Characterization of midazolam metabolism in locusts: the role of a CYP3A4-like enzyme in the formation of 1'-OH and 4-OH midazolam

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Abstract

1. The metabolism of midazolam was investigated in vivo in locusts in order to evaluate the presence of an enzyme with functionality similar to human CYP3A4/5.
2. Hydroxylated metabolites of midazolam identical to human metabolites were detected in locusts and the apparent affinities (Km values) were in the same range as reported in humans (in locusts: 7–23 and 33–85 µM for the formation of the 1'-OH and 4-OH metabolites, respectively).
3. The formation of hydroxylated metabolites could successfully be inhibited by co-administration of ketoconazole, a known CYP3A4/5 inhibitor.
4. Besides phase I metabolites, a number of conjugated metabolites were detected using high-resolution mass spectrometry. The most abundant metabolites detected were structurally identified by 1H NMR as two N-glucosides. NMR analysis strongly suggested that the glycosylation occurred at the two nitrogens (either one in each case) of the imidazole ring.
5. Distribution of midazolam and the glucose conjugates were successfully measured using desorption electrospray mass spectrometry imaging revealing time-dependent changes in distribution over time.
6. In conclusion, it appears that an enzyme with functionality similar to human CYP3A4/5 is present in locusts. However, it appears that conjugation with glucose is the main detoxification pathway of midazolam in locusts.

Introduction

The use of invertebrates in research has enabled the discovery of many fundamental biological mechanisms, e.g. apoptosis and embryogenesis and has expanded our knowledge of genetic and inheritance (Pandey & Nichols, 2011; Ségalat, 2007; Wilson-Sanders, 2011). The fruit fly, Drosophila melanogaster (Dm), is one of the most commonly used invertebrates and several reviews have been published covering the use of this invertebrate in life science research (Pandey & Nichols, 2011; Ségalat, 2007; Wilson-Sanders, 2011).

Dm has the advantage of a complete genome sequenced, but as it is a relatively small insect, it is difficult to handle. Recently, the desert locust (Scistocerca gregaria) was proposed as a model insect for brain permeation studies (Andersson et al., 2013, 2014). Locusts are larger insects than Dm, and a full pharmacokinetic profile can be obtained following administration of a substrate, since multiple samples can be obtained from the same insect. Pharmacokinetic parameters such as drug half-life and clearance can thus be used for calculation of brain exposure. Furthermore, as found in humans, Cytochrome P450 (CYP) enzymes have been shown to play an important role in detoxification of xenobiotics in insects (Scott, 2008; Scott & Wen, 2001) and it was recently suggested that locusts may have an enzyme with functionality similar to the human CYP3A4/5. In humans, terfenadine is metabolized by CYP3A4/5 and it was shown, that identical metabolites were formed in locusts and human liver microsomes (HLMs), and the formation in locusts could be significantly inhibited by co-administration of ketoconazole, a specific CYP3A4/5 inhibitor (Olsen et al., 2014).

The insect CYP enzymes identified so far share little sequence identity with human CYP enzymes and even between different insect species large variation exists (Feyereisen, 2012). In order to determine whether locusts have an enzyme with functionalities similar to human CYP3A4/5, the metabolism of additional compounds specific to the enzyme should be investigated in locusts.
Besides metabolism, insects are also capable of eliminating xenobiotics from hemolymph by sequestration. Depending on the insect and the toxin, sequestration is acting as either a defence mechanism to avoid circulation of toxic chemicals from, e.g., plants, or self-defense strategy rendering the insect toxic to its predators following ingestion of toxic material (Opitz & Muller, 2009). This was investigated in the present study by desorption electrospray mass spectrometry (DESI-MS) imaging.

Midazolam is another CYP3A4/5-specific compound and is one of the most frequently used drugs to determine the activity of CYP3A4/5. In HLM, two different hydroxylated metabolites are formed, namely 1’-hydroxymidazolam (1’-OH MDZ) and 4-hydroxymidazolam (4-OH MDZ); where 1’-OH MDZ accounts for >90% of the phase I metabolites (Yuan et al., 2002).

In order to further validate the presence of an enzyme functionally similar to the human CYP3A4/5, the metabolism of midazolam in locusts was investigated. Furthermore, the tissue distribution of parent compound and metabolites was investigated using DESI-MS imaging.

Materials and methods

Animals

Desert locusts (S. gregaria) were purchased from a commercial breeder (Petra Aqua, Prague, Czech Republic) and housed under crowded conditions with a 12:12-h light/dark cycle. Animals were purchased from a commercial breeder (Petra Aqua, Prague, Czech Republic) and housed under crowded conditions with a 12:12-h light/dark cycle. They were fed Chinese cabbage and wheat bran. All the experiments were performed on adult, male locusts.

Chemicals

All the solvents used were HPLC grade or higher. Methanol (MeOH) and acetonitrile (MeCN) were purchased from VWR. All the solvents used were HPLC grade or higher. Methanol (MeOH) and acetonitrile (MeCN) were purchased from VWR. acetonitrile (MeCN) were purchased from VWR. acetonitrile (MeCN) were purchased from VWR. formic acid, quinidine, α-naphthoflavone, sulphaphenazole, nootkatone, ketoconazole, amitriptyline, carboxy-methyl-cellulose sodium salt and methanol-d₄. Dimethylsulfoxide (DMSO) was purchased from Sigma–Aldrich (St. Louis, MO): 1’-OH MDZ, 4-OH MDZ, formic acid, quinidine, α-naphthoflavone, sulphaphenazole, nootkatone, ketoconazole, amitriptyline, carboxy-methyl-cellulose sodium salt and methanol-d₄. Dimethylsulfoxide (DMSO) was purchased from Sigma–Aldrich (St. Louis, MO).

Midazolam (Dormicum®) 5 mg/ml was obtained from F. Hoffmann-La Roche AG (Basel, Schweiz). Milli-Q water (MQ) for mobile phases and solutions was water purified with the Direct-Q 3 UV system (Millipore, Billerica, MA). Midazolam (Dormicum®) 5 mg/ml was obtained from F. Hoffmann-La Roche AG (Basel, Schweiz). Milli-Q water (MQ) for mobile phases and solutions was water purified with the Direct-Q 3 UV system (Millipore, Billerica, MA).

Locust experiments

To identify possible CYP enzymes involved in the phase I metabolism of midazolam, 40 μl of a 1-mM solution of midazolam (dissolved in 0.1% lactic acid, 5% DMSO) was administered with and without inhibitor (2.5 mM) of selected CYP iso-enzymes. Midazolam was administered to locusts in co-solution with an inhibitor (one at a time) specific to each of the five most important drug metabolizing CYP enzymes in humans, namely 1A2 (α-naphthoflavone), 2C9 (sulphaphenazole), 2C19 (nootkatone), 2D6 (quinidine) and 3A4/5 (ketoconazole). Hemolymph (20 μl) was sampled using an end-to-end micro pipette (Vitrex Medical, Herlev, Denmark) 60 min after injection. The hemolymph sample was precipitated with 60 μl 2% ZnSO₄ in 50% MeOH containing 500 mM amitriptyline as internal standard. The concentration of DMSO was 5% resulting in a hemolymph concentration of <1%. In case an inhibitor had significant effect on the amount of metabolite formed, a full pharmacokinetic profile with and without the inhibitor was obtained. Ten microliters of hemolymph was sampled at 1, 5, 10, 20, 30, 45, 60 and 120 min after injection from the same animal (80 μl from each animal in total). To increase the amount of metabolite for tentative identification, feces of four-dosed locusts were collected and analyzed 24 h after dosing (n = 3).

Estimation of Michaelis–Menten parameters for the formation of metabolites was done by injecting locusts with 40 μl of five different concentrations of midazolam (500 μM, 1 mM, 2 mM, 3 mM and 5 mM in MQ water) and after 1 min, a hemolymph sample was taken (20 μl) and precipitated with 80 μl 2% ZnSO₄ in 50% MeOH containing 500 mM amitriptyline as internal standard. Protein precipitation was done on Waters Sirocco™ (Milford, MA) protein precipitation plates. The plates were centrifuged at 2000 × g for 1 min and the clear filtrate was analyzed using liquid chromatography-high-resolution mass spectrometry (LC-HRMS).

LC–HRMS method

The chromatographic separation was performed on a Kinetex C18 (50 mm × 2.1 mm i.d.) column (Phenomenex) with 1.7-μm particles using a Dionex UltiMate 3000 RS ultra high-performance liquid chromatography system (Thermo Scientific, Waltham, MA) coupled to a high-resolution MS instrument [Q Exactive Orbitrap with a HESI-II interface (Thermo Scientific)]. Separation was performed with gradient elution using 0.1% formic acid in Milli-Q water as mobile phase A and MeOH with 0.1% formic acid added as mobile phase B with a flow rate of 0.5 ml/min. The mobile phase B was kept at 5% for 0.2 min followed by a linear gradient of 5–90% B over 6 min, 90% B for 1 min, followed by re-equilibration for 2 min. The column compartment was adjusted to 30°C, and the samples were kept at 5°C. UV detection was performed at 210 nm.

Detection of metabolites was performed in positive ionization mode using data-dependent tandem MS (MS²) on the top five most abundant ions in each scan with a dynamic exclusion of 5 s. Quantification of midazolam and 1’-OH and 4-OH midazolam was performed in targeted MS². The linear range of the standard curves was 2–400 nM for the metabolites and 40–4000 nM for midazolam. Other MS setting are described in Table 1. The lock mass that was used (214.0896 m/z) originated from a plasticizer, N-butyl benzenesulfonamide. Spray voltage was set to 3.3 kV, capillary temperature 350°C, sheath gas 30 arbitrary units, auxiliary gas 10 arbitrary units, probe heater 250°C and S-lens RF level 50. Thermo Scientific Mass Frontier 7.0 Spectral Interpretation Software (San Jose, CA) was used for identification of metabolites.

HPLC-HRMS-SPE-NMR

Locusts were injected with 8 mg/kg midazolam (1 mM, 40 μl) each day for several days in order to produce sufficient amounts of feces containing high amounts of metabolites for structural elucidation. The metabolites were extracted from...
feeces (~3 g) with 10 ml 100% MeOH. The extract was centrifuged at 15 000 × g for 10 min and the supernatant was filtered through 0.45 μm Milllex-HV syringe filters (Millipore). The supernatant was evaporated to dryness under nitrogen and residue was re-dissolved in 0.5 ml MeOH. The metabolites were separated using an Agilent 1200 system (Santa Clara, CA) comprising a photodiode-array detector, an auto sampler, a quaternary pump, a degasser and a thermostatted column compartment. The separation was performed at 25 °C using a Phenomenex C18(2) Luna column (150 mm × 4.6 mm i.d., 3 μm particle size, 100 Å pore size) (Phenomenex Inc., Torrance, CA). The flow was maintained at 0.5 ml/min using a binary gradient mixture of acetonitrile:water (5:95, v/v, solvent A) and acetonitrile:water (95:5, v/v, solvent B); both acidified with 0.1% formic acid. The elution gradient was as follows: 0 min, 20% B; 2 min, 20% B; 20 min, 30% B; 30 min, 50% B; 31 min, 20% B, followed by 10 min equilibration prior to the subsequent injection. A small proportion (1%) of the HPLC eluate was directed to a micrOTOF-Q II mass spectrometer (Bruker Daltonik GmbH, Bremen, Germany) equipped with an electrospray ionization interface. Mass spectra were acquired in positive ionization mode, using drying temperature of 200 °C, capillary voltage of 4100 V, nebulizer pressure of 2.0 bar and drying gas flow of 7 l/min. A solution of sodium formate clusters was automatically injected in the beginning of each run to enable internal mass calibration. The rest of the HPLC eluate was directed to the photo-diode array detector, and subsequently to a Prospekt 2 SPE-unit (Spark Holland, Emmen, The Netherlands). Cumulative SPE trappings of individual analytes were performed for 10 consecutive injections (20 μl/injection) using extracted ion chromatograms of the individual metabolites m/z to trigger analyte trapping. Chromatography, peak trapping and analyte transfer from the SPE unit were controlled with HyStar ver. 3.2 software (Bruker Biospin GmbH, Rheinstetten, Germany). The HPLC eluate was diluted with Milli-Q water at a flow rate of 1.0 ml/min, prior to trapping on 10 mm × 2 mm i.d. Resin GP (general purpose, 5–15 μm, spherical shape, polydivinylbenzene phase) SPE cartridges from Spark Holland (Emmen, The Netherlands). After drying for 45 min with pressurized nitrogen gas, analytes were automatically eluted into 1.7 mm o.d. NMR tubes (96 position tube racks) with methanol-d4 using a Gilson Liquid Handler controlled by PrepGilon software Version 1.2 (Bruker Biospin GmbH, Rheinstetten, Germany). NMR experiments were performed with a Bruker Avance III system (1H operating frequency of 600.13 MHz) equipped with a Bruker SampleJet samplechanger and a cryogenically cooled gradient inverse triple-resonance 1.7 mm TCI probe-head (Bruker Biospin) optimized for 1H and 13C observation. Icon NMR (version 4.2, Bruker Biospin) was used for controlling automated acquisition of NMR data and processing of NMR data was performed using Topspin (version 3.2, Bruker Biospin). All NMR spectra were recorded in methanol-d4 at 300 K, and 1H chemical shifts were referenced to the residual solvent signal (δ 3.31). One-dimensional 1H NMR spectra were acquired in automation (temperature equilibration, optimization of lock parameters, gradient shimming and setting of receiver gain) with 30°-pulses, 3.66 s inter-pulse intervals, 64 k data points and multiplied with an exponential function corresponding to line-broadening of 0.3 Hz prior to Fourier transform.

**DESI-MS imaging**

Locusts were dosed by intrahemolymphic injection with 8 mg/kg midazolam (40 μl, 1 mM). The locusts were left for 30 min or 2 h before they were euthanized by freezing. The dead locusts were embedded in an aqueous 5% carboxymethylcellulose gel on a cryotome sample holder at −20 °C. The experiments were carried out on a total of n = 3 animals, and the results presented here are representative for these experiments.

Sagittal 50 μm thick whole-body cryosections were prepared using a Leica CM3050S cryomicrotome (Leica Microsystems, Wetzlar, Germany), thaw-mounted on microscope glass slides (VWR, Herlev, Denmark) and stored at −80 °C until analysis. The sample slides were placed in a desiccator for 10 min prior to DESI-MS analysis. DESI imaging of the locusts was performed on a Thermo LTQ XL linear ion trap mass spectrometer (Thermo Scientific, Bremen, Germany), equipped with a custom built DESI imaging as described in details elsewhere (Thunig et al., 2011). Data conversion was made with an imzML converter (available from www.maldi-msi.org) (Schramm et al., 2012), and Data Cube Explorer (AMOLF, Amsterdam) was used for image generation.

The imaging analysis was performed in the positive ion mode with scan range m/z 150–900, using a 5-μl/min flow of methanol and water (95:5) and a nebulizer gas pressure of 9 bar. All the images were acquired at 240-μm spatial resolution, using the displaced dual mode imaging method (Janfelt et al., 2013) for simultaneous acquisition of full-scan and MS/MS images. The sample was moving in the x-dimension at a speed corresponding to 240 μm per spectrum acquisition time. In the y-dimension, the row distance was 80 μm and three different scan modes were used in a cycle, such that three separate imaging experiments were running in parallel, each with 240 μm row distance in the y-dimension. In this way, a full scan imaging experiment and two additional MS/MS experiments (one for the parent drug and one for a selected metabolite) were obtained in alignment on the same sample.

To confirm the findings of the DESI-MS imaging experiments, the crop content of locusts dosed with midazolam was separated from the remaining intestine and extracted with 50% MeOH and treated like fecal extracts and analyzed with LC-HRMS.

**Results**

The influence of specific CYP inhibitors was investigated in locusts by injecting midazolam to locusts in co-solution with
an inhibitor (one at a time) specific to human CYP1A2 (α-naphthoflavone), 2C9 (sulphaphenazole), 2C19 (nootkatone), 2D6 (quinidine) and 3A4/5 (ketoconazole). One hour after administration, hemolymph was sampled and the formation of 1'-OH and 4-OH MDZ was measured. The findings are depicted in Figure 1.

Both of the human CYP3A4/5-mediated specific midazolam metabolites (1'-OH-MDZ and 4-OH-MDZ) were detected in locust hemolymph. As can be seen in Figure 1, only ketoconazole could significantly inhibit the formation of the two hydroxylated metabolites.

A pharmacokinetic profile of the elimination of midazolam and formation of metabolites was obtained with and without co-administration of the inhibitor ketoconazole. No significant difference in the elimination profile of midazolam was seen (Figure 2). However, the formation of 1'-OH and 4-OH midazolam was significantly inhibited. As can be seen in Figure 2, formation of metabolites increase in the initial 20–30 min after which the amount decreases for both metabolites. The area under curve (AUC) was calculated without and with co-administration of ketoconazole. The results are listed in Table 2.

An attempt was made to calculate an apparent $K_m$ value for the formation of both hydroxylated metabolites formed in vivo in locusts. Locusts were injected with different concentrations of midazolam, and the initial formation rate of metabolites was estimated. From Figure 2, it can also be seen that the formation of the two hydroxylated metabolites is only linear in the initial couple of minutes and thus, the initial rate should be measured within this time range. The parameters were estimated based on the concentration of midazolam (plotted on the X-axis) and concentration of metabolite in the hemolymph after only 1 min. The $K_m$ values were calculated by non-linear regression (Figure 3). $K_m$ for the 1'-OH metabolite in locusts was calculated to be $15 \pm 8 \mu M$ and the $K_m$ for 4-OH in locusts was calculated to be $59 \pm 26 \mu M$.

In order to assess the metabolites excreted from locusts, feces of dosed animal were collected and analyzed. Based on the responses in the MS and UV, it was seen, that midazolam is mostly metabolized and only to a minor degree excreted as the original drug (data not shown). The most abundant metabolites tentatively identified in hemolymph and in feces were two different glucose conjugates of midazolam.
The structures were confirmed with NMR (see Supplementary Material), and based on the chemical shifts the glycosylation was found to occur on the nitrogens in the imidazole ring (Figure 4). Besides the two glucosides, a number of other conjugates were detected. The most abundant metabolites detected in locusts dosed with midazolam are shown in Figure 4. The metabolites M3 and M4 were only tentatively identified based on exact mass and fragmentation patterns.

The identities of M3 and M4 in Figure 4 were determined from exact mass and the MS2 spectrum as shown in Figure 5. The compound with m/z 568.105 gave a characteristic chlorine pattern in full scan and identical fragments of the compound and midazolam were obtained. Besides the fragments characteristic for midazolam, an additional fragment with m/z 98.98 was seen corresponding to H4PO4+ with a mass accuracy within 5 ppm on the fragment. The mass accuracy of the glucose-phosphate conjugate (m/z 568.105) was within 1 ppm. The absence of hydroxyl groups on midazolam suggests that phosphorylations occur on the glucose moiety. The quality of NMR data obtained in the HPLC-HRMS-SPE-NMR mode did not allow for unambiguous identification of metabolite M3 and M4.

Several additional phase I and phase II metabolites of midazolam were detected in locusts, all containing the characteristic chlorine pattern. Some of these could be tentatively identified based on exact mass and fragmentation patterns and included an additional hydroxylated metabolite (m/z 342) and glucose conjugates of the hydroxylated metabolites (m/z 504). No further attempt to structurally identify these metabolites based on fragmentation pattern was performed.

The distribution of midazolam and the glucoside metabolites in locust tissues was measured 30- or 120-min post-administration, using DESI-MS imaging; the results are presented in Figure 6(b)–(e) (30-min post-administration) and Figure 6(g)–(j) (120-min post-administration). Figure 6(a) and (f) show a picture of the locusts taken during cryosectioning. Figure 6(b) and (g) show the extracted m/z 156 (betaine) from a full scan and Figure 6(c) and (h) show m/z 782 (phosphatidyl choline), respectively. These masses are endogenous compounds and show the shape of the imaged locust and the identification of these compounds have been described previously (Olsen et al., 2015). MS2 of midazolam (326->291) is shown in Figure 6(d) and (i). After 30 min (Figure 6d), midazolam was distributed in the entire locust but with highest abundance (intensity) in the lower part of the locust. After 120 min (Figure 6i), midazolam was almost exclusively detected in feces. MS2 of midazolam glucoside (488->326) is shown in Figure 6(e) and (j) was after 30 min only detected in the lower intestine. After 120 min (Figure 6j), midazolam glucoside was found primarily in the intestine in the crop (food storage), gastric caeca and feces (rectum).

The presence of midazolam and midazolam glucoside in the crop after 120 min was confirmed by LC-HRMS of crop-material extracted with 50% MeOH as described in the “Experimental” section. Other metabolites of midazolam were not detected in the crop.

Table 2. Calculated AUCs for midazolam and 1’-OH MDZ and 4-OH MDZ with and without co-administration of ketoconazole.

|           | AUC (µM min) - KTZ | AUC (µM min) + KTZ | Significant |
|-----------|-------------------|-------------------|-------------|
| Midazolam | 1148 (83)         | 1356 (130)        | No          |
| 1’-OH MDZ | 4.6 (0.5)         | 1.5 (0.3)         | Yes (p = 0.0004) |
| 4-OH MDZ | 2.7 (0.3)         | 0.5 (0.04)        | Yes (p = 0.0003) |

Mean and SEM, n = 5–6.

Discussion

In previously published studies, it was shown that an enzyme with functionality similar to human CYP3A4/5 was responsible for the phase I metabolism of terfenadine, a substrate specific to human CYP3A4/5 (Olsen et al., 2014). To confirm these findings, the metabolism of additional CYP3A4/5 compounds should be investigated in locusts. Midazolam 1’
hydroxylation is one of the most commonly used biotransformations to characterize CYP3A4/5 enzyme activity in vitro (Yuan et al., 2002). Therefore, the metabolism of midazolam in locusts was investigated to further validate the presence of an enzyme in *S. gregaria* with functionality similar to human CYP3A4/5.

Reference standards of the two human metabolites, 1-OH MDZ and 4-OH MDZ were used to identify the metabolites in locusts by comparing retention time and MS² fragmentation patterns. Hemolymph samples were collected 60 min after administration of midazolam to ensure adequate time for distribution and formation of metabolites. The major hydroxylated metabolite found in locust hemolymph was 1-OH MDZ, but both hydroxylated metabolites were found in locust hemolymph after administration and only ketoconazole out of the five enzyme inhibitors used could significantly inhibit the formation (Figures 1 and 2, Table 2). These results support the findings reported previously where terfenadine was used as model substrate (Olsen et al., 2014).

An attempt was made to determine the $K_m$ value for the formation of the two hydroxylated metabolites. Previous attempts using terfenadine as model substrate were unsuccessful as the amount of metabolite was too low resulting in very large uncertainty on the data obtained (Olsen et al., 2014). As can be seen in Figure 3, the variation in the data of present experiment was significant. In locusts the apparent $K_m$ values for the 1'-OH and 4-OH metabolites were calculated to 7–23 and 33–85 μM, respectively. The $K_m$ value in HLMs for 1'-OH reported to be 2.5–9 and 12–78 μM for the 4-OH metabolite (Ghosal et al., 1996; Kotegawa et al., 2002; Patki et al., 2003; Perloff et al., 2000; van Waterschoot et al., 2008), and thus the apparent $K_m$ values determined in locusts are relatively close to human values. Assuming that only one enzyme is converting midazolam to 1'-OH and 4-OH the affinity of the enzyme toward midazolam is present in the same range in humans and in locusts. However, comparison of data obtained in vitro with data obtained in vivo should be interpreted with caution, as multiple factors can affect the results obtained in vivo, such as distribution, phase II metabolism and elimination which may contribute to the substantial variation in the data presented in Figure 3. Estimation of additional parameters such as $V_{max}$ and intrinsic clearance was not done due to the significant variation in the data and the further conversion of the 1'-OH and 4-OH metabolites to phase II metabolites. Therefore, it would have been more accurate to assess the
kinetic parameters, $K_m$ and $V_{\text{max}}$ in microsomes isolated from locusts. Rapid elimination of both midazolam and the phase I metabolites had been observed (Figure 2), and as a consequence, initial formation rates of the metabolites was measured only 1 min after injection. One minute may not be adequate to ensure systemic circulation, however, the injection site (illustrated in Figure 6f) was placed far from the hemolymph extraction site (below the head) in order to minimize contamination from the injection. Nevertheless, large variation in the measured concentrations was observed.

From Table 2, it can be seen that the concentration of midazolam in the hemolymph is present in the μM-range, whereas the 1’ and 4-OH-metabolite concentrations are only in nM-range. Analysis of feces from dosed locusts revealed that relatively low amounts of unchanged midazolam were excreted and it was therefore expected that alternative metabolism pathways dominate the elimination of midazolam. Searching for compounds with identical fragmentation pattern compared with midazolam resulted in the detection of a number of conjugates. The fragmentation patterns of midazolam and the phase I metabolites give little information about structure, thus NMR spectroscopy was necessary in order to structurally identify the metabolites, where reference standards were not available. Due to the extensive number of different phase II metabolites of midazolam formed in locusts, only the most abundant ones have been structurally identified. The most abundant phase II metabolites were glucose and glucose-phosphate conjugates of midazolam (Figure 4) as well as glucose conjugates of various phase I metabolites. Such phase II metabolites have been reported in insects previously (Myers & Smith, 1954; Ngah & Smith, 1983; Smith, 1955, 1962, 1968) but this is the first time these metabolites have been identified after administration of midazolam. The reason for the formation of glucose-phosphate metabolites is unclear as conjugation with glucose should render the compound adequately soluble for excretion. Based on the presence of two phosphorylated glucosides, with similar ratios as the two glycosides identified by NMR, it is speculated that phosphorylations occur after the glycosylation thus having the same glycosylation sites as illustrated in Figure 4. Complete characterization of the metabolic pathways of midazolam was outside the scope of the current study. Both glucose and phosphate conjugates, but not a phosphate-glucose conjugate, were also detected following administration of terfenadine to locusts. The formation of the glucose conjugate was significantly increased when phase I metabolism was inhibited, thus ensuring elimination from hemolymph. As listed in Table 2, the AUC of midazolam is unchanged despite significantly lower amount of 1’-OH and 4-OH MDZ, suggesting that phase II metabolism compensate for the enzyme inhibition as it was seen for terfenadine (Olsen et al., 2014). Furthermore, the AUCs of the hydroxylated metabolites constitute <1% of the AUC of midazolam, and therefore the AUCs of midazolam are unlikely to be affected by co-administration of ketoconazole.

The observed elimination of midazolam may also be due to extraction of hemolymph. To obtain a pharmacokinetic profile of the elimination of midazolam, 8 samples containing 10 μl hemolymph was extracted from the same locusts. Locusts contain ~145 μl hemolymph per gram (Lee, 1961) and have an average weight of 1.6 g resulting in a total hemolymph volume of 230 μl. Administration of 40 μl results...
in a 17% volume increase. Extraction of 80 μl account for almost 1/3 of the original hemolymph volume, and thus, the circulation may be compromised. This was not investigated further, but this constitutes one of the major limitations using locusts as experimental animal. A pharmacokinetic analysis of the clearance of midazolam was not performed as the total metabolism of midazolam is very different from the metabolism reported in humans and other mammals. The focus of the present work was to validate the presence of an enzyme with functionality similar to human CYP3A4/5.

The rapid elimination of midazolam from locust hemolymph could be, besides metabolism, due to distribution and binding to specific tissue. This was observed in locusts dosed with terfenadine (Olsen et al., 2015). Two hours after injection terfenadine was detected primarily in the gastric caeca, whereas the metabolite (terfenadine acid) was detected in the lower intestine. In Figure 6, the distribution of midazolam and the midazolam glucoside metabolites are shown 30 and 120 min after injection. Midazolam was diffusely distributed after 30 min and practically eliminated from hemolymph after 2 h at least below the detection limit and was found primarily in the rectum (lower intestine), and thus, the fast elimination from hemolymph does not appear to be caused by distribution into tissue as it was observed for terfenadine. Interestingly, the midazolam glucosides were, after 2 h, also detected in the crop of the locusts. This part of the locust functions as food storage and only expected to contain digestive enzymes (Klowden, 2007). Glucosidase enzyme activity has been shown to be relatively high in the crop (Kikal & Smith, 1959), and it can be speculated if midazolam glucosides are formed in the intestine and then transported by malphigian tubules from the intestine and into the hemolymph and actively taken up in the crop in order for the locust to recycle the glucose. LC-MS analysis of the crop content did reveal low amounts of unchanged midazolam, possibly cleaved from glucose, even though the injection site is relatively far away from this area. Another explanation for the presence of glucosides in the crop could be that the grasshoppers are coprophagous, however, such behavior was not observed.

**Conclusion**

The results obtained in the present study confirm that an enzyme with functionality similar to human CYP3A4/5 is present in locusts. Furthermore, the $K_m$ values for the formation of human metabolites *in vivo* in locusts are in the same concentration range as values reported in humans, and further assessment of the pharmacokinetic parameters *in vitro* in microsomes isolated from locusts would be valuable. The major metabolites detected in locust hemolymph and feces were, however, not known to be formed in humans. Glucose and glucose-phosphate conjugates were tentatively identified as the major metabolites, and metabolism rather than tissue distribution was identified as the major reason for elimination. The differences in metabolic pathways make the use of locusts as a model for human clearance is questionable, however, locusts may be used for production of metabolites in high quantities. Each insect can be dosed repeatedly and feces containing high concentrations of metabolite can be collected for purification of metabolites.

**Declaration of interest**

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Supplementary material available online
Supplementary Information