans, for several drugs, including tocainide (Ronfeld et al., 1982), sertraline (Obach et al., 2005), rimantadine (Brown et al., 1990), and varenicline (Obach et al., 2006). Some drugs that contained tertiary amines, for instance SK&F86466 (Straub et al., 1988) and mirtazapine (Delbressine et al., 1990), are initially N-dealkylated to yield a secondary amine that is subsequently metabolized to a carbamate glucuronide.

The N⁺-glucuronide and the carbamoylglucuronide formation are likely due to the nucleophilic nature of the nitrogen in asenapine. It is instructive to compare these human biotransformation pathways of asenapine with mirtazapine and mianserin, drugs with a similar structure to asenapine. All three form the N⁺-glucuronide, whereas the carbamoylglucuronide was found only for mirtazapine and asenapine. These biotransformation routes are more dominantly present for asenapine, followed by mirtazapine and then mianserin, in line with the nucleophilic character of the nitrogen (asenapine>mirtazapine>mianserin). Moreover, due to this chemical property, the formation of the N-oxide metabolites as found in some urine and fecal samples are either generated by an enzymatically catalyzed process or formed spontaneously during the sample treatment.

Asenapine metabolism includes next to reactions at the nitrogen also oxidative (phase I) reactions at various positions. Formation of phase I metabolites, as investigated with
individual cDNA-expressed major human cytochrome P450s (CYP1A2, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1, 3A4 and 3A5) incubated with asenapine and human liver microsomes incubated with asenapine and specific enzyme inhibitors (data on file, 1998), was shown to be catalyzed mainly by CYP1A2 with contributions of CYP3A4 and CYP2D6. In humans, the 11-hydroxylated asenapine metabolite was mainly present in conjugated form. The mono-hydroxylated intermediate could be further hydroxylated into a 10, 11 di-hydroxy intermediate. This diol was either conjugated at the 10 or 11 position, occasionally combined with methylation of the remaining hydroxy moiety. The formation of all these regio-isomeric conjugates, the glucuronides present as diastereoisomers, in combination with demethylation or N-oxidation resulted in many structurally related metabolites, which, under the conditions applied, could hardly be separated. Spectral (MS and NMR) analyses often showed that the HPLC fractions consisted of a mixture of metabolites. For that reason and because of the lack of the corresponding reference compounds, no attempts were made to quantify the individual metabolites formed from asenapine.

Metabolites observed in humans were also observed in laboratory animals at least on the basis of retention time comparison (data on file and van den Wildenberg et al., 1990). In addition, all metabolic pathways as observed in human have been observed in preclinical species which confirm that the choice of preclinical species used in safety assessment was acceptable.

In summary, the overall disposition and metabolism of asenapine has been determined in male volunteers. Asenapine, after a single sublingual radiolabeled dose under steady-state conditions, is absorbed rapidly and substantially. Total excretion averaged 90% with excretion being slightly higher in urine than in feces. Metabolism appeared to be extensive and resulted in several regio-isomeric hydroxylated and conjugated metabolites. The metabolic reactions occurred mainly at the nitrogen, resulting in direct glucuronidated and
demethylated metabolites which do not contribute to the pharmacological effects of asenapine.
Acknowledgments

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Conducted experiments: E. Spaans, PAM Peeters, GJ Kemperman and SFM van de Wetering-Krebbers

Contributed new reagents or analytical tools: GJ Kemperman

Performed data analysis: PL Jacobs and SFM van de Wetering-Krebbers

Wrote or contributed to the writing of the manuscript: PL Jacobs, MLPS van Iersel, LPC Delbressine and SFM van de Wetering-Krebbers
References

(2010) Saphris® (asenapine) sublingual tablets. Full Prescribing Information, Schering-Plough, a subsidiary of Merck & Co., Inc., Whitehouse Station, NJ.


Footnotes

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Legends for Figures

Figure 1. Structure of asenapine maleate.

Figure 2. Mean plasma concentration versus time curves.

Figure 3. Mean values of cumulative excretion of [14C]-radioactivity after multiple sublingual dosing of unlabeled asenapine followed by a single sublingual dose of [14C]-asenapine to healthy male volunteers.

Figure 4. Proposed metabolic routes of asenapine. * very low levels of asenapine N-oxide is detected by LC-MS analysis in plasma. In urine this metabolite was only found in combination with other metabolic reactions. ** Only found in combination with other metabolic reactions. *** asenapine is a racemic mixture.

Figure 5. HPLC metabolite profiles in pooled plasma (1.5–12 h, Subjects 1-4) of healthy male volunteers after multiple sublingual dosing of unlabeled asenapine followed by a single sublingual dose of [14C]-asenapine. P1, asenapine 11-O-sulphate and some other hydroxylated and conjugated metabolites; P2/3, asenapine N+-glucuronide (diastereoisomeric mixture); P4, N-desmethysenapine-N-carbamoylglucuronide; P5, N-desmethyl-asenapine; P6, asenapine. The structures of the other peaks remain unidentified.

Figure 6. HPLC metabolite profile in urine (0–48 h, Subject 1) after multiple sublingual dosing of unlabeled asenapine followed by a single sublingual dose of [14C]-asenapine. U1, methoxy, glucuronide of 10, 11-dihydroxy-N-desmethysenapine in which the position of
the methoxy and glucuronide is 10,11 and the reverse and a mono glucuronide of 10,11-dihydroxy-N-desmethylasenapine; U2/3, methoxy, sulphate of 10,11-dihydroxy-N-desmethylasenapine in which the position of the methoxy and sulphate is 10,11 or the reverse, the glucuronide of 11-hydroxy-N-desmethylasenapine, the asenapine 11-O-glucuronide plus some undefined conjugated metabolites (sulphates and glucuronides); U4/5 contained some undefined conjugated metabolites (sulphates and glucuronides); U6/7 is identified as asenapine 11-O-sulphate plus some other undefined conjugated metabolites (sulphates and glucuronides) of most probably the asenapine N-oxide; U8/9 is identified as asenapine N’-glucuronide; U10, is identified as N-desmethylasenapine-N-carbamoylglucuronide. The structures of the other peaks remain unidentified.

**Figure 7.** HPLC metabolite profile in feces (48–72 h, Subject 1) after multiple sublingual dosing of unlabeled asenapine followed by a single sublingual dose of \(^{14}\text{C}\)-asenapine to healthy male volunteers. F1/2, 10,11-dihydroxy-N-desmethylasenapine and 10,11-dihydroxyasenapine; F8 co-elutes with the N-desmethylasenapine; F9, asenapine; F10, 11-hydroxy-N-formylasenapine; F11, X-hydroxy-N-formylasenapine (most probably the 6-hydroxy). The structures of the other peaks remain unidentified.
Table 1. Dosing schedule<sup>a</sup>

<table>
<thead>
<tr>
<th>Day -1</th>
<th>Day 1</th>
<th>Day 2</th>
<th>Day 3</th>
<th>Day 4</th>
<th>Day 5</th>
<th>Day 6</th>
<th>Day 7</th>
<th>Day 8</th>
<th>Day 9</th>
<th>Day 10</th>
</tr>
</thead>
<tbody>
<tr>
<td>Placebo</td>
<td>0.3 mg</td>
<td>1 mg</td>
<td>3 mg</td>
<td>5 mg</td>
<td>10 mg</td>
<td>10 mg</td>
<td>10 mg</td>
<td>10 mg</td>
<td>10 mg</td>
<td>10 mg+&lt;sup&gt;14&lt;/sup&gt;C</td>
</tr>
</tbody>
</table>

<sup>a</sup>All doses were given twice daily, except on day 10.
Table 2. Reference compounds used for identification of metabolites isolated from plasma, urine, and feces after sublingual dosing of unlabeled asenapine followed by a single sublingual dose of $^{[14C]}$-asenapine

<table>
<thead>
<tr>
<th>Org code</th>
<th>Short metabolite name</th>
<th>Chemical name</th>
</tr>
</thead>
<tbody>
<tr>
<td>Org 5222</td>
<td>asenapine</td>
<td>(3aRS,12bRS)-5-Chloro-2-methyl-2,3,3a,12b-tetrahydro-1H-dibenz[2,3:6,7]-oxepino[4,5-c]pyrrole (2Z)-2-butenedioate (1:1)</td>
</tr>
<tr>
<td>Org 30526</td>
<td>N-desmethylasenapine</td>
<td>trans-5-chloro-2-H-2,3,3a,12b tetrahydro-1H-dibenzo[2,3:6,7]-oxepino[4,5-c]pyrrole maleate</td>
</tr>
<tr>
<td>Org 31437</td>
<td>asenapine N-oxide</td>
<td>trans-5-chloro-2,3,3a,12b-tetrahydro-2-methyl-1H-dibenzo[2,3:6,7]-oxepino[4,5-c]pyrrole 2-oxide</td>
</tr>
<tr>
<td>Org 207652-0</td>
<td>N-formylasenapine</td>
<td>trans-5-chloro-2-formyl-2,3,3a,12b-tetrahydro-1H-dibenzo[2,3:6,7]-oxepino[4,5-c]pyrrole</td>
</tr>
<tr>
<td>Org 213772-0</td>
<td>11-hydroxyasenapine</td>
<td>trans-5-chloro-11-hydroxy-2-methyl-2,3,3a,12b-tetrahydro-1H-dibenzo[2,3:6,7]-oxepino[4,5-c]pyrrole</td>
</tr>
<tr>
<td>Org 213913-1</td>
<td>11-hydroxy-N-</td>
<td>trans-5-chloro-11-hydroxy-2,3,3a,12b-tetrahydro-1H-dibenzo[2,3:6,7]-oxepino[4,5-c]pyrrole hydrochloride</td>
</tr>
<tr>
<td>Org 214025-0</td>
<td>asenapine 11- O-sulfate</td>
<td>trans-5-chloro-11-sulfoxy-2-methyl-2,3,3a,12b-tetrahydro-1H-dibenzo[2,3:6,7]-oxepino[4,5-c]pyrrole</td>
</tr>
<tr>
<td>Org 216761-0</td>
<td>asenapine N$^+$-glucuronide</td>
<td>trans-5-chloro-2(ß-D-glucopyranuronosyl)-2-methyl-2,3,3a,12b-tetrahydro-1H-dibenzo[2,3:6,7]-oxepino[4,5-c]pyrrolium</td>
</tr>
<tr>
<td>Org</td>
<td>Name</td>
<td>Formula</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------------------------------------</td>
<td>-------------------------------------------------------------------------</td>
</tr>
<tr>
<td>220473-0</td>
<td>7-hydroxyasenapine</td>
<td>trans-5-chloro-7-hydroxy-2-methyl-2,3,3a,12b-tetrahydro-1H-dibenzo[2,3:6,7]-oxepino[4,5-c]pyrrole</td>
</tr>
<tr>
<td>213979-0</td>
<td>11-hydroxy-N-formylasenapine</td>
<td>trans-5-chloro-2-formyl-11-hydroxy-2,3,3a,12b-tetrahydro-1H-dibenzo[2,3:6,7]-oxepino[4,5-c]pyrrole</td>
</tr>
<tr>
<td>237498-0</td>
<td>11-methoxyasenapine</td>
<td>trans-5-chloro-2-methyl-11-methoxy-2,3,3a,12b-tetrahydro-1H-dibenzo[2,3:6,7]-oxepino[4,5-c]pyrrole</td>
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<tr>
<td>225789-0</td>
<td>11-hydroxyasenapine N-oxide</td>
<td>trans-5-chloro-2,3,3a,12b-tetrahydro-11-hydroxy-2-methyl-1H-dibenzo[2,3:6,7]-oxepino[4,5-c]pyrrole 2-oxide</td>
</tr>
<tr>
<td>191475-0</td>
<td>N-desmethylasenapine-N-carbamoylg glucuronide</td>
<td>1-((3aR,12bR)-rel-5-chloro-2,3,3a,12b-tetrahydro-1H-dibenzo[2,3:6,7]-oxepino[4,5-c]pyrrole-2-carboxylate)-D-glucopyranuronic acid</td>
</tr>
</tbody>
</table>
Table 3. Major MS-MS product ions of proposed metabolite structures for the main biotransformation routes of asenapine, as shown in Figure 4

<table>
<thead>
<tr>
<th>Metabolite ID</th>
<th>Proposed structure</th>
<th>Precursor Ion</th>
<th>Product Ions (Positive Ion Electrospray)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1 U6/7A</td>
<td>asenapine 11- O-sulphate</td>
<td>382 (QqTOF)</td>
<td>302 (loss of SO₃)</td>
</tr>
<tr>
<td>P2/3 U8/9A+B</td>
<td>asenapine N⁺-glucuronide</td>
<td>462 (QqTOF)</td>
<td>286, 159, 131</td>
</tr>
<tr>
<td>P4 U10</td>
<td>N-desmethylasenapine- N-carbamoylglucuronide</td>
<td>492 (QqTOF)</td>
<td>316, 272</td>
</tr>
<tr>
<td>P5 F8</td>
<td>N-desmethylasenapine</td>
<td>272 (QqTOF)</td>
<td>229, 215, 201, 166</td>
</tr>
<tr>
<td>Metabolite</td>
<td>Structure</td>
<td>MRM Transition</td>
<td>MRM Peaks</td>
</tr>
<tr>
<td>------------</td>
<td>-----------</td>
<td>----------------</td>
<td>-----------</td>
</tr>
<tr>
<td><strong>P6</strong> asenapine</td>
<td><img src="image" alt="Structure" /></td>
<td>286 (QqTOF)</td>
<td>229, 215, 201, 166</td>
</tr>
<tr>
<td><strong>F9</strong> 10, 11-dihydroxy N-desmethylasenapine</td>
<td><img src="image" alt="Structure" /></td>
<td>304 (QqTOF)</td>
<td>287, 275, 261, 252 (no Cl), 233, 156</td>
</tr>
<tr>
<td><strong>F1/2A</strong> 10, 11-dihydroxy of asenapine</td>
<td><img src="image" alt="Structure" /></td>
<td>318 (QqTOF)</td>
<td>261, 44</td>
</tr>
<tr>
<td><strong>F10</strong> 11-hydroxy-N-formylasenapine</td>
<td><img src="image" alt="Structure" /></td>
<td>316 (IT)</td>
<td>288, 271, 259, 245, 236 (no Cl), 176 (no Cl), 165</td>
</tr>
<tr>
<td><strong>F11</strong> most probably 6-hydroxy-N-formylasenapine</td>
<td><img src="image" alt="Structure" /></td>
<td>316 (IT)</td>
<td>288, 271, 259, 245, 236 (no Cl), 176 absent, 165</td>
</tr>
</tbody>
</table>

*Additional proposed metabolite structures are not described in this table but only in the results paragraph and Figure 4.*
Figure 4