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Unraveling the metabolic transformation of tetrazepam to diazepam with mass spectrometric methods

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Abstract The metabolic transformation pathways of the 1,4-benzodiazepine tetrazepam ($C_{16}H_{17}ClN_2O$, average mass: 288.772) were studied with capillary LC-QqTOF-MS and -MS/MS by analyzing human plasma and urine samples collected from healthy volunteers. Each volunteer took 50 mg of tetrazepam, given in the form of one tablet of Myolastan (Sanofi-Synthelabo, Vienna, Austria). Accurate molecular mass measurements in full-scan mode (scan range: 50–700) were used to survey the collected samples for putative metabolic transformation products. Full-scan fragment ion mass spectra were collected in subsequent LC/MS/MS experiments. Each spectrum was matched to a spectral library containing 3759 MS/MS-spectra of 402 compounds, including eighteen different benzodiazepines, to prove the structural relatedness of a tentative metabolite to tetrazepam. This "similarity search" approach provided a rapid and powerful tool to exclude non-drug-related species, even without any knowledge of the fragmentation chemistry. Interpretation of tandem mass spectrometric data was only required in order to elucidate the site of transformation. Using this strategy, 11 major classes of tetrazepam metabolites were identified. Possible metabolic routes from tetrazepam to diazepam ($C_{16}H_{13}ClN_2O$, average mass: 284.740) via repeated hydroxylation and dehydration of the cylohexenyl moiety were discovered. No evidence for extensive hydroxylation of tetrazepam at position 3 of the diazepine ring was found. In contrast to what is commonly believed, this distinct transformation reaction may be of only minor importance. Furthermore, the occurrence of demethylation, hydration, and glucuronidation reactions was proven.

Keywords Mass spectrometry · Liquid chromatography · Electrospray ionization · Tetrazepam · Metabolism · Library search

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Introduction

Tetrazepam ($C_{16}H_{17}ClN_2O$, average mass: 288.772) belongs to the family of 1,4-benzodiazepines, but it has a peculiar structure, as the typical 5-phenyl moiety is substituted by a cyclohexenyl group (Fig. 1). This substance exerts different effects than other benzodiazepines; it acts as an anxiolytic and muscle relaxant at considerably lower doses than are needed to induce other typical benzodiazepine effects, such as sedation and ataxia [1]. This means that tetrazepam provokes a dissociation between sedating and muscle-relaxing effects, despite being an agonist to both central and peripheral benzodiazepine-binding sites [2]. Tetrazepam has been established as a treatment for muscle spasms of different origins, including whiplash injury, muscular rheumatism and slipped spinal disks, and is commonly prescribed. Normal daily dosages vary between 50 and 200 mg. Tetrazepam is rapidly and almost completely absorbed if given orally, and is bound to plasma proteins at levels of approximately 70–90%. Its elimination half-life is about 14.9 h [3]. According to the manufacturer, tetrazepam should be demethylated to nortetrazepam and hydroxylated to 3-hydroxytetrazepam in the body, the latter being the main metabolite in urine after glucuronidation (Fig. 1). Biotransformability of tetrazepam to diazepam ($C_{16}H_{13}ClN_2O$, average mass: 284.740) was recently reported [4] and confirmed [5]. Although it was suggested that tetrazepam

Fig. 1 Assumed metabolic transformations of tetrazepam. Tetrazepam should be demethylated to nortetrazepam and hydroxylated to 3-hydroxytetrazepam, the latter being the main metabolite in urine after glucuronidation
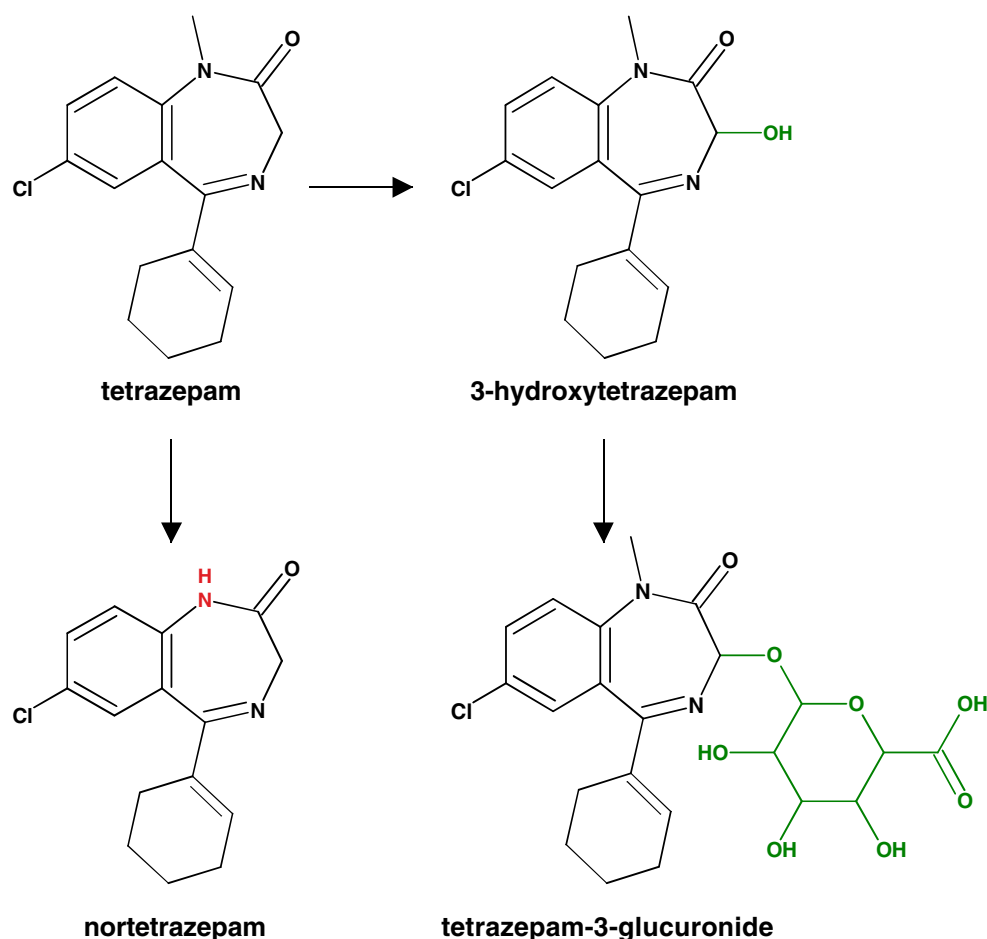
might be transformed via repeated hydroxylation and dehydration of the cylohexenyl moiety to diazepam, the metabolic transformation pathways between tetrazepam and diazepam have not been elucidated yet.

Beside other techniques, mass spectrometry (MS) plays an invaluable role in the elucidation of biotransformation pathways of drugs by identifying circulatory and excretory metabolites [6–8]. In particular, liquid chromatography (LC) hyphenated to electrospray ionization (ESI) MS has become a powerful tool for the rapid detection, structural elucidation, and quantification of drug-derived species within various biological specimens. LC enables the clean-up and fractionation of compounds prior to mass spectrometric analysis. Thus, adverse effects on the mass spectrometric detectability of metabolites caused by co-ionization with matrix compounds or transformation products are reduced, which facilitates the identification of possible metabolites. Furthermore, based on various chromatographic properties, LC can be used to separate and thus to identify isomeric species. MS can be operated in different scan modes. For targeted approaches, various combinations of product ion, precursor ion and neutral loss scans are applied in order to screen a sample for predicted metabolic transformation

products. Targeted analysis is specific and sensitive, but suffers from the problem that uncommon or unexpected transformation pathways may be ignored. Full-scan MS represents a more versatile acquisition strategy which should enable the detection of most ionizable metabolites. Low- and high-resolution MS can be applied for the detection of transformation products by analyzing test and control samples. For the structural elucidation of tentative metabolites, full-scan tandem mass spectrometric (MS/MS) data is acquired and interpreted. Typically, a lot of expert knowledge of gas-phase fragmentation chemistry is necessary in order to unequivocally prove the structural relatedness of a metabolite and its precursor drug. Thus, metabolite identification is often a difficult and time-consuming process.

Here, we report on the use of different stages of mass spectrometry for the identification of tetrazepam metabolites in human specimens (plasma, urine). LC/MS was used to generate a list of putative tetrazepam metabolites, for which full-scan tandem mass spectra were acquired in subsequent LC/MS/MS experiments. Each spectrum was matched to a spectral library containing 3759 MS/MS-spectra of 402 compounds, including eighteen different benzodiazepines, to prove the structural relatedness of a

tentative metabolite to tetrazepam. This "similarity search" approach provided a rapid and powerful tool to exclude non-drug-related species, even without any knowledge of the fragmentation chemistry. It was only necessary to interpret tandem mass spectrometric data in order to elucidate the site of transformation.

Experimental section

Subjects and conditions

A total of ten healthy volunteers, five males and five females, participated in the study. All individuals gave their written informed consent, and the study was conducted according to the guidelines of the ethical committee of the Innsbruck Medical University. (The following data are given as mean ± standard deviation, if not indicated otherwise.) Participants were aged from 21 to 39 years (29.7±4.9), with body weight varying between 59 and 100 kg (69.9±12.2), and were between 168 and 193 cm in height (176.6±7.4). Each volunteer took 50 mg of tetrazepam, given in the form of one tablet of Myolastan (Sanofi-Synthelabo, Vienna, Austria). No other medication was taken during the experiments, as confirmed by urine analyses. Food and (non-alcoholic) beverage intake was not regulated, in order to obtain practical conditions within the experiments. 1–2 h after tetrazepam intake, a 5 ml blood sample was taken from an indwelling catheter in the cubital vein, using a blood collection system without preservatives (Monovette, Sarstedt, Wiener Neudorf, Austria). Blood samples were centrifuged immediately. The plasma was separated. Urine samples were collected several times up to 24 hours after the intake of tetrazepam. All samples were stored at −18 °C until analysis.

Sample preparation

One milliliter of sample was diluted with 3 ml of distilled water and vortexed. After centrifugation (5 min, 4500 turns/min), the supernatant was used for solid-phase extraction (SPE). SPE columns (SPE-ED Scan ABN, Applied Separations, Allentown, PA, USA) were conditioned with 2 ml methanol and 2 ml 0.1 M phosphate buffer (pH 6.0). Samples were rinsed through the columns. Next, the columns were washed with 3 ml distilled water, 1 ml 1 M acetic acid, and an aqueous methanol solution (5% v/v), centrifuged for 5 min (4500 turns/min), and dried with a nitrogen stream to remove any possible remaining aqueous washing solvent. Elution was performed with 2 ml of an anhydrous solvent mixture of dichloromethane, isopropanol and ammonia. The eluate was evaporated to dryness at 60 °C under a nitrogen stream and reconstituted in 30 μl 0.05% acetic acid solution containing 5.0% acetonitrile (Sigma-Aldrich, St. Louis, MO, USA).

Tetrazepam quantification

Quantitative analyses were performed using gas chromatography combined with electron capture detection (GC-ECD) on an Autosystem XL device (Perkin Elmer, Vienna, Austria). A HP-5 MS column (30 m×0.25 mm i.d. ×0.25 μm film thickness; J&W Scientific, Folsom, CA, USA) was used; operating conditions: carrier gas (hydrogen) at 1.5 ml/min; injection temperature 250 °C, injection volume 1 μl; oven: 100 °C, 20 °C/min to 220 °C, hold for 15 min, 20 °C/min to 300 °C, hold for 5 min, ECD temperature 350 °C. Tetrazepam standard (1.0 mg/ml) was obtained from Cerilliant (Round Rock, TX, USA). Calibration for tetrazepam quantification was performed by spiking tetrazepam-free samples with the analyte, resulting in calibration levels of 0, 100, 250, 500, 750 and 1000 μg/l. A linear calibration was achieved. The limit of detection (LOD) was calculated (B.E.N. 2.01 provided by Herbold and Schmitt, Heidelberg, Germany) as 20 μg/l. In all measurement series, positive controls at two concentration levels and blank matrix used as the negative control were prepared and analyzed.

Determination of creatinine

Creatinine in urine samples was measured with an immunological assay on a Hitachi 902B Automatic Analyzer (Hitachi Science Systems, Ibaraki, Japan) using CEDIA reagents (Microgenics, Passau, Germany) according to the manufacturer's protocol.

Capillary LC

An Ultimate fully integrated capillary HPLC system (LC Packings, Amsterdam, The Netherlands) was used for all experiments. A Famos micro autosampler (LC Packings) equipped with a 1.0 μl loop was used for sample injection. The chromatographic column was prepared as described elsewhere [9]. At one end of a 25 cm long polyimide-coated fused-silica capillary tubing (350 μm o.d. and 200 μm i.d., Polymicro Technologies, Phoenix, AZ, USA), a retaining frit was created by sintering a thin slug of 5.0 μm silica particles (Spherisorb S5W, Phase Sep, Queensferry, Clwyd, UK) wetted with a small droplet of sodium silicate solution (Sigma-Aldrich). Eurospher 100-5 C18 particles (Knauer, Berlin, Germany) were used as stationary phase. The particles were suspended in isobutanol (Sigma-Aldrich). The column was packed with methanol (Sigma-Aldrich) at a constant pressure of 40 MPa until the length of the packed section was 150 mm. Finally, the column was trimmed to a length of 100 mm. No frit was made at the inlet end of the

column. The chromatographic separation was accomplished using a gradient of 5.0% to 95.0% acetonitrile (Sigma-Aldrich) in 0.05% acetic acid solution within 10 min. HPLC-grade water (Merck, Darmstadt, Germany) was used to prepare the HPLC mobile phase. The column was thermostatted at 50 °C. The flow rate was set to 2.0 μl/min. The column outlet was connected online to the ESI source.

Mass spectrometry

ESI-MS was performed in the positive ion mode on a QSTAR XL mass spectrometer (Applied Biosystems, Foster City, CA, USA) using a modified TurboIonSpray source. The modifications included the replacement of the PEEK tubing transfer line and the stainless steel sprayer capillary with fused silica capillaries (transfer line: 375 μm o.d., 20 μm i.d., sprayer capillary: 90 μm o.d., 20 μm i.d., Polymicro Technologies). Mass calibration and optimization of instrumental parameters was achieved by the infusion of a mixture of 1.0 mg/l caffeine and 1.0 mg/l reserpine dissolved in 0.05% aqueous acetic acid solution containing 50% acetonitrile (v/v) at a flow rate of 2.0 μl/min. The spray voltage was set to 4.0 kV. Gas flows of 1–3 arbitrary units (nebulizer gas) and 40 arbitrary units (turbo gas) were employed. The temperature of the turbo gas was adjusted to 200 °C. The accumulation time was set to 1.0 s and four time bins were summed. Mass spectra were collected in the range between 50 and 700. For MS/MS, the Q1 resolution was set to unit resolution. The collision gas (N_2) flow was set to five arbitrary units. Collision-energy values of between 25 eV and 35 eV were applied to generate product ion mass spectra. Total ion chromatograms and mass spectra were recorded on a personal computer with the Analyst QS software (1.0, service pack 8, Applied Biosystems).

Similarity search

The reference library was established on a QqTOF instrument (Qstar XL, Applied Biosystems) and contained 3759 MS/MS-spectra of 402 compounds [10]. For each reference compound, product-ion spectra were acquired at ten different collision energy values between 5 eV and 50 eV. Because of possible saturation effects, and to avoid false-positive matching of the precursor ion with product ions originating from alternative compounds, all signals within a ±4.0 window around the m/z of the precursor ion were deleted from the reference spectra obtained. To further increase the specificity, reference spectra were filtered. Only those signals with a relative intensity of >0.01% which were observed at least in two spectra collected at different collision energies were regarded as being suitable for identification. The remaining mass peaks were deleted from the reference spectra.

Tandem mass spectra of tentative tetrazepam metabolites were centroided and exported as txt files. All signals within a ±4.0 window around the m/z of the precursor ion were deleted from the spectra obtained. Each txt file contained information about the precursor ion mass and a list of the observed fragment ions [mass-to-charge ratios (m/z) and the corresponding relative intensities]. All spectra were matched against the established reference library. Automated library searches were performed with a program written in ActivePerl 5.6.1 (Active State Corporation, Vancouver, Canada). Calculations were performed on a personal computer running under the Windows XP operating system (1.7 GHz Pentium, 1.0 GB RAM). The principles of the library search procedure applied have been described elsewhere [10].
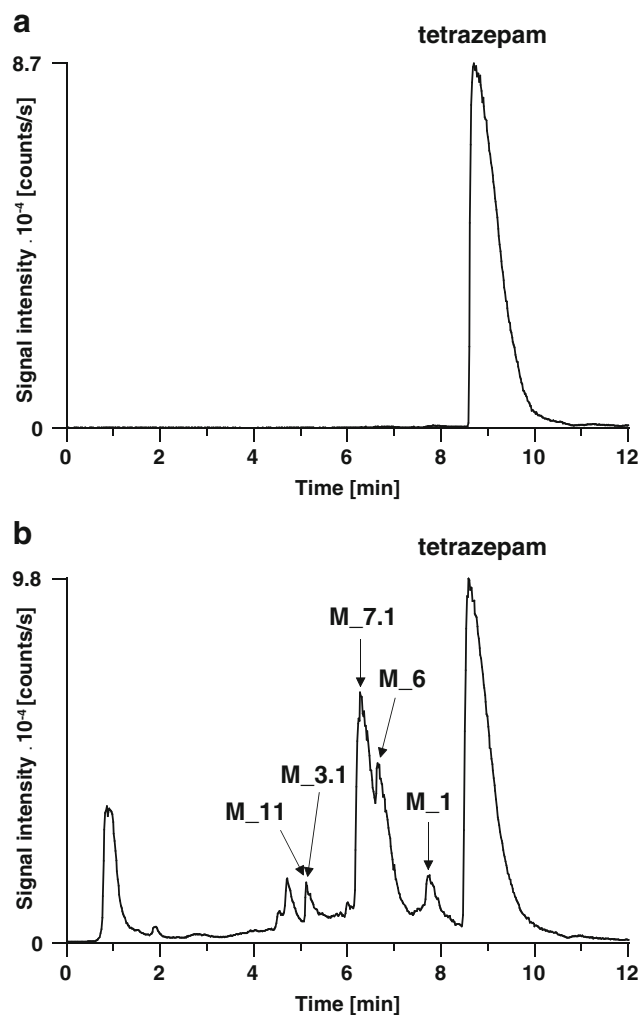

Fig. 2 Reconstructed total ion current chromatograms from the LC/MS analysis of (**a**) 40 ng of the pharmaceutical specialty Myolastan and (**b**) a representative urine sample collected 6.33 h after the intake of 50 mg Myolastan

Results and discussion

On the strategy used to identify tetrazepam metabolites

Tetrazepam metabolism was characterized in specimens of human origin (plasma and urine) using different stages of MS. Solid-phase extraction was used to prepare the samples for LC/MS(/MS). Firstly, a tentative list of metabolites was created during a set of LC/MS experiments. Accurate molecular mass measurements obtained in full-scan mode (scan range: 50–700) on the applied time-of-flight mass analyzer were used to survey the collected samples. Species which were not observed in blanks (i.e., urine or plasma samples collected prior to the intake of tetrazepam) and which showed mass differences that would have been characteristic for metabolic transformation products were selected as precursor ions for LC/MS/MS experiments. The fragment ion mass spectra were collected in full-scan mode (scan range: 50–500), and used to evaluate the degree of structural similarity between a tentative metabolite and its possible precursor tetrazepam. Each single spectrum was matched to a spectral library containing 3759 MS/MS spectra of 402 compounds. Spectra of eighteen different benzodiazepines, including tetrazepam, diazepam, temazepam and nordiazepam, were present in the collection. The library, however, did not contain any specific spectra for any of the putative intermediates between tetrazepam and diazepam. Thus, the direct identification of tetrazepam metabolites via an "identity search" was impossible. In cases where the query compound is not included in the library, database systems can be used to find entries which possess structures or structural features that are similar to those of the query compound. Such an approach is called a "similarity search." Typically, only selected parts of a compound's structure are changed during the course of the metabolic transformation process. Thus, a large number of identical fragment ions might be observed in the fragment ion mass spectra obtained from a compound and its metabolite. Only those fragment ions that contain the transformed moiety will differ in mass. Thus, a library search performed with a tandem mass spectrum of a metabolite often yields a high score for the corresponding precursor drug. In many cases, the precursor itself (or at least a compound showing a large degree of structural similarity with it) will be the compound that represents the best match. In this way, the structural relatedness between the tentative metabolite and the drug is proven, and metabolites are distinguished quickly and efficiently from non-drug-related species.

The drawback of the "similarity search" approach, however, is the fact that it is difficult to use in order to directly elucidate the structure of a metabolite. Manual interpretation of tandem mass spectrometric data is necessary in order to more closely inspect the metabolite's structure. The collision-induced dissociation of benzodiazepines results in rich MS/MS spectra. The fragmentation chemistry is well known, and the occurrence of distinct fragmentation reactions can provide an indication of the site of structural change when metabolites of benzodiazepines are encountered [11, 12]. In our case, the manual interpretation of tandem mass spectrometric data was of particular importance when determining the preferred site of tetrazepam hydroxylation.

Table 1 Summary of the degradation products (D) detected in the pharmaceutical specialty Myolastan

(a) Results of the LC/MS experiments

	Retention time (min)	Measured m/z	Mass difference to tetrazepam	Tentative transformation of tetrazepam	Calculated m/z of the tentative transformation product	Difference from the measured mass
D_1	7.89	275.095	−14.015	− CH_2	275.095	0.000
D_2.1	6.40	305.112	16.002	+ O	305.116	0.004
D_2.2	6.74	305.105	15.995	+ O	305.116	0.011
D_2.3	7.91	305.109	15.998	+ O	305.116	0.007
D_3.1	7.93	307.130	18.020	+ H_2O	307.127	−0.003
D_3.2	8.60	307.123	18.012	+ H_2O	307.127	0.004

(b) Summary of tandem mass spectral data used to elucidate a compound's relatedness to tetrazepam and its structure

	Similarity search result	− CO	− (CO + RCN)	− RCN	− H_2O	− (CO + H_2O)	− (H_2O + RCN)
D_1	Tetrazepam	×	×	×	−	−	−
D_2.1	Tetrazepam	×	×	×	×	×	−
D_2.2	Tetrazepam	×	×	×	×	×	−
D_2.3	Tetrazepam	×	×	×	×	×	−
D_3.1	Tetrazepam	×	×	×	×	×	−
D_3.2	Tetrazepam	×	×	×	×	×	−

Characterization of the pharmaceutical specialty Myolastan

Each pharmaceutical formulation is degraded to some extent during storage, mainly via oxidation, hydrolysis, or hydration. After intake of the pharmaceutical specialty, the resulting degradation products may be misinterpreted as metabolites of the drug. Thus, to exclude the possibility that a putative transformation product detected within a specimen of human origin was actually part of the Myolastan formulation, we decided to initially check a tablet for the presence of degradation products. Aside from the parent drug, however, only minute amounts of tetrazepam-related compounds were detected (Fig. 2a, Table 1). These species originated from demethylation (D_1), oxidation (D_2), and hydration (D_3)

Table 2 Summary of the tetrazepam metabolites (M) identified in human plasma and urine

(a) Results of the LC/MS experiments

	Retention time (min)	Found in plasma	Found in urine	Measured m/z	Mass difference from tetrazepam	Tentative transformation of tetrazepam	Calculated m/z of the tentative transformation product	Difference from measured m/z
M_1	7.84	×	×	275.094	−14.016	$- CH_2$	275.095	0.001
M_2	8.05		×	285.076	−4.035	$- 2 H_2$	285.079	0.003
M_3.1	5.19	×	×	287.094	−2.016	$- H_2$	287.095	0.001
M_3.2	6.38	×	×	287.094	−2.017	$- H_2$	287.095	0.001
M_3.3	7.69		×	287.099	−2.011	$- H_2$	287.095	−0.004
M_3.4	8.33	×		287.095	−2.015	$- H_2$	287.095	0.000
M_4	5.80		×	291.092	1.982	$- CH_2 + O$	291.126	0.034
M_5	6.78		×	301.073	11.963	$- 2 H_2 + O$	301.074	0.001
M_6	6.77		×	303.088	13.978	$- H_2 + O$	303.089	0.001
M_7.1	6.11		×	305.107	15.997	$+ O$	305.116	0.009
M_7.2	6.38	×	×	305.099	15.989	$+ O$	305.116	0.017
M_7.3	6.72	×		305.099	15.989	$+ O$	305.116	0.017
M_8	7.89	×		307.120	18.010	$+ H_2O$	307.127	0.007
M_9.1	5.36		×	321.106	31.996	$+ O_2$	321.100	−0.006
M_9.2	6.30		×	321.106	31.996	$+ O_2$	321.100	−0.006
M_9.3	6.49	×		321.107	31.997	$+ O_2$	321.100	−0.007
M_9.4	8.40	×		321.116	32.006	$+ O_2$	321.100	−0.016
M_10.1	5.31		×	323.111	34.001	$+ O_2 + H_2$	323.116	0.005
M_10.2	5.68		×	323.117	34.007	$+ O_2 + H_2$	323.116	−0.001
M_11	5.19		×	481.144	192.034	$+$ glucuronic acid $- H$	481.137	−0.007

(b) Summary of the tandem mass spectral data used to elucidate a compound's relatedness to tetrazepam and its structure

|	Similarity search result	$- CO$	$- (CO + RCN)$	$- RCN$	$- H_2O$	$- (CO + H_2O)$	$- (H_2O + RCN)$
M_1	Tetrazepam	×	×	×	−	−	−
M_2	Diazepam	×	×	×	−	−	−
M_3.1	Tetrazepam	×	×	×	−	−	−
M_3.2	Tetrazepam	×	×	×	−	−	−
M_3.3	Tetrazepam	×	×	×	−	−	−
M_3.4	Tetrazepam	×	×	×	−	−	−
M_4	Tetrazepam	×	×	×	×	×	−
M_5	Diazepam	×	×	×	×	×	−
M_6	Tetrazepam	×	×	×	×	×	−
M_7.1	Tetrazepam	×	×	×	×	×	−
M_7.2	Tetrazepam	×	×	×	×	×	−
M_7.3	Tetrazepam	×	×	×	×	×	−
M_8	Tetrazepam	×	×	×	×	×	−
M_9.1	Tetrazepam	×	×	×	×	×	−
M_9.2	Tetrazepam	×	×	×	×	×	−
M_9.3	Tetrazepam	×	×	×	×	×	−
M_9.4	Tetrazepam	×	×	×	×	×	−
M_10.1	Tetrazepam	×	×	×	×	×	−
M_10.2	Tetrazepam	×	×	×	×	×	−
M_11	Tetrazepam	×	−	−	×	×	−

of tetrazepam. Ion traces at characteristic *m/z* values for each of these compounds were extracted from the reconstructed ion current chromatogram. The resulting chromatographic peaks were integrated, and the peak areas were determined. Peak area ratios were used to estimate the extent of tetrazepam degradation. It was found that less than 0.1% of the tetrazepam had been transformed. Due to the low content of degradation products in the tablet, transformation reactions

that occurred prior to the intake of the tablet were ruled out as major source of tetrazepam-related species detected in human specimens.

Characterization of tetrazepam metabolites

Plasma as well as urine samples were screened for tetrazepam metabolites. In a first set of experiments,

Fig. 3 Characteristic MS/MS fragmentation of (**a**) 3-hydrogen- and (**b**) 3-hydroxybenzodiazepines [11, 12]
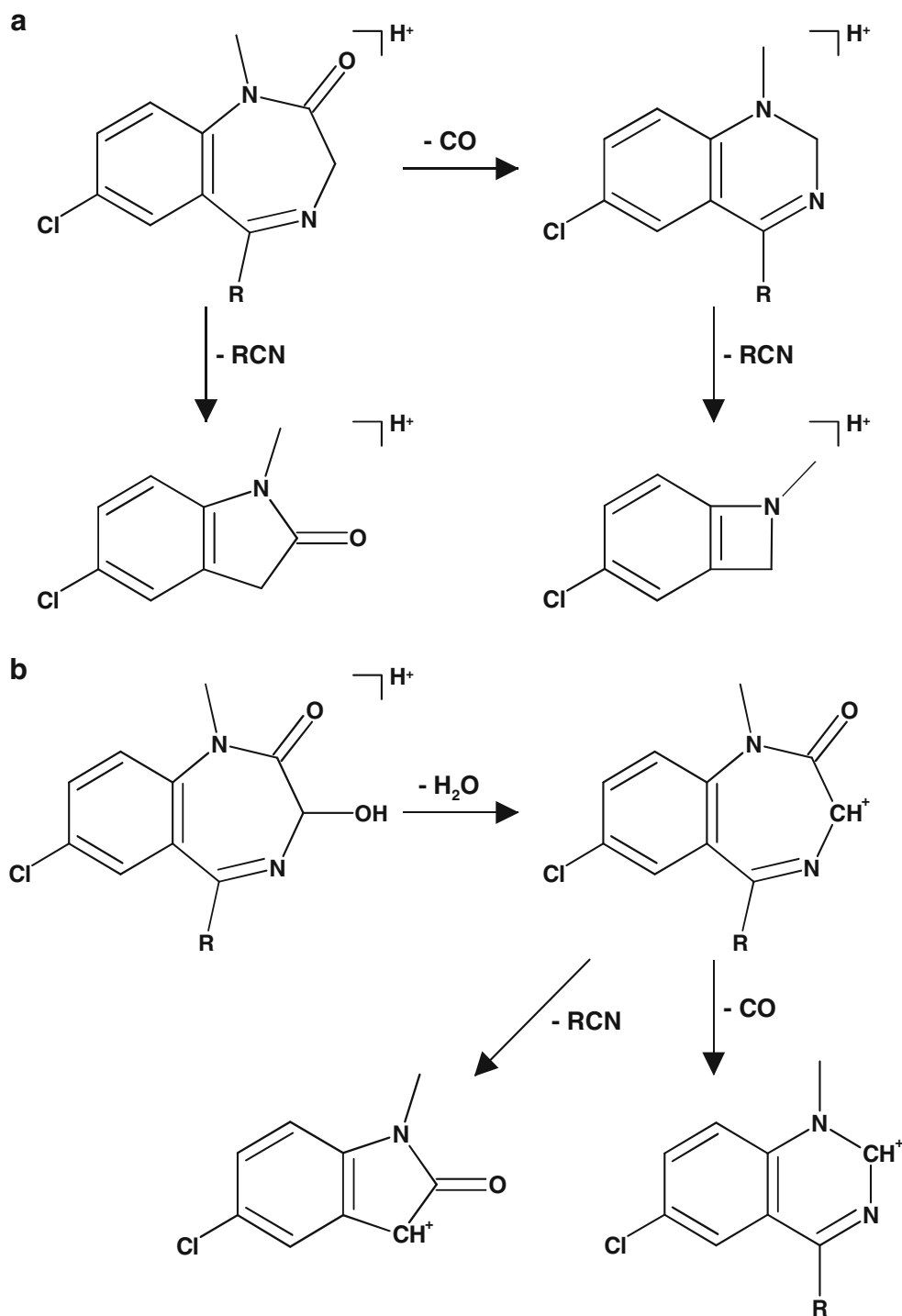
extracted samples were surveyed for putative tetrazepam metabolites by LC/MS. A representative reconstructed ion current chromatogram obtained from the analysis of an extracted urine sample is shown in Fig. 2b. The parent drug tetrazepam eluted after 8.6 min. Putative transformation products eluted between 5.2 and 8.4 min from the chromatographic column (Fig. 2b). The corresponding m/z values were extracted from the mass spectrometric data to generate a list of putative tetrazepam metabolites. In the course of this study, plenty of putative metabolites were detected either in urine, in plasma, or in both matrices (Table 2a). In a set of LC/MS/MS experiments, fragment ion mass spectra were acquired for each of these species. The spectra were used as inputs for similarity searches within the spectral library. Eighteen species were matched to tetrazepam (Table 2b). For these compounds, a high degree of structural relatedness to tetrazepam was demonstrated, and this clearly suggests that these species represent true metabolites of tetrazepam. Two species were matched to diazepam. One of these compounds was most probably

diazepam itself (M_2), while the other one was probably an oxidation product of diazepam (M_5). Taken together, the mass spectrometric information indicated that demethylation, hydration, and hydroxylation combined with dehydration or glucuronidation were metabolic transformation reactions.

A closer inspection of the tandem mass spectral data was conducted to localize the site of metabolic transformation. Differentiation of hydroxylation at position 3 and hydroxylation of the cyclohexenyl moiety was accomplished by interpreting the observed fragmentation chemistry. Some important fragmentation pathways of unmodified and 3-hydroxybenzodiazepines are summarized in Fig. 3 [11, 12]. Species which are hydroxylated at position 3 of the diazepine ring first lose the neutral species H_2O (Fig. 3b). Subsequently, either CO or RCN are lost from the intermediate to give either a six- or five-membered heterocyclic ring. Unfortunately, the loss of H_2O was observed for all hydroxylated benzodiazepine species irrespective of the hydroxylation site, and so this did not represent a fragmentation reaction characteristic of 3-hydroxybenzodiazepines. However, only
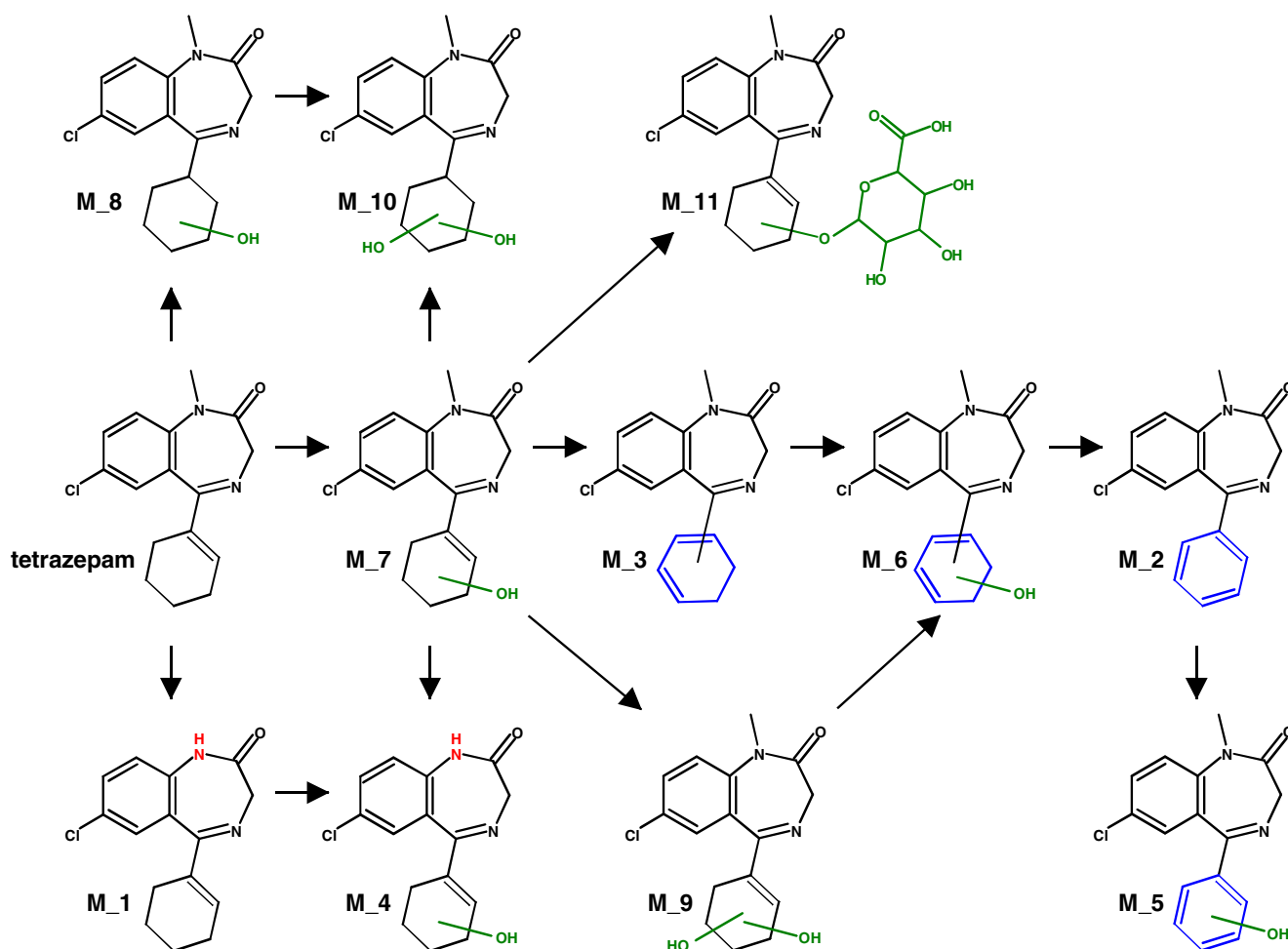

Fig. 4 Proposed metabolic transformation of tetrazepam in humans, based on the screening of urine and plasma samples for tetrazepam metabolites
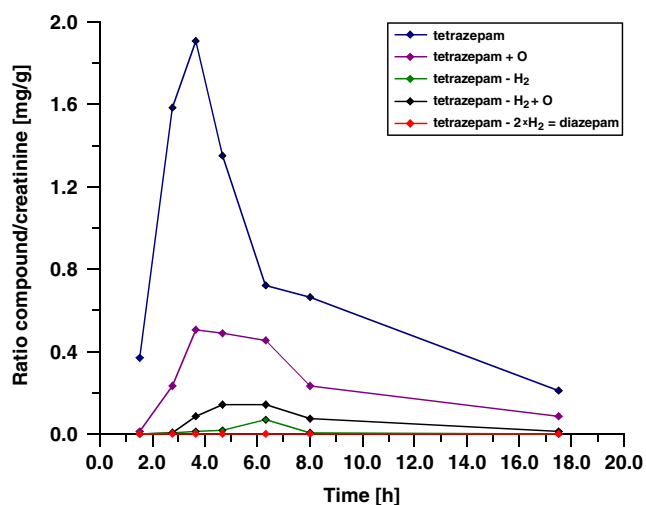
Fig. 5 Typical elimination curve of tetrazepam and selected metabolites in urine. Values were creatinine-corrected, and so do not represent the absolute measured benzodiazepine concentrations

the species that lack hydroxylation at position 3 of the diazepine ring lose the neutral species CO (Fig. 3a). The cleavage results in the contraction of the seven-membered ring to a resonance-stabilized six-membered one, and represents a unique identifier for side-chain hydroxylation. Loss of CO was observed for all tentative tetrazepam metabolites (Table 2b). This observation clearly suggested that the side chain represents the typical site of oxidation. No evidence was found for extensive hydroxylation of either tetrazepam or diazepam at position 3 of the diazepine ring, which suggests that, in contrast to what is commonly believed, this distinct transformation reaction may be of only minor importance.

Altogether, 11 major categories of tetrazepam metabolites were identified (Fig. 4). Some classes of metabolites were divided into subcategories as a result of at least partial chromatographic separation of structural isomers. These isomers could not be distinguished from each other by interpreting the corresponding fragment ion mass spectra. The use of other analytical techniques (like nuclear magnetic resonance spectroscopy) would have been necessary for a comprehensive characterization of the observed isomeric species. However, the structural information obtained from the mass spectrometric experiments was sufficient to understand the processes included in the tetrazepam metabolism. In particular, the transformation of tetrazepam to diazepam via repeated hydroxylation and dehydration reactions was elucidated successfully.

Elimination kinetics of tetrazepam and its major metabolites

To get an idea of the extent of metabolic transformation of tetrazepam, the concentrations of tetrazepam and its

metabolites were determined in urine samples collected from one person representing a typical tetrazepam metabolizer 0, 1.5, 2.75, 3.67, 4.67, 6.33, 8.0, and 17.5 h after intake of a single dose of tetrazepam (50 mg). Quantitative analysis of tetrazepam was performed with a GC-ECD method. The concentrations obtained were interpreted with respect to the creatinine content to compensate for the diuretic state. Tetrazepam metabolite concentrations were derived from LC/MS data. Ion traces at characteristic m/z values for each compound were extracted from the chromatogram. The resulting chromatographic peaks were integrated, and the peak areas were determined. The peak areas of isomeric forms belonging to a certain metabolite category were added together. The ratio of the metabolite-specific peak area to the tetrazepam peak area was used to calculate the content of the metabolite. It is important to note that, as different metabolites can vary in their sample preparation recoveries and in their ionization efficiencies, only rough estimates of individual metabolite concentrations were obtained. Thus, the quantification results provided here should be interpreted carefully.

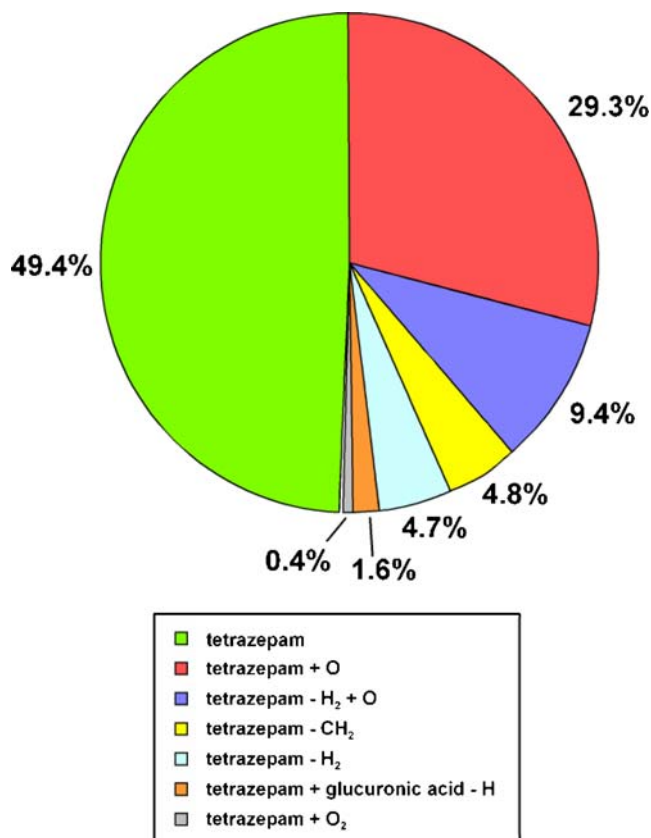Typical elimination curves observed for five tetrazepam-related species in urine are shown in Fig. 5. The highest

Fig. 6 Typical distributions of tetrazepam and its major metabolites in human urine collected 6.33 h after the intake of 50 mg Myolastan

tetrazepam concentration (1.9 mg/g creatinine) was measured 3.67 h after tetrazepam intake, which is in accordance with recently published data [4]. Tentative tetrazepam metabolites reached their maximum concentrations somewhat later, which clearly suggests that the observed transformation products do not represent any kind of artifact produced by the applied analytical method. For example, the peak concentration of diazepam (1.6 μg/g creatinine) was measured 2 h after tetrazepam had reached its maximum.

In Fig. 6, the relative contents of the main metabolite classes that were found within a selected urine sample are summarized. Six hours after intake of the tablet, more than half of the parent drug had been eliminated in the form of its metabolites. Hydroxylated species (M_7) represented nearly 30% of all detected tetrazepam-related compounds, and were therefore the dominant type of transformation product. Other important groups of metabolites were M_6 (9.4%), M_3 (4.7%), and M_9 (0.4%). These four compound classes represent intermediates along the transformation route between tetrazepam and diazepam. The fraction of diazepam was 0.03%. Other significant amounts of metabolites were produced via demethylation (4.8%) and glucuronidation (1.6%) of tetrazepam. The relative amount of nortetrazepam (M_1) agreed well with values previously determined for this kind of metabolite [3, 13].

We were able to show that the largest fraction of the tetrazepam metabolites belonged to species that were transformed at the cyclohexenyl moiety, but this feature of the tetrazepam metabolism has rarely been recognized in earlier studies [3, 14, 15]. We can only speculate about the reasons. The analytical techniques that were used to study the metabolism of tetrazepam in the 1980s were probably too insensitive to detect low-abundance tetrazepam transformation products. Moreover, the protocols applied included a hydrolysis reaction to open up the diazepine ring and to transform tetrazepam and its metabolites into acridone-related species. Often, different kinds of tetrazepam metabolites were transformed into the same acridone-related species. Thus, certain metabolites could not be distinguished using the chromatographic, spectrometric, and spectroscopic methods that were applied to characterize the hydrolyzates back then. Furthermore, the anticipation that tetrazepam might be metabolized like diazepam to the 3-hydroxy-metabolite may be a reasonable explanation as to why the biotransformation of tetrazepam has not been investigated in very much detail.

Conclusions

Tetrazepam is a drug that was developed in the 1960s. The metabolic transformation pathways that are commonly associated with this drug were established in the 1980s using the best chromatographic, spectroscopic, and spectrometric methods available at that time. Nevertheless, 20–25 years of technological progress has significantly improved our ability to detect and to characterize metabolites. We used sophisticated mass spectrometric methods which included full MS scanning and automated similarity searches with full MS/MS data to study the in vivo metabolism of tetrazepam. We were able to identify a large number of tentative metabolites. The transferability of tetrazepam to diazepam via repeated hydroxylation and dehydration of the cylohexenyl moiety was demonstrated. No evidence for the extensive hydroxylation of tetrazepam at position 3 of the diazepine ring was found. In contrast to what is commonly believed, this distinct transformation reaction may be of only minor importance. The biotransformation pathways of tetrazepam presented here differ significantly from those published in textbooks.

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