BENZODIAZEPINE METABOLISM: AN ANALYTICAL PERSPECTIVE

Roberto Mandrioli, Laura Mercolini, Maria Augusta Raggi*

Laboratory of Pharmaco-Toxicological Analysis, Faculty of Pharmacy, Department of Pharmaceutical Sciences, Alma Mater Studiorum - University of Bologna, Via Belmeloro 6, I-40126 Bologna, Italy.

* Author to whom correspondence should be addressed:

Prof. Maria Augusta Raggi

Department of Pharmaceutical Sciences

Via Belmeloro 6,

I-40126 Bologna

Italy

Tel. +39 051 2099700

Fax: +39 051 2099740

E-mail: mariaaugusta.raggi@unibo.it

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ABSTRACT

Benzodiazepines are currently among the most frequently prescribed drugs all over the world. They act as anxiolytics, sedatives, hypnotics, amnesics, antiepileptics and muscle relaxants. Despite their common chemical scaffold, these drugs differ in their pharmacokinetic and metabolic properties. In particular, they are biotransformed by different cytochrome P450 isoforms and also by different UDP-glucuronosyltransferase subtypes. The most important studies on the metabolic characteristics of several 1,4-benzodiazepines, carried out from 1998 onwards, are reported and briefly discussed in this review. Moreover, the analytical methods related to these studies are also described and commented upon and their most important characteristics are highlighted. Most methods are based on liquid chromatography, which provides wide applicability and good analytical performance granting high precision, accuracy and feasibility. Mass spectrometry is gaining widespread acceptance, particularly if the matrix is very complex and variable, such as human or animal blood. However, spectrophotometric detection is still used for this purpose and can grant sufficient selectivity and sensitivity when coupled to suitable sample pre-treatment procedures.

A monograph is included for each of the following benzodiazepines: alprazolam, bromazepam, brotizolam, clotiazepam, diazepam, etizolam, flunitrazepam, lorazepam, midazolam, oxazepam and triazolam.

INTRODUCTION

Benzodiazepines (BZDs) are among the most frequently prescribed drugs due to their anxiolytic, hypnotic, sedative, amnesic, antiepileptic and muscle relaxant properties [1]. They are also much safer than the older barbiturates in case of overdose [2]. Most BZDs share the 5-phenyl-1,3-dihydrobenzo[*e*][1,4]diazepine nucleus, with different possible substitutents on the 1, 2, 3, 7 and 2' positions; some (such as clobazam, arfendazam and lofendazam) are based on the 5-phenyl-2,3-dihydrobenzo[b][1,5]diazepine nucleus instead, while others have different aromatic rings fused to the diazepine ring: e.g., clotiazepam is a thiophenediazepine. BZDs are usually divided into three groups according to their duration of action: less than 12h for short-acting BZDs, 12-24 h for intermediate-acting BZDs and more than 24 h for long-acting BZDs [3]. It should be noted that the different durations of action of the various BZDs are mainly due to the presence of one or more metabolites with notable pharmacological activity. Some of these metabolites (oxazepam, lorazepam, nordazepam) are commercialised as drugs on their own. Short-acting BZDs are usually prescribed for the treatment of insomnia and related sleep disturbances, since they allow the patient to sleep rapidly and cause limited somnolence the following morning. On the contrary, intermediate- and long-acting BZDs are usually prescribed for anxiety-related disorders, to keep the symptoms under control for the whole day with a single dose or even less frequent administration [4].

BZD metabolism involves two main pathways: oxidation and glucuronidation. Usually, those BZDs which first undergo oxidation, mainly by action of cytochrome P450 (CYP), generate active metabolites which have then to be glucuronidated in order to be excreted, thus their duration of action can be very long. On the contrary, direct glucuronidation of the drug produces inactive, rapidly excreted metabolites; those BZDs that are mainly glucuronidated generally have a short duration of action [5].

Despite having first been introduced onto the market almost fifty years ago, BZDs are still considered the most safe and efficacious anxiolytic-hypnotics currently available. Their use is extremely widespread and they are also often prescribed as adjuvants during antidepressant and antipsychotic therapies [6-8]. Most BZDs have been available for more than twenty years, thus their pharmacokinetic and metabolic characteristics are generally very well known. However, this is not to say that these topics are not studied anymore. On the contrary, in the last few years several advances have been made in the knowledge of, e.g., the specific enzymes or enzyme subtypes involved in their metabolism, or the pharmacological interactions with other drugs. In this paper, the most relevant studies published in the last ten years (i.e., since 1998) on BZD metabolism and interactions will be reviewed. The analytical methods used in these studies for the determination of BZDs and their metabolites in biological matrices will also be described in detail and commented upon. This can be a useful support in evaluating the exact analytical needs of the study and the most suitable techniques and procedures to obtain reliable and complete data.

A bibliographic search has been carried out, regarding the most frequently used 1,4-BZDs: alprazolam, bromazepam, chlordiazepoxide, clotiazepam, delorazepam, diazepam, etizolam, lorazepam, oxazepam and prazepam among those mainly used as anxiolytics; brotizolam, flunitrazepam, flurazepam, lormetazepam, midazolam, nordazepam, temazepam and triazolam among those mainly used as hypnotics.

Of these, however, chlordiazepoxide, delorazepam, flurazepam, lormetazepam, nordazepam, prazepam and temazepam are not included as monographs. In fact, it has been found that very few scientific papers (or none at all) have been published in the last ten years dealing with the metabolism of these compounds, despite their relatively frequent use in some countries. Nordazepam and temazepam are two important diazepam metabolites, thus they are often included in studies regarding the parent drug. For this reason, they will be briefly cited in this role in the diazepam monograph.

ANXIOLYTIC BENZODIAZEPINES

ALPRAZOLAM

Alprazolam (8-chloro-1-methyl-6-phenyl-4*H*-[1,2,4]triazolo[4,3-a][1,4]benzodiazepine) is an intermediate-acting triazolobenzodiazepine mainly known as Xanax, or Alpravecs, Alprazig, Frontal, Niravam and Valeans. An extended-release form is available as Xanax XR. Its main therapeutic uses include the treatment of panic disorder, with or without agoraphobia, and the short-term treatment of severe acute anxiety [9]. Doses are usually 0.25-0.5 mg three times a day, with a total maximum of 3 mg.

- Metabolism and pharmacokinetics

Alprazolam is well absorbed after oral administration [10]; however, when it is administered as an orally disintegrating tablet, the presence of food can delay the time needed to reach maximum blood concentration (t_{max}) by 1.5 hours and lower the maximum plasma concentration (C_{max}) by 25%. No significant effect on the area under the time - blood concentration curve (AUC) has been found and thus, presumably, on clinical efficacy [11]. Age does not seem to have a significant effect on the main pharmacokinetic parameters of alprazolam, such as half-life ($t_{1/2}$), t_{max}, C_{max}, distribution volume (V_d) and apparent clearance [12]. It is well known that alprazolam is a substrate of cytochrome P450 (CYP) subtype 3A4 [13]. The main phase I metabolite thus formed is 4-hydroxyalprazolam, while α-hydroxyalprazolam is formed by the action of CYP3A5 [14, 15] (Figure 1). However, it has been found that constant ingestion of grapefruit juice (an inhibitor of CYP3A4) does not cause any change in the pharmacokinetic or pharmacodynamic properties of alprazolam; this has been attributed to its high bioavailability [16]. Some recent studies suggest that CYP3A5 genetic polymorphism affects the disposition of alprazolam and provides a plausible

explanation for interindividual variation in the disposition of this drug [17]. However, these results are still a matter for discussion [18, 19]. Alprazolam has been suggested as a possible probe for CYP3A phenotyping; plasma concentration ratios obtained between 1 h and 48 h after administration correlated significantly to the corresponding AUC(0-∞) ratios for both alprazolam/4-hydroxyalprazolam and alprazolam/α-hydroxyalprazolam [20].

After i.v. administration, the plasma levels of alprazolam decay biexponentially; after oral dosing, instead, a double peak has been observed in the time-concentration curve, for both the parent drug and the two hydroxylated metabolites [21]. A possible explanation for this fact is a reduction in gastric motility due to the muscle relaxant effect of the drug [21].

Several papers have been published in the last ten years, regarding possible pharmacological interactions between alprazolam and other drugs. Alprazolam therapy can increase the oral bioavailability of lithium carbonate in bipolar patients with generalised anxiety; this is probably due to a decrease in gastro-intestinal symptoms [22]. Interactions between alprazolam and antidepressants have been particularly studied, due to the high frequency of co-administration, and most second-generation antidepressants, such as SSRIs (selective serotonin reuptake inhibitors), have been tested for this purpose. No interaction has been found between alprazolam and the SSRIs paroxetine [23] and sertraline [24] in healthy volunteers. Fluoxetine, an SSRI antidepressant and an inhibitor of CYP3A4, prolongs by 16% the half-life and increases by 32% the AUC of alprazolam [23]. The SSRI fluvoxamine, an inhibitor of CYP2C19, caused very evident increases (up to 100% and more) in alprazolam plasma concentrations; very high interindividual variations were found, probably related to different CYP2C19 genotypes [25]. The SNRI (serotonin and norepinephrine reuptake inhibitor) antidepressant venlafaxine has been found to lower the AUC of alprazolam by about 30% and to increase the drug clearance in healthy volunteers, however the clinical consequences of this fact seem to be unclear [26]. A low-hyperforin St. John's wort extract (used as an antidepressant as well) showed no influence on alprazolam plasma levels; since hyperforin is a CYP3A4 inducer, the lack of interaction could be attributed to the low content of this active principle [27].

- Analytical methods

Most analytical methods used in the last few years for the determination of alprazolam and its main metabolites in human plasma for pharmacokinetic purposes are based on high-performance liquid chromatography with mass spectrometry detection (usually tandem mass - MS/MS - for added selectivity) [11, 28, 29, 30]. This technique has the advantages of very high selectivity, high sensitivity (lower limit of quantification: 40-100 pg/mL, depending on the analyte) and short run times (typically less than five minutes for one chromatographic run); only precision is not completely satisfactory (CV < 20%). The sample pre-treatment based on liquid-liquid extraction (LLE) [28, 29] significantly increases the total analysis time (it requires agitation, centrifugation, phase separation, drying and redissolution). An aliquot of 500 μL of plasma is sufficient for one analysis and the analytes are concentrated 2.5 times during the pre-treatment procedure. On the contrary, the solid-phase extraction (SPE) procedure [30] on hydrophilic-lipophilic balance (HLB) cartridges requires 1 mL of plasma and concentrates the analytes 20 times before the injection. The double peak in alprazolam time-concentration curve after oral administration was observed using an analytical method published some years before [31], which is based on HPLC with UV detection at 230 nm. Simple LLE is used as a sample pre-treatment and sensitivity is lower that that of HPLC-MS methods (LOQ = 5 ng/mL), as one can expect.

The interaction studies typically analyse alprazolam only, not the metabolites; the analytical method for the alprazolam-paroxetine study [23] is based on HPLC-MS/MS after liquid-liquid extraction; although the Authors claim that the method had been validated, no reference to a complete analytical paper is reported and only limited details on method performance (LOQ = 100 pg/mL) are reported. The method used for the venlafaxine-alprazolam study [26] is based on HPLC-MS/MS as well, however it allows analysing the two main metabolites as well and the paper reports a LOQ of 100 pg/mL, RSD values lower than or equal to 6.8% and accuracy in the 93-117% range. The methods used in the alprazolam-fluoxetine and in the alprazolam-fluvoxamine studies, on the contrary, are based on HPLC-UV [25, 32], with limits of detection of 0.6-0.8 ng/mL, thus significantly higher than those of HPLC-MS methods.

Other benzodiazepines, such as midazolam, etizolam and triazolam, have been used as the internal standards (ISs) of these analytical methods. Obviously, this does not pose any particular problem for pharmacokinetics and interaction studies, where drug administration is tightly controlled, but could potentially cause analytical interference if the methods are used for other purposes, such as therapeutic drug monitoring (TDM).

BROMAZEPAM

Bromazepam (7-bromo-1,3-dihydro-5-(2-pyridinyl)-2*H*-1,4-benzodiazepin-2-one) is known as Brixopan, Calmepam, Compendium, Creosedin, Durazanil, Lectopam, Lexaurin, Lexilium, Lexomil, Lexotan, Lexotanil, Normoc, Novepam, Somalium or Lexatin. It is a pyridinylbenzodiazepine mainly used as an anxiolytic, however it possesses strong sleep-inducing properties and is also used (in association with an antimuscarinic agent) for gastro-intestinal spasms and enuresis [33]. Usual doses are 1.5-12 mg three times per day, with a maximum daily dose of 120 mg.

- Metabolism and pharmacokinetics

Only a few papers have been recently published dealing with topics related to the pharmacokinetics of bromazepam. A study has found that rectal administration of bromazepam increases the drug AUC by 70% and its C_{max} by 60% with respect to oral administration [34]. The Authors suppose that this effect is due to a lack of degradation of bromazepam by this route. The drug metabolism presumably involves cytochrome P450, since it is inhibited by fluvoxamine [35], however the specific subtype has not been clearly identified. In fact, fluvoxamine is a potent inhibitor of CYP1A2, a less potent CYP3A4, 2C9 and 2C19 inhibitor, and a negligible inhibitor of CYP2D6. However, CYP3A4 probably is not the responsible enzyme, since itraconazole does not affect

bromazepam plasma concentrations [36]. The main phase I metabolite of bromazepam is 3-hydroxybromazepam, which is pharmacologically active and whose half-life is approximately equal to that of bromazepam (about 17 hours) [37] (Figure 2).

Recently, a paper has been published on the urinary metabolites of bromazepam in four different species (human, dog, rat and mouse) [38]. The most abundant urinary metabolite in humans is 3-hydroxybromazepam glucuronide, followed by 2-(2-amino-5-bromo-3-hydroxybenzoyl)pyridine and 2-(2-amino-5-bromobenzoyl)pyridine.

A prolonged release formulation of bromazepam has been developed and it has been found bioequivalent to normal capsules in one study [39] on 24 healthy volunteers.

In recent years, azole antifungals have been the main subjects of interaction studies with bromazepam. As already said, itraconazole administration does not seem to have any effect on bromazepam pharmacokinetics [36]. Similar results have been obtained with fluconazole [34].

It has been also observed that bromazepam does not impair the uptake of iron from $Fe(OH)_3$ polymaltose complexes used as a source of soluble, non-ionic iron [40].

- Analytical methods

The analytical method used to determine bromazepam plasma levels in the fluconazole study [34] is a slight modification of a previously published method [41] and is based on HPLC with UV detection at 240 nm. Plasma pre-treatment is carried out by liquid-solid extraction with dichloromethane on a diatomaceous earth column. While sensitivity (LOQ = 1 ng/ml) and precision (RSD < 8%) appear to be satisfactory, run times are very long (more than 40 minutes). This could be understandable for the original method, which analysed the main metabolites in urine as well, however it is a major drawback for the analysis of only one compound. The itraconazole-bromazepam study [35] was carried out using a further modification of the same method, with a double solvent extraction and slightly lower sensitivity (LOQ = 2 ng/mL); the mobile phase modification was presumably made to decrease the retention times of the analyte and the IS

(alprazolam).

As a comparison, some methods based on HPLC-MS have been published in the last few years, specifically developed for pharmacokinetic studies [42]. One of these deals with the choice of the most suitable ion source (ESI or API) and analyser (triple quadrupole or ion trap) [42]. While the two sources were substantially equivalent, higher sensitivity was obtained with the triple quadrupole analyser. In this case, sensitivity is similar to that of UV detection (LOQ = 2.5 ng/mL), but run times are many times shorter, i.e., about 2 minutes. Sample pre-treatment is carried out by protein precipitation with acetonitrile. Another paper proposes the use of HPLC-MS/MS preceded by on-line SPE [43]: this allows the automated pre-treatment and analysis of the biological samples for pharmacokinetics and bioequivalence purposes in about 3 minutes. The cartridge sorbent is a C18 resin and 100 μL of sample are sufficient for one analysis. Another paper describes a method based on HPLC-MS/MS for bioequivalence studies [44]. It reports the use of LLE for the sample pre-treatment, a run time of 2.5 min and a LOQ of 1 ng/mL.

For bromazepam, again, most methods use other benzodiazepines, such as nitrazepam, lorazepam or alprazolam, as the ISs.

CLOTIAZEPAM

Clotiazepam (5-(2-chlorophenyl)-7-ethyl-1,3-dihydro-1-methyl-2*H*-thieno[2,3-e]-1,4-diazepin-2-one) is commercially known as Trecalmo, Rizen or Tienor. Usual daily doses for the control of anxiety range from 5 to 30 mg.

Only a few papers have been published on clotiazepam metabolism and pharmacokinetics since the 1970-80s, when the original pre-clinical and clinical studies were carried out on this drug.

The early studies on healthy volunteers established that the drug is rapidly absorbed after oral administration. The two main metabolites are α-hydroxyclotiazepam and *N*-desmethylclotiazepam

(Figure 3), which appear and disappear approximately in parallel with the parent compound. The mean elimination half-lives of clotiazepam, the hydroxy metabolite and the desmethyl metabolite are 6.5, 7.0 and 17.8 h, respectively [45]. The analytical technique used in this study was gas-liquid chromatography (GLC) with electron capture detection. Electron capture detector was used since it is selective towards compounds containing very electronegative atoms and in particular towards halogens. Recently, an *in vitro* study has been published on the contribution of different CYP isoforms to the metabolism of some psychotropic drugs (among which the benzodiazepines diazepam, clotiazepam, tofisopam and etizolam) [46]. The metabolism of clotiazepam was catalyzed, in order of increasing metabolic activity, by CYP2B6, CYP3A4, CYP2C18 and CYP2C19. However, since CYP3A4 is more abundant than CYP2B6 in the human liver [47], the former seems to be the main metabolising cytochrome of clotiazepam [46]. In this paper, the analytical method is based on gradient HPLC with UV detection at 242 nm, however the biological matrix in this case is not a real biological sample, but purified liver microsomes or recombinant cytochromes. A typical chromatographic run lasts 12 minutes, diazepam is used as the IS and the LOQ is about 15 ng/mL.

DIAZEPAM

Diazepam (7-chloro-1,3-dihydro-1-methyl-5-phenyl-2*H*-1,4-benzodiazepin-2-one) has been the second BZD ever introduced onto the market (after chlordiazepoxide) and is still one of the most widely used BZDs in the whole world. It is used for the treatment of anxiety, seizures and for preoperative anaesthesia induction. It is generally known as Valium, however it is also commercialised under names such as Ansiolin, Diazemuls, Noan, Tranquirit and Valeans.
The typical dose for anxiety is 6 mg/day in three administrations, but it can be increased up to 30 mg/day. The rectal administration is used for acute anxiety and agitation, at 500 μg/kg every 12 h

until the symptoms are under control.

- Metabolism and pharmacokinetics

Diazepam is primarily metabolized by hepatic enzymes, with very little unchanged drug eliminated in the urine [48]. Hepatic *N*-demethylation results in the formation of the active metabolite desmethyldiazepam (also known as nordazepam). This metabolite is hydroxylated to form oxazepam, which is also active and is metabolised to form oxazepam glucuronide. A minor active metabolite is temazepam, which can be in turn demethylated to oxazepam or glucuronidated. The main active species found in blood are diazepam and desmethyldiazepam, because oxazepam and temazepam are conjugated and excreted at almost the same rate as they are generated [49]. Another minor metabolic pathway in animals is 4'-hydroxylation by CYP2D1 [50]. In rats, it has been observed that CYP2D3 might be involved in diazepam 4'-hydroxylation, while CYP2D1 and 2D2 practically do not participate in diazepam metabolism [51]. Some Authors have hypothesised that CYP2B6 could have a role in diazepam metabolism, since anti-CYP2B6 antibodies inhibit diazepam disappearance in human hepatocytes [52]. More detailed studies on human recombinant cytochromes have shown that CYP2B6 catalyses exclusively (and 2C8, 2C9, and 2C9R144C preferentially) the *N*-demethylation of diazepam; CYP3A4 and 3A5 catalyse primarily its 3-hydroxylation (Figure 4) [53]. Other, similar studies have indicated a 60-80% contribution of CYP3A4/5 to temazepam formation [54, 55]. Nordazepam formation was inhibited by antibodies for CYP2B6 (6-23%), 2C (12-61%) and 3A4/5 (14-45%). The antibodies for CYP1A1, 1A2, 2A6, 2D6, and 2E1 did not inhibit temazepam or nordazepam formation [55]. It has been found that the genetic polymorphism of CYP2C19 can influence diazepam [56, 57] and nordazepam [58] pharmacokinetics. However, there is a only maximum 2-fold difference in the hepatic elimination rate or the AUC between the extremes of extensive metabolisers and poor metabolisers [59], thus no treatment guidelines for diazepam take pharmacogenetic data into account [60].

Diazepam has a $t_{1/2}$ of 20-50 h and nordazepam, the main active form of its metabolites, has an even longer duration of action (up to 200 h); thus, caution should be taken for possible accumulation of the drug or the metabolite. The $t_{1/2}$ values of both compounds are significantly longer in case of overdose, resulting in possible drowsiness, dysarthria, diplopia and dizziness for more than one week even after treatment with activated charcoal, diuretics and the BZD antagonist flumazenil [61].

Due to the complex metabolic profile of diazepam, several drug interactions are possible during therapy. For example, chronic administration of the antiepileptic phenobarbital powerfully induces CYP, thus notably decreasing diazepam AUC, C_{max} and bioavailability and increasing its t_{max} [62]. On the other hand, grapefruit juice (by way of intestinal CYP3A4 inhibition) [63], rifabutin (a rifampicin analogue) [64] and isoniazid [65] can have the opposite effect. Omeprazole can inhibit the formation of the three main direct metabolites of diazepam (nordazepam, temazepam, 4'hydroxydiazepam) [66].

Diazepam can be administered to treat status epilepticus and other kinds of seizures. While the preferred route of administration for this indication is rectal, it has been shown that diazepam is rapidly absorbed intranasally [67] and that it can easily reach therapeutic concentrations by this route [68].

Since neonates have a reduced capacity of the hepatic biotransformation system, serum levels of diazepam and its metabolites can be substantially elevated if this is not taken into account before i.v. or oral administration. The very long-acting nordazepam in particular can cause persistent respiratory failure, muscular hypotonia and drowsiness [69].

Diazepam and its metabolite oxazepam are carcinogens in mice but not in rats. They have also been shown to induce both CYP2B and CYP4A in mice and only CYP2B in rats; thus, it has been hypothesised that the carcinogenic effect is related to CYP2B activation, however the reason of the species-specific CYP induction is still unclear [70].

- Analytical methods

Analysis of diazepam, temazepam, nordazepam and 4'-hydroxydiazepam for metabolism studies was carried out on rat microsomes by HPLC-UV at 230 nm [50, 51]. A simple pre-treatment by LLE with ethyl acetate is carried out. The analytes and the IS (nitrazepam) are separated on a C18 column using an acidic phosphate buffer / acetonitrile / methanol mixture as the mobile phase. Unfortunately, run times are long (more than 35 min) and no other detail of the method is reported in the paper.

The in vivo assay of diazepam and nordazepam in human plasma for CYP2C19 activity [58] was carried out by GC with nitrogen-phosphorus detection, which is specific for compounds containing these heteroatoms. The sample pre-treatment consists of an LLE procedure with diethyl ether; analyte separation is achieved using a capillary column coated with 50% dimethyl - 50% diphenyl polysiloxane. According to the Authors' report, precision CVs were always lower than 10% and the LOQ was 8 ng/mL for both analytes. In this case, the tricyclic antidepressant clomipramine is used as the IS instead of another BZD.

Another study on the effect of CYP2C19 polymorphism [59] exploited a previously published analytical method for the simultaneous analysis of eleven BZDs by HPLC-UV at 254 nm [71]. It is not clear whether any changes were made to the original method, since the choice appears quite peculiar: in fact, in the cited paper diazepam was used as the IS, thus the method was not validated for this analyte (i.e., no linearity range or precision values were obtained for it). Moreover, the retention time for diazepam was more than 26 minutes; while this is acceptable for a multi-analyte method, it is decidedly too long for a single analyte, as in this case.

Interestingly, the study on pharmacokinetics interactions between phenobarbital and diazepam [62] was carried out on dog serum by means of a fluorescence polarisation immunoassay (FPIA), the TDx Benzodiazepines Serum Assay. Since the assay has an almost complete cross-reactivity between diazepam, nordazepam and oxazepam [72], it was intentionally used to obtain the total "active moiety" in serum.

The two analytical methods used to analyse diazepam and three metabolites (temazepam, nordazepam and 4'hydroxydiazepam) in the omeprazole-diazepam interaction study [66] are based on HPLC-UV and on HPLC-MS/MS. The HPLC-UV [73] method uses a C18 column and a mobile phase composed by methanol and water; a chromatographic run lasts about 25 min. The detection wavelength is 254 nm. The assay is carried out on rat microsomes or hepatocytes after incubation and LLE with ethyl acetate. This method was found not selective enough for the analyses on hepatocytes, thus HPLC-MS/MS (ESI and triple quadrupole analyser) was used for the latter. Unfortunately, the only analytical information available on this method are the mass transitions employed and the IS (triazolam).

Diazepam absorption after intranasal administration [67] was determined by a radiochemical method, administering 2-^{14}C-diazepam to rats and measuring the radioactivity of blood samples drawn from two different parts of a carotid artery (one closer to the brain and the other closer to the heart). Of course, this method cannot discriminate between diazepam and its metabolites. However, the assay was carried out to assess diazepam transfer from the nasal cavity to the brain arterial blood, thus biotransformation was not considered.

ETIZOLAM

Etizolam (4-(2-chlorophenyl)-2-ethyl-9-methyl-6*H*-thieno[3,2-f][1,2,4]triazolo[4,3-a][1,4]diazepine) is marketed under the names Sedekopan, Depas and Pasaden. It is a thienotriazolodiazepine, which, according to the manufacturer, is rapidly metabolised and incurs lower risks of accumulation with respect to other BZDs [74]. The usual doses for anxiety are 0.25-0.50 mg two or three times per day, for insomnia 1 or 2 mg before bedtime.

- Metabolism and pharmacokinetics

In humans, etizolam t_{max} is about3.2 hours, and $t_{1/2}$ is about 6.2 hours. So, it can be classified as a short-medium acting BZD. A recently published study has reported that the main cytochrome isoform responsible for etizolam hepatic metabolism is CYP3A4, with minor contributions from CYP2C18 and 2C19 [46] (Figure 5). In fact, CYP3A4 showed the fastest enzymatic rate and furthermore ketoconazole, a well-known inhibitor of CYP3A4, strongly inhibited etizolam metabolism [75]. Itraconazole, another selective CYP3A4 inhibitor, had similar effects [76]. However, the contribution of CYP2C19 can be significant. It has been observed that the single dose pharmacokinetics of etizolam are strongly influenced by mutations (CYP2C19*2 and CYP2C19*3) causing the complete loss of CYP2C19 activity, with larger AUC and longer elimination $t_{1/2}$ and corresponding prolongation of the pharmacological effect [77]. Conversely, the CYP3A4 inducer carbamazepine [78] significantly decreases the C_{max}, $t_{1/2}$ and AUC values of etizolam in healthy volunteers [79].

Recently, some studies have been carried out on possible interactions between traditional Japanese Kampo drugs and benzodiazepines [80]. In particular, a study regards the simultaneous administration of etizolam and two herbal preparations used to treat menopausal symptoms: Kamisyoyosan, used for agitated patients, and Tokisyakuyakusan, used for menoxenia and oedema. Etizolam is often prescribed together with this kind of herbal remedy to ease the anxious symptoms associated with menopause. Both herbal preparations are decoctions of 6-10 different plants, such as Angelica, Paeonia, Glycyrrhiza and Menthae; they can interact with CYP3A4 inhibiting it, although with lower potency with respect to grapefruit juice [80]. The results of the study suggest that, at normal doses, no inhibition of CYP3A4 occurs; however, an overdose of Tokisyakuyakusan (i.e. at least 20 times the normal dose) could influence, at least in theory, the pharmacokinetics of etizolam.

- Analytical methods

The study on the CYP isoform responsible for etizolam metabolism [46] was carried out by HPLC-UV, using a C18 column and a sample pre-treatment by LLE with ethyl acetate. An analytical run lasts about 12 minutes and the LOQ value is approximately 7 ng/mL. The sensitivity is not outstanding, however this method was only used for the *in vitro* evaluation of etizolam disappearance in a microsomal preparation.

The method for etizolam determination in plasma samples, used to assess the influence of either CYP2C19 mutations [77] or CYP3A4 inhibition [79], is based on HPLC-UV with column switching. In this method [81], the sample pre-treatment was carried out online on a short column with a C18 sorbent coated with albumin; the analyte is then switched to the C18 analytical column. The reported extraction yield (near 100%) and LOQ (0.6 ng/mL) values are very good for an HPLC-UV method applied to such a complex matrix as plasma.

The study of the interactions between etizolam and Japanese traditional drugs [80] was carried out by HPLC- MS with a single quadrupole detector and APCI interface. Sample pre-treatment was carried out by protein precipitation with acetone and 50 μL of plasma were needed for one analysis. Unfortunately, no other detail is reported on the performance of the analytical method, apart for a mean etizolam extraction yield corresponding to 97%. The IS used for most studies [46, 80] was diazepam.

LORAZEPAM

Lorazepam (7-chloro-5-(2-chlorophenyl)-1,3-dihydro-3-hydroxy-2*H*-1,4-benzodiazepin-2-one) is an intermediate-acting BZD with potent anxiolytic effects. Its relatively potent amnesic effect, together with its anxiolytic and sedative properties, makes lorazepam also useful as premedication

before a general anaesthetic [82]. It is marketed as Ativan, Temesta, Control, Lorans, Slipirem, Tavor, Lorzem and Zelirem. It is also one of the main metabolites of lormetazepam.
The usual dose is 1-4 mg/day for anxiety and 1-2 mg before going to bed for insomnia with anxiety. It can also be injected intramuscularly (25-30 μg/kg) to treat acute and severe panic attacks.

- Metabolism and pharmacokinetics

Lorazepam is poorly soluble in water and lipids, binds strongly to plasma proteins and its metabolism is non-oxidative, with the formation of a pharmacologically inactive 3-*O*-glucuronide [83]. UDP-glucuronosyltransferase (UGT) 2B15 genetic polymorphisms have been found to influence the clearance of lorazepam, however the effect did not notably increase when UGT2B15 was induced with rifampicin or inhibited with valproic acid [84] (Figure 6). From experiments on rabbit microsomes, it has been found that azole antifungals (fluconazole, miconazole, and ketoconazole) can competitively inhibit UGT as well as CYP enzymes, thus lorazepam metabolism can be significantly impaired by the simultaneous administration of these drugs [85].
Pregnancy changes the pharmacokinetics of lorazepam, with an increase in the apparent distribution volume, an increase in apparent oral clearance and a reduction of elimination $t_{1/2}$. The increase in oral clearance may indicate an increase in glucuronidation capacity, with a possible reduction in the plasma drug concentrations [86].
The $t_{1/2}$ of lorazepam is 8-25 hours [83], thus it is usually prescribed as one or two daily doses when taken regularly. Drug peak effects roughly coincide with peak serum levels [87], which occur up to one hour after intramuscular injection and about 120 minutes after oral administration. However, it has been observed that lorazepam can sometimes be detected in the blood of elderly patients (in a nursing home environment) for as long as six weeks after discontinuation [88]. In the same study, no clear correlation between daily dosage and plasma levels was found and this was attributed to wide interindividual variability in the drug elimination.

Due to its poor solubility, injectable formulations of lorazepam often contain a glycol (normally propylene glycol) as a solvent. This, however, can cause an accumulation of glycol in the body, causing hyperosmolar metabolic acidosis with potentially dangerous effects such as renal dysfunction, intravascular haemolysis, cardiac arrhythmias, seizures and central nervous system depression. A study has found that glycol accumulation can be observed after at least 48 h of continuous infusion, and that it is directly related to the infusion rate [89]. This kind of toxicity was also observed in patients subjected to venovenous haemofiltration [90], which is also insufficient to remove lorazepam from blood (but eliminates the glucuronidated metabolite) [91].

- Analytical methods

Quantitative determinations of lorazepam and its metabolite during pregnancy [86] were carried out by HPLC-MS/MS. The method can be applied to both plasma and urine and has a LOQ of 0.2 ng/mL; the sample pre-treatment procedure is based on LLE with *tert*-butyl ether and needs 1 mL of sample for one analysis. The metabolite is quantified as the difference between total and non-glucuronidated lorazepam, after hydrolysis with β-glucuronidase [92]. Nitrazepam is used as the IS. For the study regarding the persistence of lorazepam in blood after discontinuation [88], no analytical details were given, except that the determination was based on HPLC and that the LOQ was 5 ng/mL.

OXAZEPAM

Oxazepam (7-chloro-1,3-dihydro-3-hydroxy-5-phenyl-2*H*-1,4-benzodiazepin-2-one) is marketed under the names Alepam, Limbial, Murelax, Oxascand, Serax, Serepax, Serpax, Seresta and Sobril.

It is used to relieve the symptoms of anxiety and of alcohol withdrawal at daily doses of 45-120 mg, divided into 3-4 administrations.

Oxazepam is also a metabolite of other BZDs, such as diazepam, nordazepam, chlordiazepoxide and prazepam. It is listed by the IARC as a possible carcinogen (group 2b) [93].

- Metabolism and pharmacokinetics

Since oxazepam is one of the main metabolites of diazepam, which is much more widespread in use, the former is usually mostly included in scientific studies regarding the latter.

Oxazepam is an intermediate-acting BZD, with a $t_{1/2}$ of 5-14 h. Like lorazepam, it is mainly metabolised by 3-*O*-glucuronidation [83], thus it is less likely to accumulate and to cause adverse effects in the elderly than other BZDs, which require hepatic oxidation. Oxazepam is formulated as a racemic preparation of *(S)*- and *(R)*-stereoisomers, although the *(S)*-enantiomer is thought to be much more active as a benzodiazepine receptor agonist compared with the *(R)*-enantiomer [94]. (S)-oxazepam, but not *(R)*-oxazepam, has been shown to be polymorphically glucuronidated in humans [95]. Further studies have found that *(S)*-oxazepam is almost exclusively metabolised by the UGT2B15 isoform, while *(R)*-oxazepam is metabolised by several different UGT isoforms, mainly UGT1A9 and UGT2B7 [96] (Figure 7). This could explain why a single mutation can impair *(S)*-oxazepam, but not *(R)*-oxazepam, biotransformation. Gender also play a role, as well as the specific genotype [97].

Like diazepam, oxazepam has been shown to induce both CYP2B and CYP4A in mice, but only CYP2B in rats; this has been related to the fact that both drugs induce hepatic carcinogenesis in mice and not in rats [70].

- Analytical methods

Since glucuronic acid is a chiral molecule, the glucuronidated forms of oxazepam are diastereomers, not enantiomers. Thus, they can be chromatographically separated without the need

for a chiral selector or a chiral column. In fact, the analytical method used to study the rate of glucuronidation of the drug [98] is based on HPLC with UV detection (at 214 nm) and the separation of the glucuronides and oxazepam is achieved on a simple C18 column using a phosphate buffer / acetonitrile mixture as the mobile phase. Phenacetin is used as the IS. In this case, run times are quite long (more than 25 minutes) due to the high retention of oxazepam and the IS, however the time needed to separate the analytes is acceptable (9 minutes), considering that they are diastereomers. No sample pre-treatment procedure is reported (apart from centrifugation after incubation), since the matrices are simply human liver microsomes or purified UGTs in buffer.

SEDATIVE-HYPNOTIC BENZODIAZEPINES

BROTIZOLAM

Brotizolam (2-bromo-4-(2-chlorophenyl)-9-methyl-6*H*-thieno[3,2-f][1,2,4]triazolo[4,3-a][1,4]diazepine) is a short-acting thienotriazolodiazepine commercialised as Lendormin, Nimbisan. It is used in the short-term treatment of insomnia, although due to its short half-life it is considered to have relatively high abuse potential and so it is not a first-line treatment [99].

- Pharmacokinetics and metabolism

Brotizolam is a potent drug with a dosage of 0.5 or 1.0 mg/day and is rapidly eliminated, having an average $t_{1/2}$ of 5.1 hours [100]. It is mainly metabolised by CYP3A4 (Figure 8), as demonstrated by the increase in the drug plasma levels, AUC and $t_{1/2}$, in volunteers taking simultaneously brotizolam and the antibiotic erythromycin, a known CYP3A4 inhibitor [101]. A similar effect was observed when administering the antifungal itraconazole [102]. Conversely, rifampicin (a CYP3A4 inducer) significantly decreases C_{max} (69%), AUC (90%) and $t_{1/2}$ (79%) of the drug [103]. Therefore, the co-administration of brotizolam and these antibiotics is not recommended.

- Analytical methods

The method used to assess possible pharmacokinetic interactions between brotizolam and the antibiotics rifampicin [103] and erythromycin [101] is a slight variation of a previously published method [81] on the analysis of etizolam. The method is based on HPLC-UV with column-switching, where the first column (albumin-C8) is used for the pre-treatment of the sample, while the second column (C18) is the analytical stationary phase. The Authors report very good sensitivity for this kind of assay, i.e. a LOD value of 0.3 ng/mL and a LOQ value of 0.5 ng/mL; precision is

also very good, with a CV of 5.2% at the LOQ level. As usual in this kind of analysis, the IS is diazepam.

For the brotizolam-itraconazole study [102], another, more recent method was used [104]. It is based on HPLC after SPE. For the sample pre-treatment, C18 cartridges were used. In this case, as well, the LOD is quite low (0.5 ng/mL) and precision is satisfactory (CV < 8.9%, however the concentration level is not indicated). Triazolam is used as the IS.

FLUNITRAZEPAM

Flunitrazepam (5-(2-fluorophenyl)-1,3-dihydro-1-methyl-7-nitro-2*H*-1,4-benzodiazepin-2-one) is a nitrobenzodiazepine most commonly marketed under the trade name Rohypnol, but is also known in some countries as Darkene, Ilman, Insom, Hipnosedon, Hypnodorm, Nilium, Roipnol, Valsera and Vulbegal. Flunitrazepam is usually prescribed for severe insomnia not responsive to other hypnotics, especially in inpatients [105]. It is considered one of the most potent hypnotic BZD on effect: the usual dose is 0.5-1 mg before going to sleep, with a maximum of 2 mg. Abuse of flunitrazepam among drug addicts is considerable and possession of flunitrazepam without a valid prescription is illegal in many countries.

-Pharmacokinetics and metabolism

While 80% of flunitrazepam that is taken orally is absorbed, bioavailability in suppository form is closer to 50% [106]. Flunitrazepam has a $t_{1/2}$ of 18 - 26 h, which means that, after nighttime administration, flunitrazepam effects persist throughout the next day. Important phase I metabolites are *N*-desmethylflunitrazepam and 3-hydroxyflunitrazepam. The main CYP isoforms responsible for the formation of both metabolites are CYP3A4 and CYP2C19 [107], however the role of CYP2C19 seems to be minor and differences in its activity are unlikely to affect the clinical effects

of the drug [107]. Another study, on the other hand, has found evidence that CYP3A4 catalyses the formation of hydroxyflunitrazepam and CYP2C19 the formation of desmethylflunitrazepam (Figure 9), potentially influencing the efficacy and safety of the therapy due to its high polymorphism [108]. CYP3A5 [109] and CYP1A2 [110] have also been found to be active *in vitro* in this regard. It is still unclear which enzyme precisely is responsible for the reduction to 7-aminoflunitrazepam; recent studies suggest the involvement of NADPH-cytochrome P 450 reductase [111], at least under hypoxic conditions.

Buprenorphine is known to interact with flunitrazepam, increasing the frequency and severity of side effects. The exact mechanism of this interaction is not known, however it probably is pharmacodynamic, not pharmacokinetic: in fact, nor buprenorphine nor its main metabolite norbuprenorphine significantly inhibit CYP3A4 or CYP2C19 [112].

The simultaneous administration of alcohol inhibits flunitrazepam metabolism *in vitro* and in particular the formation of desmethylflunitrazepam (20-40%) and, in a lesser way, of hydroxyflunitrazepam (10-30%); 7-aminoflunitrazepam does not seem to be affected [113]. The association of ethanol and flunitrazepam can also cause hepatotoxicity [114].

Flunitrazepam is one of the most frequently used drugs for the purpose of administering it to unsuspecting victims and raping them while they are under the influence of this substance. Thus, its detectability in urine is important to establish the exact timeline of the crime and the responsibility of suspects and to confirm the victim's testimony. The highest concentrations of flunitrazepam and 7-aminoflunitrazepam in urine are observed 6-24 h after administration; aminoflunitrazepam levels usually are 100-200 times those of the parent drug in urine. Accordingly, flunitrazepam can be detected in this biological fluid for up to 5 days after intake and aminoflunitrazepam for up to 28 days [115].

- Analytical methods

Flunitrazepam has been analysed at relatively high concentrations (in the µg/mL range) in human

microsomes and in CYP solutions by HPLC-UV using a C18 column, 234 nm as the detection wavelength [109] and clonazepam (another nitrobenzodiazepine) as the IS. Unfortunately, no other details or references to analytical studies are reported. Another method [108] used HPLC-UV at 234 nm to analyse flunitrazepam and its metabolites in microsomes or expressed CYP within a 17-min chromatographic run. The stationary phase was a C18 column, while the mobile phase was an acetonitrile/methanol/water mixture (IS: clonazepam). The study on the involvement of CYP3A4 and 2C19 in flunitrazepam metabolism [108] was carried out on microsomes using a previously published analytical method [116], based on HPLC-UV at 210 nm. This method was specifically developed to quantify the hydroxy and desmethyl metabolites, however flunitrazepam was analysed as well. On a C18 column, and using a phosphate buffer / acetonitrile mixture as the mobile phase, an analytical run lasts about 30 min. As usual for *in vitro* experiments, flunitrazepam concentrations were relatively high (in the µg/mL range), however the LOQ for both metabolites was 200 nM, corresponding to 66 ng/mL for hydroxyflunitrazepam and 99 ng/mL for desmethylflunitrazepam. The analytes were extracted by means of *n*-hexane / diethyl ether from the alkalinised reaction mixture and lorazepam was used as the IS.

The analysis of flunitrazepam and its main metabolites in human blood has been carried out by HPLC-UV at a wavelength of 250 nm [107]. Analyte separation was achieved on a C18 column within 16 min using a mobile phase consisting of a phosphate buffer / acetonitrile / methanol / tetrahydrofuran mixture. Sample pre-treatment was carried out by LLE with *n*-hexane/dichloromethane, concentrating the analytes 12.5 times. The LOQ for all analytes was 0.5 ng/mL and again clonazepam was the IS.

Flunitrazepam and aminoflunitrazepam can be analysed in urine by means of GC-MS with extremely low LOQ values, 100 pg/mL for the parent drug and 10 pg/mL for the metabolite [115].

MIDAZOLAM

Midazolam (8-chloro-6-(2-fluorophenyl)-1-methyl-4*H*-imidazo[1,5-a][1,4]benzodiazepine) is a short-acting imidazobenzodiazepine used mainly for its action as a sedative (2-2.5 mg in one dose), before anaesthesia (30-100 μg/kg) or for sedation of intensive care patients (slow i.v. injection, 30-300 μg/kg in 1-2.5 mg doses every 2 min, then 30-200 μg/kg/h). It can also be used for the treatment of *status epilepticus* (10 mg in one dose).

It is marketed under the brand names Dormicum, Dormonid, Flormidal, Hypnovel, Ipnovel and Versed.

- Metabolism and pharmacokinetics

Midazolam is almost exclusively metabolised by CYP3A4, both in the intestine (first-pass metabolism) and in the liver [117], producing three hydroxylated metabolites, 1'-hydroxymidazolam, 4-hydroxymidazolam and 1',4-dihydroxymidazolam [118], without any appreciable contribution from CYP3A5 [119, 120], CYP2B6 or CYP2C [121]. This has led to the identification of the drug as the ideal probe for assessing CYP3A4 activity, its induction and its inhibition. Consequently, most papers published in the last few years focus on midazolam metabolism, but only as a means to individuate the potential for interactions of other drugs. Many compounds have been tested as possible CYP3A4 modulators in this way in the last ten years, using midazolam as the probe and thus highlighting possible interactions when they are co-administered with it. Table 1 summarises the main characteristics of these studies; as can be seen, many CYP3A4 inhibitors have been found, while only a few compounds induce the metabolism of midazolam. The main midazolam metabolite, 1'hydroxymidazolam, is pharmacologically active and its plasma levels are about two-thirds those of the parent drug [151]. Thus, it can significantly contribute to the pharmacological effect of midazolam administration, in both normal and intensive care patients [152]. Midazolam pharmacokinetics are strongly dose-dependent: even in patients with hepatic CYP3A induction, the half-life of midazolam increases with high doses as a result of a rise in its

(Insert Table 1 here)

volume of distribution, which is a consequence of an increase in the free fraction of the drug [153]. Recent studies have shown that both midazolam and 1'-hydroxymidazolam can undergo *N*2-glucuronidation catalysed by UGT1A4 [154], while 1'-hydroxymidazolam can also undergo 1'-*O*-glucuronidation [155] catalysed by UGT2B4 and UGT2B7 (Figure 10).

The drug can be administered to adults and children of all ages and for several purposes (sedation, seizure control, pre-surgical anaesthesia), thus its pharmacokinetic parameters have been studied in different populations and for different routes of exposure. In children and adolescents, oral bioavailability of midazolam is extremely variable (9-71%), as well as its disposition. The rate of formation of 1'-hydroxymidazolam is higher for oral administration than for i.v. administration [156], probably due to intestinal first-pass metabolism. Due to immature hepatic cytochrome CYP3A4 activity, midazolam clearance and 1'-hydroxymidazolam concentration are reduced markedly in preterm infants. For this reason, caution is advised when administering the drug in the neonatal intensive care setting [157]. Similar results, with increased $t_{1/2}$ and distribution volume, were found in neonates receiving extracorporeal membrane oxygenation, probably due to midazolam sequestration by the membrane oxygenation circuit [158]. The drug can also be sub-cutaneously administered during palliative care and when i.v. administration is not possible. Absolute drug bioavailability by this route is 96%, without significant differences, in terms of AUC, with intravenous infusion. Other experimental formulations for intranasal administration in the treatment of status epilepticus have been developed; they have high bioavailability (83%) and a metabolic pattern (1'-hydroxymidazolam formation) similar to that of i.v. administration [159]. Sublingual administration in rabbits gave almost 100% bioavailability and no significant difference from i.v. administration for the main pharmacokinetic parameters [160].

Midazolam is not eliminated by haemofiltration, thus it can be administered to patients requiring sedation and haemofiltration, e.g., those suffering from multiple organ dysfunction syndrome [161]. On the contrary, continuous venovenous haemofiltration is able to effectively remove 1'-hydroxymidazolam glucuronide [162].

- Analytical methods

Studies on the metabolism of midazolam in animal microsomes [118] have been carried out by capillary HPLC (internal column diameter: 0.8 mm) after LLE with cyclohexane/dichloromethane. Capillary HPLC allows the use of minimal amounts of solvents and sample, while maintaining good sensitivity. This method was used for the analysis of midazolam and its three main metabolites; it is a slight modification of a previously developed method [163] for the determination of midazolam and the 1'-hydroxy metabolite in human plasma. UV detection is carried out at 240 nm for the analytes and at 300 nm for the IS (diazepam) and a chromatographic run lasts about 20 min, with gradient elution. While the original method was fully validated, only a few details are reported for the second one: apparently, it uses a different IS (flunitrazepam) to shorten run times. The assays for in vitro studies on glucuronidation [155] were based on HPLC-MS/MS in positive ESI mode, with the use of an ion trap detector: this kind of analyser allows obtaining detailed information on the chemical structures of the analytes, which were then completed and confirmed by nuclear magnetic resonance (NMR) spectra. Gradient elution and a 3.5-µm particle column granted very good separations of all compounds of interest, which are structural isomers with the same molecular mass. NMR was also used to obtain quantitative results.

Other assays were carried out on human plasma [151], again with a slight modification of a previous analytical work [164]. The method is based on HPLC with UV detection (240 nm) and uses a small-particle (3 µm) column. As a consequence, very fast chromatographic separations can be obtained: the total run time for the analysis of midazolam, 1'hydroxymidazolam and the IS diazepam is less than 5 min. Sample pre-treatment is carried out by simple LLE with methyl *t*-butyl ether. Sensitivity (LOQ = 7 ng/mL) and precision (RSD < 8%) are satisfactory. On the contrary, the dose-dependence of blood levels of midazolam and its monohydroxylated metabolites [153] used an HPLC-MS method [165] with outstanding sensitivity (LOQ = 50 pg/mL for all analytes), even though precision is comparable (CV < 7.3%). The method is based on a small diameter (2.1 mm)

C18 column and gradient elution; an ion trap spectrometer allowed working in tandem mass conditions. Sample pre-treatment was carried out by LLE with hexane/dichloromethane and midazolam-d_6 was used as the IS.

Other analytical techniques have been used as well, such as GC-MS [156, 157, 158]. For the study on the use of midazolam in neonatal intensive care [157], a 0.2-μm diameter capillary bonded stationary phase (phenyl-methylpolysiloxane) was used. Only a few details of the method (and no reference to an analytical paper) are reported, such as LOQ (1 ng/mL for midazolam, 0.5 ng/mL for the 1'-hydroxy metabolite) and precision (CV < 10%) values and the use of a very small amount of sample (50 μL) for one analysis. Sample pre-treatment is carried out by SPE, however nothing is said of the procedure, or of the sorbent type.

TRIAZOLAM

Triazolam (8-chloro-6-(2-chlorophenyl)-1-methyl-4*H*-[1,2,4]triazolo[4,3-a][1,4]benzodiazepine) is a triazolobenzodiazepine commercially available as Halcion, Novodorm, Songar or Zotrilax. It is a short-acting BZD used for the treatment of insomnia. Normal doses are extremely low, in the 0.125-0.25 mg range; doses higher than 0.5 mg are generally avoided. Triazolam use has been associated with psychological side effects, such as increased aggressiveness and violent reactions [166]. For this reason, the drug was withdrawn for a period in the USA; now it has been deemed safe at low doses by the US Food and Drug Administration (FDA). However, it is still banned in some countries, such as the UK.

- Metabolism and pharmacokinetics

Triazolam is mainly metabolised in the liver by CYP3A4 [167], however none of its metabolites is pharmacologically active. Some pre-systemic metabolism seems also to occur [168], since

grapefruit juice (a known inhibitor of gastro-intestinal CYP3A4) can cause dramatic changes in the drug bioavailability, with an increase of 96% in the AUC(0-24) [169]; repeated doses of juice amplify this effect [170]. From studies on human recombinant CYP isoforms, it has been found that the action of CYP3A4 produces two hydroxylated metabolites (1'-hydroxytriazolam and 4-hydroxytriazolam, Figure 11), while CYP1A2, CYP2C9, CYP2C19, CYP2D6 and CYP2E1 do not seem to be involved [171]; the possible contribution of CYP3A5 is still uncertain [172]. The importance of CYP3A4 has been confirmed by the ineffectiveness of oral triazolam therapy during the administration of rifampicin, a potent inducer of this CYP [173]. Several other drugs have been shown to influence triazolam metabolism by way of CYP3A4 interactions; for example, the antimycobacterial isoniazid [65], the viral protease inhibitors ritornavir and amprenavir [174, 175], some reverse transcriptase inhibitors [176], some macrolide antimicrobials [177] and azole antifungals (ketoconazole, itraconazole, fluconazole) [178] inhibit triazolam biotransformation. On the contrary, the stimulant modafinil [179] induces it. The anti-ulcer ranitidine has been reported to increase triazolam AUC(0-∞); however, no increase in the 1'-hydroxytriazolam/triazolam AUC(0-∞) ratio was observed. This has led to hypothesise that ranitidine increases triazolam absorption by raising the gastric pH, since the latter drug is acid-labile [180].

Since triazolam has been associated to aggressiveness, violence and abuse, the presence of its metabolites in urine has drawn considerable attention as a possible means of ascertaining (voluntary or unsuspecting) triazolam intake. The main metabolite, 1'-hydroxytriazolam, is detectable in this fluid for up to 35 h after triazolam intake and reaches its peak urine concentration after 5-10 h [181], while the urinary excretion rate of the 4-hydroxy metabolite appears to be slower [182]. Both compounds are almost completely glucuronidated in urine.

- Analytical methods

Like for midazolam, the almost exclusive dependence of triazolam metabolism on CYP3A4 activity has let to the identification of the drug as one of the ideal probes for CYP3A4 modulation by other

agents. As a consequence, there are plenty of scientific papers (and related analytical methods) dealing with changes in triazolam concentrations to assess the potential for interactions of other drugs or active compounds. Some of these deal with matrices such as recombinant cytochromes or microsomes; we will focus on those, which deal with biological fluids such as blood or urine.

A study on the effect of grapefruit ingestion [169] used a rather complicated HPLC-UV method to determine triazolam plasma levels. In fact, the plasma sample pre-treatment consisted of protein precipitation with methanol, followed by a double preparative HPLC run with fraction collection and re-injection. Finally, the purified sample was injected into a C18 column, with a chromatographic run length of about 15 min. In any case, the method sensitivity seems to be good (LOQ = 0.5 ng/mL) and precision is acceptable, albeit with high RSD values (up to 18%). Interestingly, the chosen IS is not another BZD, but butyl *p*-hydroxybenzoate. Alternatively, for a similar purpose [170] a capillary GC method was used. In this case, sample pre-treatment was carried out by SPE on C8 cartridges and detection by electron capture, obtaining higher sensitivity (LOQ = 0.1 ng/mL) and comparable precision (RSD < 16%) [183]. While run times were long (30 min), this was due to the fact that the method was originally developed for the analysis of 15 different anxiolytic-hypnotics. Prazepam was chosen as the IS.

Two methods were developed for the analysis of triazolam metabolites in urine.

The first method [181] regards 1'-hydroxytriazolam only, which was analysed on a 30-m fused silica capillary column. The MS detector uses an electron impact source and a single quadrupole analyser. Sample pre-treatment involves hydrolysis by means of β-glucuronidase (to analyse free and glucuronidated 1'-hydroxytriazolam) and LLE with ethyl acetate after alkalinisation. A complete analytical run lasts about 13 min and, interestingly, 1'-hydroxytriazolam-d_4 is used as the IS. As previously noted, using deuterated ISs is the norm when dealing with MS detection, however most methods dealing with BZD metabolism use other BZDs instead. In this case, however, sample composition and co-administered drugs are much more unpredictable (i.e., real samples come from possible unsuspecting victims), thus analytical interferences cannot be ruled out as in a controlled

pharmacokinetic assay: hence the use of a deuterated analogue. Good sensitivity (LOQ = 0.1 ng/mL) and precision (RSD < 5%) were achieved with this assay.

It should be noted that BZDs usually are not volatile enough to allow their direct GC analysis; they have to be derivatised by trimethylsilylation first. This, of course, introduces a further pre-treatment step and a possible source of error in the procedure

The second method [182] deals with the analysis of both 1'-hydroxytriazolam and 4-hydroxytriazolam in urine by HPLC-MS. Since in this case the setting was tightly controlled, the IS was nitrazepam, another BZD as usual. Analyte separation was achieved on a C18 column using ammonium acetate /methanol as the mobile phase. MS detection was carried out by ESI and single quadrupole. Again, all samples were hydrolysed with β-glucuronidase and extracted by LLE with hexane/dichloromethane. The LOQ values were 2 ng/mL for 1'-hydroxytriazolam and 1 ng/mL for 4-hydroxytriazolam; precision values, expressed as RSD, were always under 10.5%.

CONCLUSION

In this review, an overview has been presented of the recent advances (1998-today) in the knowledge of metabolism and possible interactions of the most widely used BZDs. Furthermore, details have been given and comparisons have been made regarding the analytical methods used to obtain the corresponding data. The reliability of the latter is of the utmost importance, since any clinical or therapeutic evaluation is only as good as the data on which it is based.

A plethora of analytical methods, based on several different techniques, has been published even in very recent times on the analysis of one or more BZDs and/or metabolites in human biological fluids [184-190]. However, most of them were not specifically developed (or adapted and used) with the aim of evaluating metabolic parameters, thus their suitability for this purpose remains unknown.

On the contrary, this review was focused on those methods, which have been tested on metabolism or pharmacokinetic studies. Reversed phase LC is by far the most frequently used technique, followed by GC, represented only by a few cases. This is a long established trend in the drug analysis field, due to the extreme flexibility of HPLC techniques, their ease of coupling to a wide variety of detectors and their applicability to most analytes. GC is mostly used for particular analytes, or when an established method and existing instrumentation are at hand.

For both separation techniques, coupling to MS detection is increasing in frequency. MS is almost ubiquitous for GC and only a few methods use other detection means (such as electron capture and nitrogen-phosphorus). HPLC-MS, on the other hand, is rapidly gaining widespread acceptance, however UV detection is still very common. While the superior sensitivity and selectivity of MS is a matter of fact, UV detection has still several attractive features: extremely wide availability, much lower entrance and maintenance costs, ease of operation and low number operative variables. Thus, UV detection can be a very attractive alternative to MS to carry out a less expensive study, especially when the matrix is simple (e.g., recombinant cytochromes) or sensitivity is not an issue.

Of course, when structural information on the analytes is needed, UV is not useful and MS is the technique of choice.

With regard to sample pre-treatment procedures, several have been used (protein precipitation, column switching, preparative HPLC, LLE, SPE), however LLE and SPE are by far the most frequent. LLE in particular seems to be still very widespread, probably because it results in very simple procedures. SPE is gaining importance, due to the low consumption of organic solvents and to the possibility of automation; furthermore, method development is more straightforward and usually sample cleaning and extraction yields are better than those of LLE. However, SPE can be somewhat more expensive due to the cost of cartridges.

FIGURE LEGENDS

Figure 1. Main metabolic pathways of alprazolam.

Figure 2. Main metabolic pathways of bromazepam.

Figure 3. Main metabolic pathways of clotiazepam.

Figure 4. Main metabolic pathways of diazepam.

Figure 5. Main metabolic pathways of etizolam.

Figure 6. Main metabolic pathways of lorazepam.

Figure 7. Main metabolic pathways of oxazepam.

Figure 8. Main metabolic pathways of brotizolam.

Figure 9. Main metabolic pathways of flunitrazepam.

Figure 10. Main metabolic pathways of midazolam.

Figure 11. Main metabolic pathways of triazolam.

REFERENCES

[1] Greenblatt, D.J. (1992) *J. Clin. Psychiat.,* **53**(Suppl. 1), 7-13.

[2] Nemeroff, C.B. (2003) *J. Clin. Psychiat.,* **64**(Suppl. 3), 3-6.

[3] Charney, D.S.; Mihic, S.J. and Harris, R.A. (2005) in *Goodman & Gilman's The Pharmacological Basis of Therapeutics, 11th Edition,* (Brunton, L.; Lazo, J.; Parker, K. Eds.), McGraw-Hill, Cloumbus, pp. 401-428.

[4] Erman, M.K. (2006) *J. Clin. Psychiat.,* **67**(Suppl. 13), 9-12.

[5] Chouinard, G.; Lefko-Singh, K. and Teboul E. (1999) *Cell. Mol. Neurobiol.,* **19**(4), 533-552.

[6] Whitmore, J. (2000) Psychiat. Bull., **24**, 113-115.

[7] Birkenhäger, T.K.; Moleman, P. and Nolen, W.A. (1995) *Int. Clin. Psychopharmacol.,* **10**(3), 181-195.

[8] Gaillard, R.; Ouanas, A.; Spadone, C.; Llorca, P.-M.; Loo, H. and Bayle, F.-J. (2006) *Encephale,* **32**(6), 1003-1010.

[9] Verster, J.C. and Volkerts, E.R. (2004) *CNS Drug Rev.,* **10**(1), 45-76.

[10] Smith, R.B.; Kroboth, P.D.; Vanderlugt, J.T.; Phillips, J.P. and Juhl, R.P. (1984) *Psychopharmacology,* **84**, 452-456.

[11] Erdman, K.; Stypinski, D.; Combs, M.; Witt, P.; Stiles, M. and Pollock, S. (2007) *Pharmacotherapy,* **27**(8), 1120-1124.

[12] Kaplan, G.B.; Greenblatt, D.J.; Ehrenberg, B.L.; Goddard, J.E.; Harmatz, J.S. and Shader, R.I. (1998) *J. Clin. Pharmacol.,* **38**(1), 14-21.

[13] Yasui, N.; Otani, K.; Kaneko, S.; Ohkubo, T.; Osanai, T.; Sugawara, K.; Chiba, K. and Ishizaki, T. (1996) *Clin. Pharmacol. Ther.,* **59**(5), 514-519.

[14] Williams, J.A.; Ring, B.J.; Cantrell, V.E.; Jones, D.R.; Eckstein, J.; Ruterbories, K.; Hamman, M.A.; Hall, S.D. and Wrighton, S.A. (2002) *Drug Metab. Dispos.,* **30**(8), 883-891.

[15] Hirota, N.; Ito, K.; Iwatsubo, T.; Green, C.E.; Tyson, C.A.; Shimada, N.; Suzuki, H. and

Sugiyama, Y. (2001) *Biopharm*. *Drug Dispos*., **22**(2), 53-71

[16] Yasui, N.; Kondo, T.; Furukori, H.; Kaneko, S.; Ohkubo, T.; Uno, T.; Osanai, T.o; Sugawara, K. and Otani, K. (2000) *Psychopharmacology,* **150**(2), 185-190.

[17] Park, J.-Y.; Kim, K.-A.; Park, P.-W.; Lee, O.-J.; Kang, D.-K.; Shon, J.-H.; Liu, K.-H. and Shin, J.-G. (2006) *Clin. Pharmacol. Ther.,* **79**(6), 590-599.

[18] Venkatakrishnan, K. and Gibbs, M.A. (2006) *Clin. Pharmacol. Ther.,* **80**(6), 719-720.

[19] Park, J.-Y.; Kim, K.-A. and Shin, J.-G. (2006) *Clin. Pharmacol. Ther.,* **80**(6), 720-720.

[20] Wennerholm, A.; Allqvist, A.; Svensson, J.-O.; Gustafsson, L.L.; Mirghani, R.A. and Bertilsson, L. (2005) *Eur. J. Clin. Pharmacol.,* **61**(2), 113-118.

[21] Lau, C.E. (1999) *Drug Metab. Dispos.,* **27**(8), 855-859.

[22] Gupta, B.; Chopra, S.C.; Gupta, C.; Mahajan, R.; Uppal, B.; Minocha, K.B. (2006) *Indian J. Pharmacol.,* **38**(2), 133-134.

[23] Calvo, G.; Garcia-Gea, C.; Luque, A.; Morte, A.; Dal-Re, R. and Barbanoj, M. (2004) *J. Clin. Psychopharmacol.,* **24**(3), 268-276.

[24] Preskorn, S.H.; Greenblatt, D.J. and Harvey, A.T. (2000) *J. Clin. Psychopharmacol.,* **20**(5), 585-586.

[25] Suzuki, Y.; Shioiri, T.; Muratake, T.; Kawashima, Y.; Sato, S.; Hagiwara, M.; Inoue, Y.; Shimoda, K. and Someya, T. (2003) *Eur. J. Clin. Pharmacol.,* **58**(12), 829-833.

[26] Amchin, J.; Zarycranski, W.; Taylor, K.P.; Albano, D. and Klockowski, P.M. (1998) *Psychopharmacol. Bull.,* **34**(2), 211-219.

[27] Arold, G.; Donath, F.; Maurer, A.; Diefenbach, K.; Bauer, St.; Henneicke-von Zepelin, H.-H.; Friede, M. and Roots, I. (2005) *Planta Med.,* **71**(4), 331-337.

[28] Toyo'oka, T.; Kumaki, Y.; Kanbori, M.; Kato, M. and Nakahara, Y. (2003) *J. Pharmaceut. Biomed.,* **30**(6), 1773-1787.

[29] Crouch, D.J.; Rollins, D.E.; Canfield, D.V.; Andrenyak, D.M. and Schulties, J.E. (1999) *J.*

Anal. Toxicol., **23**, 479-485.

[30] Allqvist, A.; Wennerholm, A.; Svensson, J.-O. and Mirghani, R.A. (2005) *J. Chromatogr. B,* **814**(1), 127-131.

[31] Jin, L. and Lau, C.E. (1994) *J. Chromatogr. B,* **654**(1), 77-83.

[32] Hall, J.; Naranjo, C.A.; Sproule, B.A.; Herrmann, N. (2003) *J. Clin. Psychopharmacol.,* **23**(4), 349-357.

[33] Yajima, T.; Ohno, T.; Nakamura, K. and Nakamura, K. (1984) *Folia Pharmacol. Jpn.,* **83**(3), 37-48.

[34] Ohtani, Y.; Kotegawa, T.; Tsutsumi, K.; Morimoto, T.; Hirose, Y. and Nakano, S. (2002) *J. Clin. Pharmacol.,* **42**(2), 183-191.

[35] van Harten, J.; Holland, R.L. and Wesnes, K. (1992) *Eur. Neuropsychopharmacol.,* **2**(3), 381-381.

[36] Oda, M.; Kotegawa, T.; Tsutsumi, K.; Ohtani, Y.; Kuwatani, K. and Nakano, S. (2003) *Eur. J. Clin. Pharmacol.,* **59**(8-9), 615-619.

[37] "Lexotan" Product Information, approved 6 October 2004, amended 22 February 2007. Available through http://www.pbs.gov.au/pi/roplexot10207.pdf (last access: 20 July 2008).

[38] Schwartz, M.A.; Postma, E.; Kolis, S. and Leon, A.S. (2006) *J. Pharm. Sci.,* **62**, 1776-1779.

[39] Lerner, F.E.; Schere, D.; Batafarano, N.; Casas, F. and Glancszpigel, R. (2001) *Arzneimittel Forschung,* **51**(12), 955-958.

[40] Funk, F.; Canclini, C. and Geisser, P. (2007) *Arzneimittel Forschung,* **57**(6a), 370-375.

[41] Heizmann, P.; Geschke, R. and Zinapold, K. (1984) *J. Chromatogr.,* **310**, 129-137.

[42] Albu, F.; Georgita, C.; Tache, F.; Mutihac, L.; Medvedovici, A. and David, V. (2007) *J. Liq. Chromatogr. R.T.,* **30**(18), 2699-2715.

[43] Saraiva Goncalves, J.C.; Monteiro, T.M.; de Miranda Neves, C.S.; da Silva Gram, K.R.; Volpato, N.M.; Silva, V.A.; Caminha, R.; do Rocio Bencke Goncalves, M.; Dos Santos, F.M.; Estolano Da Silveira, G. and Noel, F. (2005) *Ther. Drug Monit.,* **27**(5), 601-607.

[44] Andraus, M. H.; Wong, A.; Silva, O.A.; Wada, C.Y.; Toffleto, O.; Azevedo, C.P. and Salvadori, M.C. (2004) *J. Mass Spectrom.,* **39**(11), 1348-1355.

[45] Arendt, R.; Ochs, H.R. and Greenblatt, D. J. (1982) *Arzneimittel Forschung,* **32**(4), 453-455.

[46] Niwa, T.; Shiraga, T.; Ishii, I.; Kagayama, A. and Takagi, A. (2005) *Biol. Pharm. Bull.,* **28**(9), 1711-1716.

[47] Shimada, T.; Yamazaki, H.; Mimura, M.; Inui, Y. and Guengerich, F.P. (1994) *J. Pharmacol. Exp. Ther.,* **270**, 414-423.

[48] Greenblatt, D.J.; Divoll, M.K.; Soong, M.H.; Boxenbaum, H.G.; Harmatz, J.S. and Shader, R.I. (1988) *J. Clin. Pharmacol.,* **28**(9), 853-859.

[49] Baselt, R.C. and Cravey, R.H. (1995) in *Disposition of toxic drugs and chemicals in man, 4th ed.,* Chemical Toxicology Institute, Foster City, 1995, p. 211.

[50] Saito, K.; Sakai, N.; Kim, H.-S.; Ishizuka, M.; Kazusaka, Akio. and Fujita S. (2004) *Drug Metab. Dispos.,* **32**(9), 959-965.

[51] Sakai, N.; Saito, K.; Kim, H.-S.; Kazusaka, A.; Ishizuka, M.; Funae, Y. and Fujita, S. (2005) *Drug Metab. Dispos.,* **33**(11), 1657-1660.

[52] Yang, T.J.; Krausz, K.W.; Shou, M.; Yang, S.K.; Buters, J.T.M.; Gonzalez, F.J. and Gelboin, H.V. (1998) *Biochem. Pharmacol.,* **55**(10), 1633-1640.

[53] Yang, T.J.; Shou, M.; Korzekwa, K.R.; Gonzalez, F.J.; Gelboin, H.V. and Yang, S.K. (1998) *Biochem. Pharmacol.,* **55**(6), 889-896.

[54] Mei, Q.; Tang, C.; Assang, C.; Lin, Y.; Slaughter, D.; Rodrigues, A.D.; Baillie, T.A.; Rushmore, T.H. and Shou, M. (1999) *J. Pharmacol. Exp. Ther.,* **291**(2), 749-759.

[55] Yang, T.J.; Krausz, K.W.; Sai, Y.; Gonzalez, F.J. and Gelboin, H.V. (1999) *Drug Metab. Dispos.,* **27**(1), 102-109.

[56] Fukasawa, T.; Suzuki, A. and Otani, K. (2007) *J. Clin. Pharm. Ther.,* 32(4), 333-341.

[57] Zhou, H.-H. (2001) *Clin. Chim. Acta,* **313**(1-2), 203-208.

[58] Qin, X.-P.; Xie, H.-G.; Wang, W.; He, N.; Huang, S.-L.; Xu, Z.-H.; Ou-Yang, D.-S.; Wang, Y.-J. and Zhou, H.-H. (1999) *Clin. Pharmacol. Ther.,* **66**(6), 642-646.

[59] Inomata, S.; Nagashima, A.; Itagaki, F.; Homma, M.; Nishimura, M.; Osaka, Y.; Okuyama, K.; Tanaka, E.; Nakamura, T.; Kohda, Y.; Naito, S.; Miyabe, M. and Toyooka, H. (2005) *J. Clin. Pharm. Ther.,* **78**(6), 647-655.

[60] Klotz, U. (2007) *Clin. Pharmacokin.,* **46**(4), 271-279.

[61] de Haro, L.; Valli, M.; Bourdon, J.H.; Iliadis, A.; Hayek-Lanthois, M. and Arditti, J. (2001) *Vet. Hum. Toxicol.,* **43**(3), 174-175.

[62] Wagner, S.O.; Sams, R.A. and Podell, M. (1998) *J. Vet. Pharmacol. Ther.,* **21**(5), 335-341.

[63] Ozdemir, M.; Aktan, Y.; Boydag, B. S.; Cingi, M. I. and Musmul, A. (1998) *Eur. J. Drug Metab. Ph.,* **23**(1), 55-59.

[64] Reinach, B.; de Sousa, G.; Dostert, P.; Ings, R.; Gugenheim, J. and Rahmani, R. (1999) *Chem.-Biol. Interact.,* **121**(1), 37-48.

[65] Desta, Z.; Soukhova, N.V.; Flockhart, D.A. (2001) *Antimicrob. Agents Ch.,* **45**(2), 382-392.

[66] Jones, H.M.; Hallifax, D. and Houston, J.B. (2004) *Drug Metab. Dispos.,* **32**(5), 572-580.

[67] Einer-Jensen, N. and Larsen, L. (2000) *Pharmacol. Toxicol.,* **87**(6), 276-278.

[68] Platt, S.R.; Randell, S.C.; Scott, K.C.; Chrisman, C.L.; Hill, R.C. and Gronwall, R.R. (2000) *Am. J. Vet. Res.,* **61**(6), 651-654.

[69] Peinemann, F. and Daldrup, T. (2001) *Eur. J. Pediatr.,* **160**(6), 378-381.

[70] Parkinson, A.; Leonard, N.; Draper, A. and Ogilvie, B.W. (2006) *Drug Metab. Rev.,* **38**(1-2), 235-259.

[71] Tanaka, E.; Terada, M.; Misawa, S. and Wakasugi, C. (1996) *J. Chromatogr. B,* **682**, 173-178.

[72] Mealey, K. and Boothe, D. (1995) *Vet. Pharmacol. Ther.,* **18**, 72-74.

[73] Reilly, P.E.B.; Thompson, D.A.; Mason, S.R. and Hooper, W.D. (1990) *Mol. Pharmacol.* **37,** 767-774.

[74] "Depas" Product Information, approved 16 September 1999. Available through http://www.carloanibaldi.com/terapia/schede/DEPAS.htm#SottoTitolo_11 (last access: 20 July 2008).

[75] Niwa, T.; Shiraga, T.; Ishii, I.; Kagayama, A. and Takagi, A. (2005) *Biol. Pharm. Bull.,* **28**(9), 1711-1716.

[76] Araki, K.; Yasui-Furukori, N.; Fukasawa, T.; Aoshima, T.; Suzuki, A.; Inoue, Y.; Tateishi, T. and Otani, K. (2004) Eur. *J. Clin. Pharmacol.,* **60**(6), 427-430.

[77] Fukasawa, T.; Yasui-Furukori, N.; Suzuki, A.; Inoue, Y.; Tateishi, T. and Otani, K. (2005) *Eur. J. Clin. Pharmacol.,* **61**(11), 791-795.

[78] Yue, Q.Y., Tomson, T. and Sawe, J. (1994) *Pharmacogenetics,* **4**, 193-198.

[79] Kondo, S.; Fukasawa, T.; Yasui-Furukori, N.; Aoshima, T.; Suzuki, A.; Inoue, Y.; Tateishi, T. and Otani, K. (2005) *Eur. J. Clin. Pharmacol.,* **61**(3), 185-188.

[80] Makino, T.; Inagaki, T.; Komatsu, K. and Kano, Y. (2005) *Biol. Pharm. Bull.,* **28**(2), 280-284.

[81] Hikida, K., Inoue, Y., Nouchi, E. and Ohkura, Y. (1990) *Jpn. J. Clin. Chem.,* **19**, 354-359.

[82] Ghanchi, F.D. and Khan, M.Y. (1997) *J. Cataract Refract. Surg.,* **23**(10), 1581-1584.

[83] Greenblatt, D.J. (1981) *Clin. Pharmacokinet.,* **6**(2), 89-105.

[84] Chung, J.-Y.; Cho, J.-Y.; Yu, K.-S.; Kim, J.-R.; Jung, H.-R.; Lim, K.-S.; Jang, I.-J. and Shin, S-G. (2005) *Clin. Pharmacol. Ther.,* **77**(6), 486-494.

[85] Sawamura, R.; Sato, H.; Kawakami, J. and Iga, T. (2000) Biol. Pharm. Bull., **23**(5), 669-671.

[86] Papini, O.; Pereira Da Cunha, S.; Do Carmo Da Silva Mathes, A.; Bertucci, C.; Dantas Moises, E.C.; De Barros Duarte, L.; De Carvalho Cavalli, R. and Lanchote, V.L. (2006) *J. Pharmaceut. Biomed.,* **40**(2), 397-403.

[87] Greenblatt, D.J.; Schillings, R.T.; Kyriakopoulos, A.A.; Shader, R.I.; Sisenwine, S.F.; Knowles, J.A. and Ruelius, H.W. (1976) *Clin. Pharmacol. Ther.,* **20**(3), 329-341.

[88] Cohen-Mansfield, J.; Taylor, L.; Woosley, R.; Lipson, S.; Werner, P. and Billig, N. (2000) *Ther. Drug Monit.,* **22**(6), 688-694.

[89] Arroliga, A.C.; Shehab, N.; McCarthy, K. and Gonzales, J.P. (2004) *Crit. Care Med.,* **32**(8), 1709-1714.

[90] Al-Khafaji, A.H.; Dewhirst, W.E. and Manning, H.L. (2002) *Anesth. Analg.,* **94**(6), 1583-1585.

[91] Swart, E.L.; de Jongh, J.; Zuideveld, K.P.; Danhof, M.; Thijs, L.G. and Strack van Schijndel, R.J.M. (2005) *Am. J. Kidney Dis.,* **45**(2), 360-371.

[92] Papini, O.; Bertucci, C.; Pereira da Cunha, S.; Guinaim dos Santos, N.A. and Lanchote, V.L. (2006) *J. Pharmaceut. Biomed.,* **40**(2), 389-396.

[93] "Some Pharmaceutical Drugs", IARC Monographs vol. 66 (1996), p. 115. Available through http://www.inchem.org/documents/iarc/vol66/oxazepam.html (Last access: 20 July 2008).

[94] Mohler, H.; Okada, T.; Heitz, P. and Ulrich, J. (1978) *Life Sci.,* **22**, 985-995.

[95] Patel, M.; Tang, B.K. and Kalow, W. (1995) *Pharmacogenetics* **5**, 43-49.

[96] Court, M.H.; Duan, S.X.; Guillemette, C.; Journault, K.; Krishnaswamy, S.; Von Moltke, L.L. and Greenblatt, D.J. (2002) *Drug Metab. Dispos.,* **30**(11), 1257-1265.

[97] Court, M.H.; Hao, Q.; Krishnaswamy, S.; Bekaii-Saab, T.; Al-Rohaimi, A.; von Moltke, L.L. and Greenblatt, D.J. (2004) *J. Pharmacol. Exp. Ther.,* **310**(2), 656-665.

[98] Greenblatt, D.J. (1981) *Clin. Pharmacokinet.,* **6**, 89-105.

[99] Stockhaus, K. (1986) *Arzneimittel Forschung,* **36**(3a), 601-605.

[100] Jochemsen, J.; Wesselman, J.G.J.; Hermans, J.; van Boxtel, C.J. and Breimer, D.D. (1983) *Br. J. Clin. Pharmacol.,* **16**(Suppl 2), 285-290.

[101] Tokairin, T.; Fukasawa, T.; Yasui-Furukori, N.; Aoshima, T.; Suzuki, A.; Inoue, Y.; Tateishi, T. and Otani, K. (2005) Br. *J. Clin. Pharmacol.,* **60**(2), 172-175.

[102] Osanai, T.; Ohkubo, T.; Yasui, N.; Kondo, T. and Kaneko, S. (2005) *Br. J. Clin. Pharmacol.,* **58**(5), 476-481

[103] Ujiie, Y.; Fukasawa, T.; Yasui-Furukori, N.; Suzuki, A.; Tateishi, T. and Otani, K. (2006) *Ther. Drug Monit.,* **28**(3), 299-302.

[104] Osanai, T. and Ohkubo T. (2003) *Anal. Lett.,* **36**, 3025–3034.

[105] Druid, H.; Holmgren, P. and Ahlner, J. (2001) *Forensic Sci. Int.,* **122**(2-3), 136-141.

[106] Cano, J. P.; Soliva, M.; Hartmann, D.; Ziegler, W. H. and Amrein, R. (1977) *Arzneimittel Forschung,* **27**(12), 2383-2388.

[107] Gafni, I.; Busto, U.E.; Tyndale, R.F.; Kaplan, H.L. and Sellers, E.M. (2003) *J. Clin. Psychopharmacol.,* **23**(2), 169-175.

[108] Kilicarslan, T.; Haining, R.L.; Rettie, A.E.; Busto, U.; Tyndale, R.F. and Sellers, E.M. (2001) *Drug Metab. Dispos.,* **29**(4), 460-465.

[109] Huang, W.; Lin, Y.S.; McConn, D.J. II,; Calamia, J.C.; Totah, R.A.; Isoherranen, N.; Glodowski, M. and Thummel, K.E. (2004) *Drug Metab. Dispos.,* **32**(12), 1434-1445.

[110] Coller, J.K.; Somogyi, A.A. and Bochner, F. (1999) *Xenobiotica,* **29**(10), 973-986

[111] Peng, F.-C.; Chaing, H.-H.; Tang, S.-H.; Chen, P.-C. and Lu, S.-C. (2004) *J. Toxicol. Env. Health,* **67**(2), 109-124.

[112] Kilicarslan, T. and Sellers, E.M. (2000) *Am. J. Psychiat.,* 157(7), 1164-1166.

[113] Tanaka, E.; Nakamura, T.; Terada, T.; Shinozuka, T. and Honda, K. (2005) *Basic Clin. Pharmacol. Toxicol.,* **96**(1), 88-90.

[114] Assaf, M.S. and Abdel-Rahman, M.S. (1999) *Toxicol. in Vitro,* **13**(3), 393-401.

[115] Negrusz, A.; Moore, C.M.; Stockham, T.L.; Poiser, K.R.; Kern, J.L.; Palaparthy, R.; Le, N.L.T.; Janicak, P. G. and Levy, N.A. (2000) *J. Forensic Sci.,* **45**(5), 1031-1040.

[116] Coller, J.K.; Somogyi, A.A. and Bochner, F. (1998) *J. Chromatogr. B,* **719**(1-2), 87-92.

[117] Fisher, J.M.; Wrighton, S.A.; Watkins, P.B.; Schmiedlin-Ren, P.; Calamia, J.C.; Shen, D.D.; Kunze, K.L. and Thummel, K.E. (1999) *J. Pharmacol. Exp. Ther.,* **289**(2), 1134-1142.

[118] Eeckhoudt, S.L.; Horsmans, Y. and Verbeeck, R.K. (2002) *Xenobiotica,* **32**(11), 975-984.

[119] Shih, P.-S. and Huang, J.-D. (2002) *Drug Metab. Dispos.,* **30**(12), 1491-1496.

[120] Kharasch, E.D.; Walker, A.; Isoherranen, N.; Hoffer, C.; Sheffels, P.; Thummel, K.; Whittington, D. and Ensign, D. (2007) *Clin. Pharmacol. Ther.,* **82**(4), 410-426.

[121] Hamaoka, N.; Oda, Y.; Hase, I. and Asada, A. (2001) *Br. J. Anaesth.,* **86**(4), 540-544.

[122] Pinto Amar, G.; Wang, Y.-H.; Chalasani, N.; Skaar, T.; Kolwankar, D.; Gorski, J.C.; Liangpunsakul, S.; Hamman, M.A.; Arefayene, Million. and Hall, S.D. (2005) *Clin. Pharmacol. Ther.,* **77**(3), 178-188.

[123] Gorski, J.C.; Jones, D.R.; Haehner-Daniels, B.D.; Hamman, M.A.; O'Mara, E.M. and Hall, S.D. (1998) *Clin. Pharmacol. Ther.,* **64**(2), 133-143.

[124] Quinney, S.K.; Haehner, B.D.; Rhoades, M.B.; Lin, Z.; Gorski, J.C. and Hall, S.D. (2008) *Br. J. Clin. Pharmacol.,* **65**(1), 98-109.

[125] Zhang, W.; Tan, T.M.C. and Lim, L.-Y. (2007) *Drug Metab. Dispos.,* **35**(1), 110-115.

[126] Takedomi, S.; Matsuo, H.; Yamano, K.; Ohtani, H.; Sawada, Y. (2001) *J. Pharm. Pharmacol.,* **53**(5), 643-651.

[127] Oda, Y.; Mizutani, K.; Hase, I.; Nakamoto, T.; Hamaoka, N. and Asada, A. (1999) *Br. J. Anaesth.,* **82**(6), 900-903.

[128] Quintieri, L.; Palatini, P.; Nassi, A.; Ruzza, P. and Floreani, M. (2008) *Biochem. Pharmacol.,* **75**(6), 1426-1437.

[129] Ahonen, J.; Olkkola, K.T.; Takala, A.; Neuvonen, P.J. (1999) *Acta Anaesth. Scand.,* **43**(5), 509-514.

[130] Kashuba, A.D.; Nafziger, A.N.; Kearns, G.L.; Leeder, J.S.; Gotschall, R.; Rocci, M.L.Jr; Kulawy, R.W; Beck, D.J. and Bertino, J.S.Jr (1998) *Clin. Pharmacol. Ther.,* **64**(3), 257-268.

[131] Lam, Y.W.F.; Alfaro, C.L.; Ereshefsky, L.; Miller, M. (2003) *J. Clin. Pharmacol.,* **43**(11), 1274-1282.

[132] Veronese, M.L.; Gillen, L.P.; Burke, J.P.; Dorval, E.P.; Hauck, W.W.; Pequignot, E.; Waldman, S.A. and Greenberg, H.E. (2003) *J. Clin. Pharmacol.,* **43**(8), 831-839.

[133] Mc Donnell, C.G.; Shorten, G. and Van Pelt, F.N.A.M. (2005) *Anaesthesia,* **60**(8), 747-753.

[134] Backman, J.T.; Kivisto, K.T.; Olkkola, K.T. and Neuvonen, P.J. (1998) *J. Clin. Pharmacol.,* **54**(1), 53-58.

[135] Tsunoda, S.M.; Velez, R.L.; Von Moltke, L.L. and Greenblatt, D.J. (1999) *Clin. Pharmacol. Ther.,* **66**(5), 461-471.

[136] Ito, K.; Ogihara, K.; Kanamitsu, S.-I.; Itoh, T. (2003) *Drug Metab. Dispos.,* **31**(7), 945-954.

[137] Hamaoka, N.; Oda, Y.; Hase, I.; Mizutani, K.; Nakamoto, T.; Ishizaki, T. and Asada, A. (1999) *Clin. Pharmacol. Ther.,* **66**(2), 110-117.

[138] Nakajima, M.; Suzuki, T.; Sasaki, T.; Yokoi, T.; Hosoyamada, A.; Yamamoto, T. and Kuroiwa, Y. (1999) *Ther. Drug Monit.*, **21**(5), 507-513.

[139] Huwyler, J.; Wright, M.B.; Gutmann, H. and Drewe, J. (2006) *Curr. Drug Metab.,* **7(**2), 119-26.

[140] Hosagrahara, V.P.; Hansen, L.K. and Remmel, R.P. (1999) *Drug Metab. Dispos.,* **27**(12), 1512-1518.

[141] Wang, Z.; Gorski, J.C.; Hamman, M.A.; Huang, S.M.; Lesko, L.J. and Hall, S.D. (2001) *Clin. Pharmacol. Ther.,* **70**(4), 317-326.

[142] Qi, J.W.; Nakamura, K.; Hosokawa, S.; Okada, Y.; Horiuchi, R.; Yamamoto, K. (2005) *Biol. Pharmaceut. Bull.,* **28**(8), 1467-1471.

[143] Backman, J.T.; Maenpaa, J.; Belle, D.J.; Wrighton, S.A.; Kivisto, K.T. and Neuvonen, P.J. (2000) *Clin. Pharmacol. Ther.,* **67**(4), 382-390.

[144] Lilja, J.J.; Backman, J.T. and Neuvonen, P.J. (2007) *Clin. Pharmacol. Ther.,* **81**(6), 833-839.

[145] Wang, J.-S.; Backman, J.T.; Kivisto, K.T. and Neuvonen, P.J. (2000) *Eur. J. Clin. Pharmacol.,* **56**(8), 555-559.

[146] Kashuba, A.D.M.; Bertino, J.S.Jr; Rocci, M.L.Jr; Kulawy, R.W.; Beck, D.J. and Nafziger, A.N. (1998) *Clin. Pharmacol. Ther.,* **64**(3), 269-277.

[147] Kharasch, E.D.; Mautz, D.; Senn, T.; Lentz, G.; Cox, K. (1999) *J. Clin. Pharmacol.,* **39**(3), 275-280.

[148] Dostalek, M.; Jurica, J.; Pistovcakova, J.; Hanesova, M.; Tomandl, J.; Linhart, I. and Sulcova, A. (2007) *Xenobiotica,* **37**(12), 1355-1366.

[149] Wang, J.-S.; Backman, J.T.; Kivisto, K.T. and Neuvonen, P.J. (2000) *Eur. J. Clin. Pharmacol.,* **56**(8), 555-559.

[150] Pentikis, H.S.; Connolly, M.; Trapnell, C.B.; Forbes, W.P. and Bettenhausen, D.K. (2007) *Pharmacotherapy,* **27**(10), 1361-1369.

[151] Johnson, T.N.; Rostami-Hodjegan, A.; Goddard, J.M.; Tanner, M. S. and Tucker, G.T. (2002) *Br. J. Anaesth.,* **89**(3), 428-437.

[152] Boulieu, R.; Lehmann, B.; Salord, F.; Fisher, C. and Morlet, D. (1998) *Eur. J. Drug Metab. Ph.,* **23**(2), 255-258.

[153] Bodmer, M.; Link, B.; Grignaschi, N.; Kummer, O.; Ruegg, S.; Haschke, M. and Kraehenbuehl, S. (2008) *Ther. Drug Monit.,* **30**(1), 120-124.

[154] Zhu, B.; Bush, D.; Doss, G.A.; Vincent, S.; Franklin, R.B. and Xu, S. (2008) *Drug Metab. Dispos.,* **36**(2), 331-338.

[155] Klieber, S.; Hugla, S.; Ngo, R.; Arabeyre-Fabre, C.; Meunier, V.; Sadoun, F.; Fedeli, O.; Rival, M.; Bourrie, M.; Guillou, F.; Maurel, P. and Fabre, G. (2008) *Drug Metab. Dispos.,* **36**(5), 851-862.

[156] Reed, M.D.; Rodarte, A.; Blumer, J.L.; Khoo, K.-C.; Akbari, B.; Pou, S. and Kearns, G.L. (2001) *J. Clin. Pharmacol.,* **41**(12), 1359-1369.

[157] de Wildt, S.N.; Kearns, G.L.; Hop, W.C.J.; Murry, D.J.; Abdel-Rahman, S.M. and van den Anker, J.N. (2001) *Clin. Pharmacol. Ther.,* **70**(6), 525-531.

[158] Mulla, H.; McCormack, P.; Lawson, G.; Firmin, R.K. and Upton, D.R. (2003) *Anesthesiology,* **99**(2), 275-282.

[159] Knoester, P.D.; Jonker, D.M.; van der Hoeven, R.T.M.; Vermeij, T.A.C.; Edelbroek, P.M.; Brekelmans, G.J. and de Haan, G.J. (2002) *Br. J. Clin. Pharmacol.,* **53**(5), 501-507.

[160] Odou, P.; Barthelemy, C.; Chatelier, D.; Luyckx, M.; Brunet, C.; Dine, T.; Gressier, B.; Cazin, M.; Cazin, J.C. and Robert, H. (1999) *Eur. J. Drug Metab. Ph.,* **24**(1), 1-7.

[161] Tsubo, T.; Sakai, I.; Okawa, H.; Ishihara, H. and Matsuki, A. (2001) *Intens. Care Med.,* **27**(6), 1087-90.

[162] Swart, E.L.; de Jongh, J.; Zuideveld, K.P.; Danhof, M.; Thijs, L.G. and Strack van Schijndel, R.J.M. (2005) *Am. J. Kidney Dis.,* **45**(2), 360-371.

[163] Eeckhoudt, S.L.; Desager, J.-P.; Horsmans, Y.; De Winne, A.J. and Verbeeck, R.K. (1998) *J. Chromatogr. B,* **710**, 165-171.

[164] Carrillo, J.A.; Ramos, S.I.; Agundez, J.A.; Martinez, C. and Benitez, J. (1998) *Ther. Drug Monit.,* **20**(3), 19-24.

[165] Link, B.; Haschke, M.; Wenk, M.; Krahenbuhl, S. (2007) *Rapid Commun. Mass Spectrom.,* **21**(9),1531-1540.

[166] Berman, M.E. and Taylor, S. (1995) Exp. Clin. Psychopharmacol., 3(4), 411-416.

[167] Warrington, J.S.; Greenblatt, D.J. and Von Moltke, L.L. (2004) *Xenobiotica,* **34**(5), 463-471.

[168] Arayne, M.S.; Sultana, N. and Bibi, Z. (2005) *Pak. J. Pharm. Sci.,* **18**(4), 45-57.

[169] Sugimoto, K.; Araki, N.; Ohmori, M.; Harada, K.; Cui, Y.; Tsuruoka, S.; Kawaguchi, A. and Fujimura, A. (2006) *Eur. J. Clin. Pharmacol.,* **62**(3), 209-215.

[170] Lilja, J.J.; Kivisto, K.T.; Backman, J.T.; Neuvonen, P.J. (2000) *Eur. J. Clin. Pharmacol.,* **56**(5), 411-415.

[171] Shinozuka, T.; Kurihara, K.; Yamamoto, K.; Tanaka, E. and Terada, M. (2005) *Jpn. J. Forensic Toxicol.,* **23**(3), 205-208.

[172] Patki, K.C.; von Moltke, L.L. and Greenblatt, D.J. (2003) *Drug Metab. Dispos.,* **31**(7), 938-944.

[173] Niemi, M.; Backman, J.T.; Fromm, M.F.; Neuvonen, P.J.; Kivisto, K.T. (2003) *Clin. Pharmacokin.,* **42**(9), 819-850.

[174] Greenblatt, D.J.; von Moltke, L.L.; Harmatz, J.S.; Durol, A.L.B.; Daily, J.P.; Graf, J.A.; Mertzanis, P.; Hoffman, J.L. and Shader, R.I. (2000) *JAIDS - J. Acq. Imm. Def.,* **24**(2), 129-136.

[175] von Moltke, L.L.; Durol, A.L.; Duan, S.X. and Greenblatt, D.J. (2000) *Eur. J. Clin. Pharmacol.,* **56**(3), 259-261.

[176] von Moltke, L.L.; Greenblatt, D.J.; Granda, B.W.; Giancarlo, G.M.; Duan, S.X.; Daily, J.P.; Harmatz, J.S. and Shader, R.I. (2001) *J. Clin. Pharmacol.,* **41**(1), 85-91.

[177] Greenblatt, D.J.; Von Moltke, L.L.; Harmatz, J.S.; Counihan, M.; Graf, J.A.; Durol, A.L.B.; Mertzanis, P.; Duan, S.X.; Wright, C.E. and Shader, R.I. (1998) *Clin. Pharmacol. Ther.,* **64**(3), 278-285.

[178] Von Moltke, L.L.; Greenblatt, D.J.; Duan, S.X.; Harmatz, J.S. and Shader, R.I. (1998) *Pharm. Pharmacol. Commun.,* **4**(9), 443-445.

[179] Robertson, P.Jr; Hellriegel, E.T.; Arora, S. and Nelson, M. (2002) *Clin. Pharmacol. Ther.,* **71**(1), 46-56.

[180] O'Connor-Semmes, R.L.; Kersey, K.; Williams, D.H.; Lam, R. and Koch, K.M. (2001) *Clin. Pharmacol. Ther.,* **70**(2), 126-131.

[181] Lin, D.-L.; Huang, T.-Y.; Liu, H.-C. and Yin, R.-M. (2005) *J. Anal. Toxicol.,* **29**(2), 118-123.

[182] Tsujikawa, K.; Kuwayama, K.; Miyaguchi, H.; Kanamori, T.; Iwata, Y.; Inoue, H. and Kishi, T. (2005) *J. Anal. Toxicol.,* **29**(4), 240-243.

[183] Gaillard, Y., Gay-Montchamp, J.P. and Ollagnier, M. (1993) *J. Chromatogr.,* **622**(2), 197-208.

[184] Moore, C.; Coulter, C.; Crompton, K. and Zumwalt, M. (2007) *J. Anal. Toxicol.,* **31**(9), 596-600.

[185] Ngwa, G.; Fritch, D.; Blum, K. and Newland, G. (2007) *J. Anal. Toxicol.,* **31**(7), 369-376.

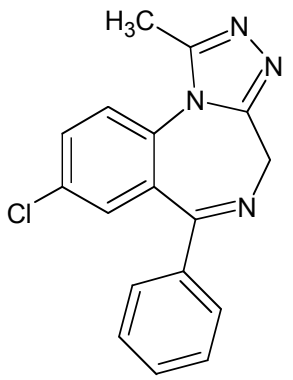
[186] Gil Tejedor, A.M.; Fernandez Hernando, P. and Durand Alegria, J.S. (2007) *Anal. Chim. Acta,* **591**(1), 112-115.

[187] Samanidou, V.F.; Pechlivanidou, A.P. and Papadoyannis, I.N. (2007) *J. Sep. Sci.,* **30**(5), 679-687.

[188] Blas, M.; McCord, B.R. (2008) *Electrophoresis,* **29**(10), 2182-2192.

[189] Anderson, R.A.; Ariffin, M.M.; Cormack, P.A.G. and Miller, E.I. (2008) *Forensic Sci. Int.,* **174**(1), 40-46.

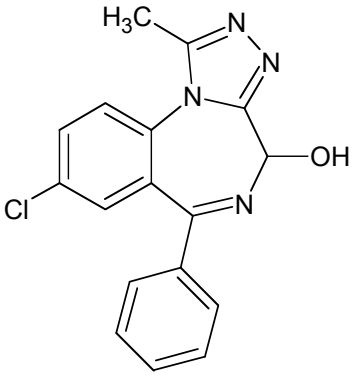
[190] Mercolini, L.; Mandrioli, R.; Amore, M. and Raggi, M.A. (2008) *J. Sep. Sci.,* **31**(14), 2619-2626.



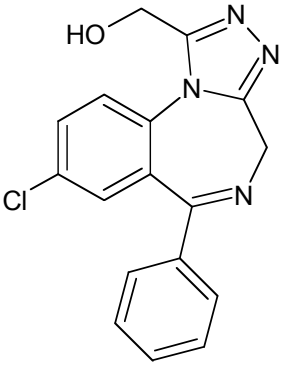
Alprazolam

CYP3A4

CYP3A5



4-Hydroxyalprazolam



α-Hydroxyalprazolam

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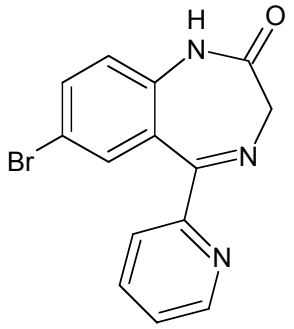
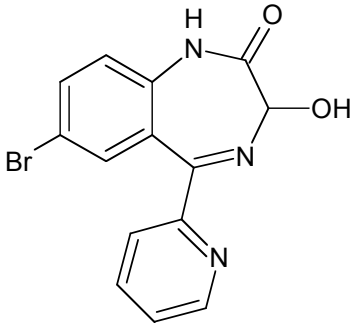
Figure 1



Bromazepam

CYP1A2?
CYP2D6?



3-Hydroxybromazepam

UGT

3-Hydroxybromazepam
glucuronide

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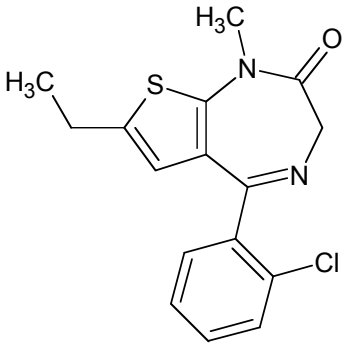
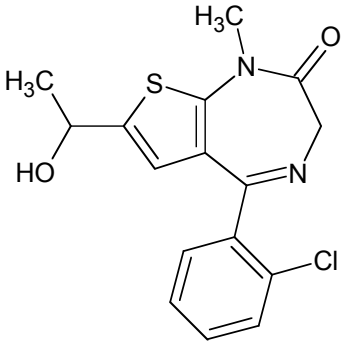
Figure 2

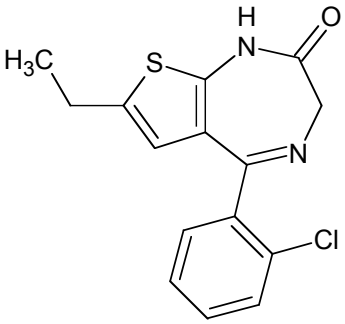


Clotiazepam

CYP3A4
CYP2B6
CYP2C18?
CYP2C19?



α-Hydroxyclotiazepam



N-Desmethylclotiazepam

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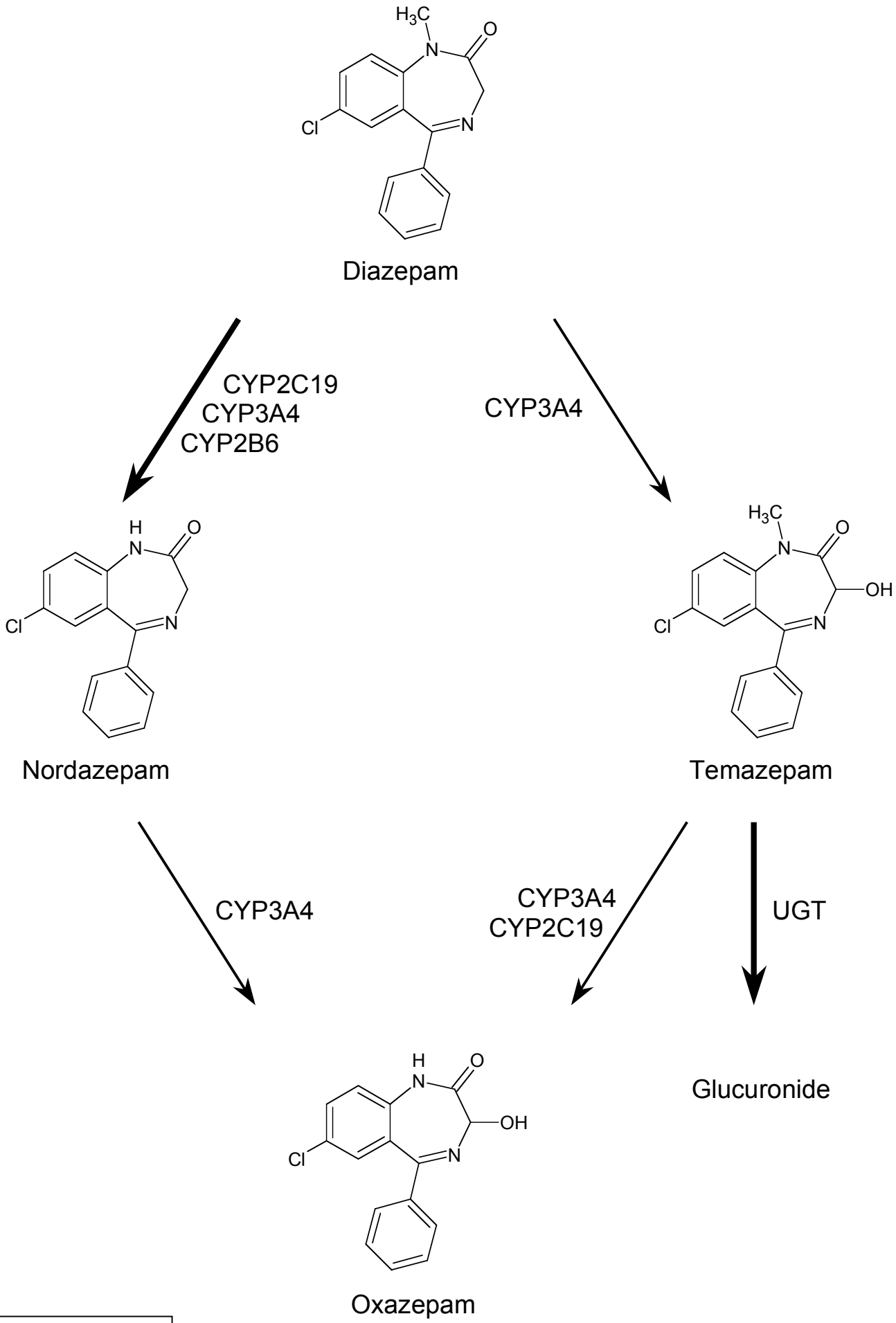
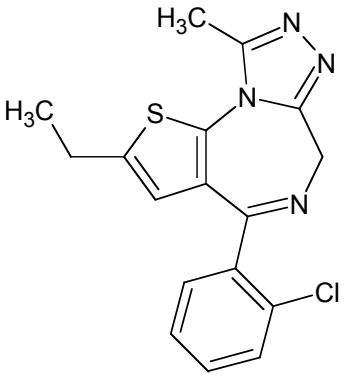
Figure 3

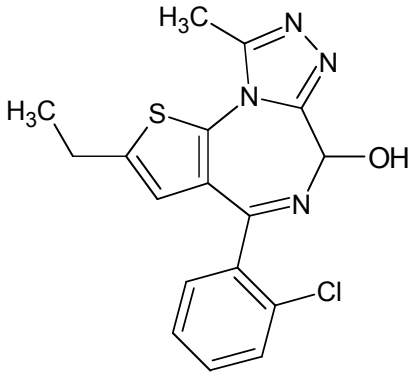


R. Mandrioli *et al.*
Figure 4

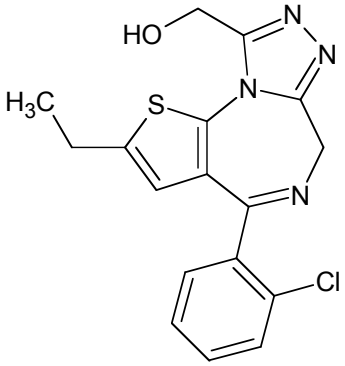


Etizolam

CYP3A4
CYP2C19
CYP2C18?



6-Hydroxyetizolam



α-Hydroxyetizolam

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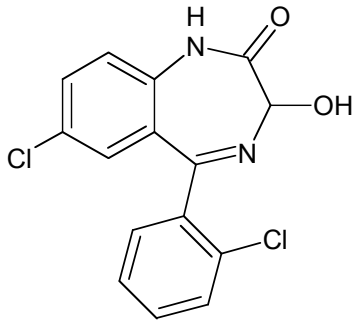
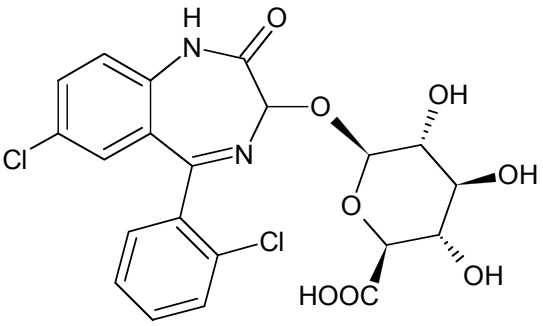
Figure 5



Lorazepam

UGT2B15?



Lorazepam 3-*O*-glucuronide

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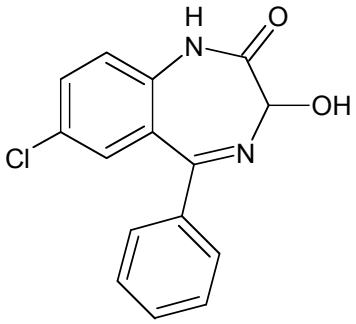
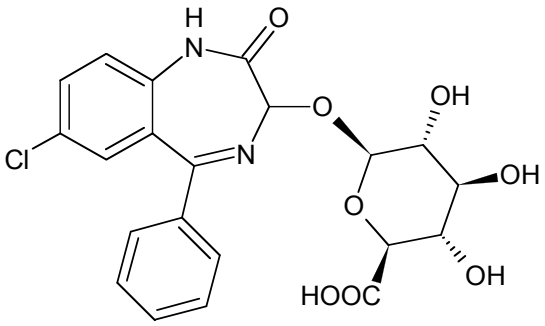
Figure 6



Oxazepam

UGT2B15 (S-oxazepam)
UGT1A9 (R-oxazepam)
UGT2B7 (R-oxazepam)



Oxazepam 3-*O*-glucuronide

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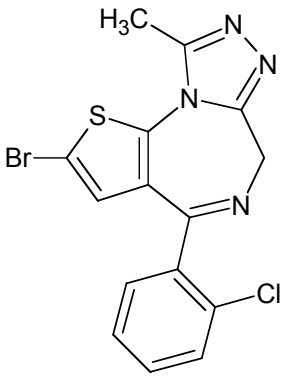
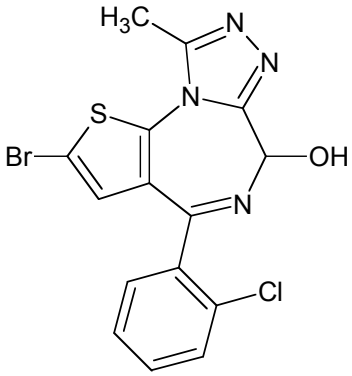
Figure 7

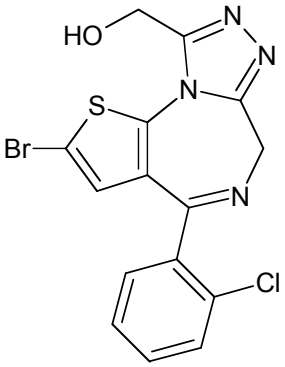


Brotizolam

CYP3A4



6-Hydroxybrotizolam



α-Hydroxybrotizolam

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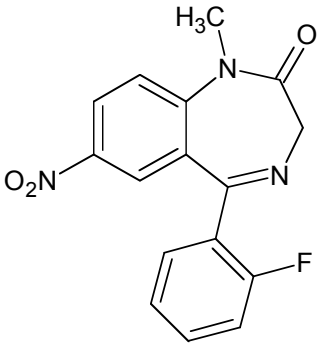
Figure 8

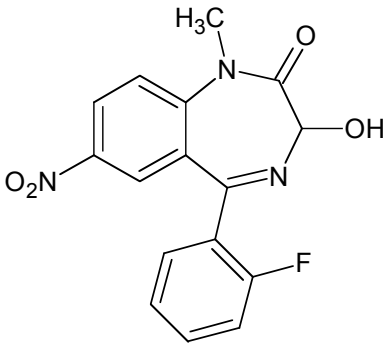


Flunitrazepam

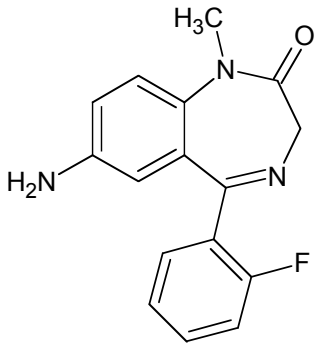
CYP3A4

NADPH-
cytochrome
P450 reductase?

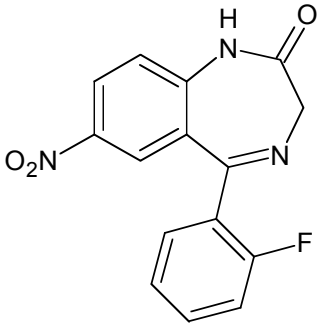
CYP2C19



3-Hydroxyflunitrazepam



7-Aminoflunitrazepam



N-Desmethylflunitrazepam

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Figure 9

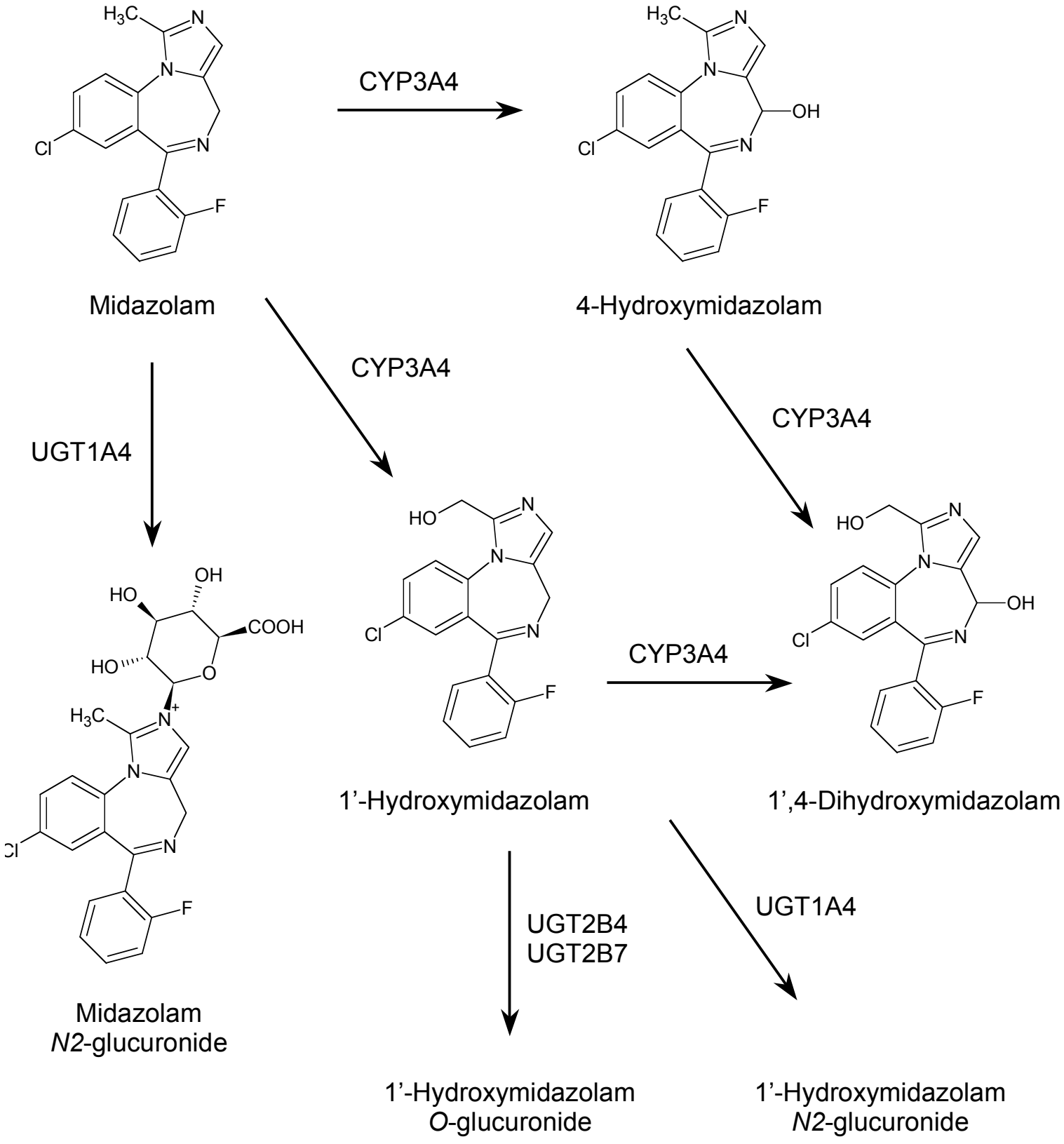

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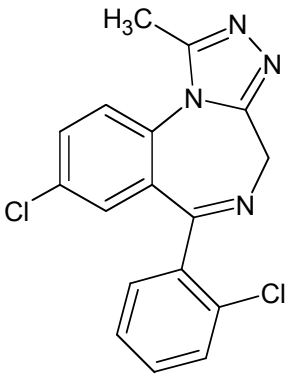
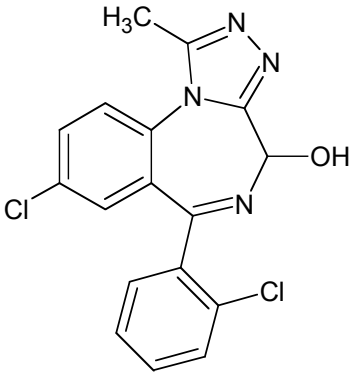
Figure 10



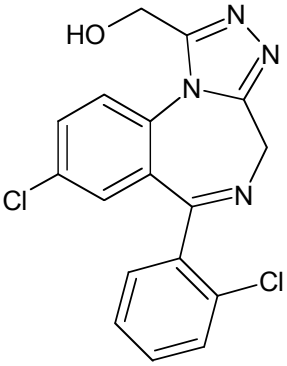
Triazolam

CYP3A4
CYP3A5?

CYP3A4
CYP3A5?



4-Hydroxytriazolam



1'-Hydroxytriazolam

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Figure 11

Table 1. Studies of midazolam interaction with respect to CYP3A4 (1998-today).

CYP3A4 interaction	Compound, class or condition	Species	Matrix	Notes	Reference
Inhibition	Clarithromycin	Human	Blood, intestinal wall homogenate	Inhibition extent proportional to baseline CYP3A4 activity	[120]
Inhibition	Clarithromycin	Human	Blood, urine	Important involvement of intestinal CYP3A4	[121]
Inhibition	Clarithromycin	Human (elderly)	Blood, urine	Important involvement of intestinal CYP3A4	[122]
Inhibition	Curcumin	Rat	Blood, liver, kidney, intestine	Inhibition of intestinal CYP3A4 is the main effect	[123]
Inhibition	Erythromycin	Rat	Blood	Only after repeated doses. No changes in total CYP content.	[124]
Inhibition	Fentanyl	Human	Liver microsomes, recombinant CYP	CYP1A2, 2A6, 2C9, 2C19, 2D6, 2E1 not involved in midazolam metabolism	[125]
Inhibition	Flavones (luteolin, diosmetin)	Human	Recombinant CYP3A4	Only polyhydroxylated flavones are CYP3A4 inhibitors	[126]
Inhibition	Fluconazole	Human	Blood	Increased effect in renal failure	[127]
Inhibition	Fluvoxamine	Human	Blood, urine	-	[128]
Inhibition	Fluvoxamine, nefazodone, ketoconazole	Human	Blood	-	[129]
Inhibition	Grapefruit juice	Human	Blood	Effect is proportional to dose and frequency	[130]
Inhibition	Hypolipidemics (atorvastatin, fluvastatin)	Human	Liver microsomes	-	[131]
Inhibition	Itraconazole	Human	Blood	-	[132]
Inhibition	Ketoconazole	Human	Blood	More intestinal than hepatic inhibition	[133]
Inhibition	Macrolide antibiotics (clarithromycin, azithromicin)	Human	Liver microsomes	-	[134]
Inhibition	Propofol	Human (intensive care)	Blood, liver microsomes, recombinant CYP3A4	-	[135]
Induction	Chronic glucocorticoid	Human	Blood,	The observed increase of midazolam metabolism	[136]

			urine	could also be due to UGT induction	
Induction	Flucloxacillin	Rat	Liver microsomes	-	[137]
Induction	Rifampicin	Human	Blood	-	[132]
Induction	Rifampicin	Pig	Cultured hepatocytes	-	[138]
Induction	St. John's wort	Human	Blood	No change in CYP2C9, CYP1A2, or CYP2D6 activity	[139]
Induction	St. John's wort	Rat	Blood, liver microsomes	-	[140]
Induction	Tangeretine	Human	Liver microsomes, recombinant CY3A4, blood	No effect of tangerine juice	[141]
No effect	Cranberry juice	Human	Blood	No change in CYP2C9 or CYP1A2 activity	[142]
No effect	Ethinylestradiol + gestodene	Human	Blood	Study on an oral contraceptive preparation. Limited inhibition, no clinical effect.	[143]
No effect	Fluoxetine	Human	Blood	-	[129]
No effect	Gender, menstrual cycle phase	Human	Blood	-	[144]
No effect	Menstrual cycle phase	Human	Blood	-	[145]
No effect	Methamphetamine	Rat	Isolated perfused liver	-	[146]
No effect	Metronidazole	Human	Liver microsomes, blood	-	[147]
No effect	Rifaximin	Human	Blood	-	[148]