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ORIGINAL ARTICLE

Medicolegal aspects of tetrazepam metabolism

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Abstract The benzodiazepine tetrazepam is primarily muscle relaxant with comparably lower central sedating effects and is therefore commonly prescribed for muscle spasms of different origins. To evaluate tetrazepam metabolism, a study was conducted with ten healthy volunteers. Blood and urine samples were regularly collected after the intake of 50 mg tetrazepam. Toxicological analyses revealed that tetrazepam is also metabolized to diazepam and further to nordazepam, which has not yet been reported. Tetrazepam and diazepam could be detected in urine samples at least 72 h after intake, the diazepam concentration being 33% (±14% SD), on average, of the tetrazepam concentration. On the basis of three case histories, the importance of the detection of these newly described metabolites is shown as necessary to prevent false accusations and potential negative legal consequences for examined persons.

Keywords Tetrazepam · Diazepam · Nordazepam · Forensic toxicology

Introduction

Our Institute was recently commissioned to give a forensic expert opinion and carry out confirmatory analyses on urine samples from three detainees, which had originally been found to be immunologically positive for benzodiazepines. All three men claimed that the positive results were due to the intake of Myolastan tablets (containing 50 mg tetrazepam) prescribed by the prison physician. A consumption of additional benzodiazepines during a furlough, which would lead to stricter conditions of imprisonment, was denied by all three accused. In case 1, our GC–MS analysis of the urine sample revealed nordazepam, while in cases 2 and 3 tetrazepam and diazepam were additionally detected.

Tetrazepam (empirical formula C_{16}–H_{17}–Cl–N_2–O) belongs to the 1,4 benzodiazepines, but has a peculiar structure as the typical 5-phenyl moiety is substituted by a cyclohexenyl group (Fig 1). This substance has different effects compared to other benzodiazepines, being an anxiolytic and muscle relaxant, in 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 of sedating and muscle relaxing effects, despite being an agonist to both central and peripheral benzodiazepine binding sites [2]. Mechanisms of tetrazepam action on spasticity were further analyzed [3] and its effectiveness as a muscle relaxant was proved [4–6]. Tetrazepam was considered to have a desirable benefit–risk ratio [7], has therefore been established as a treatment of muscle spasms of different origins including whiplash injury, muscular rheumatism, or slipped spinal disks, and is commonly prescribed. Tetrazepam is only available as Myolastan 50 mg tablets in Austria (Sanofi-Synthelabo, Vienna) and is currently a constituent of different preparations in Germany, for example Musaril (Sanofi-Synthelabo) and of a couple of generics [8]. Normal daily dosages vary between 50 and 200 mg.

Tetrazepam is rapidly and nearly completely absorbed if given orally and is bound to plasma proteins to approximately 70–90%. Due to its elimination half-life of about

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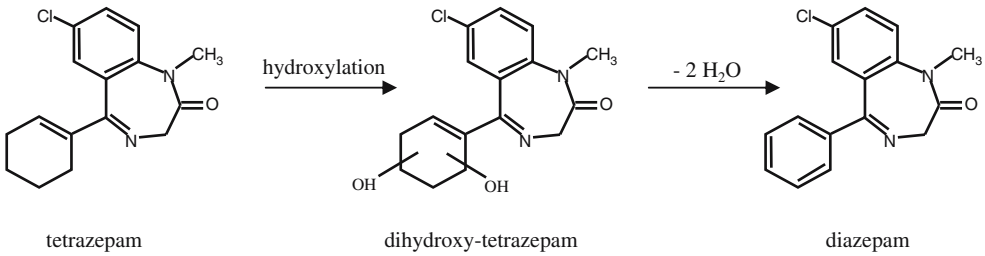

Fig. 1 Suggested metabolism pathway of tetrazepam to diazepam

14.9 h [9], it can be classified as a benzodiazepine with a medium elimination half-life. This is considerably shorter than, e.g., the frequently used diazepam [10] or nordazepam. According to the manufacturer, tetrazepam is demethylated to nor-tetrazepam and hydroxylated to 3-hydroxy-tetrazepam in the body, the latter being the main metabolite in urine after glucuronidation. In addition, hydroxy-tetrazepam, nor-hydroxytetrazepam, and some isomeric derivatives were suggested as metabolites [11], and in monkeys further hydrolysis products were described, even though not all of them were identified [12]. A biotransformation to other therapeutically used benzodiazepines has not yet been reported. Therefore, an intake of at least one further benzodiazepine in addition to the claimed Myolastan should have been stated in the commissioned expert opinion in all three cases according to the literature. Because of the particular circumstances of these cases, the metabolism of tetrazepam was further evaluated. For this purpose, a study with ten healthy volunteers was conducted, including analyses of blood samples collected regularly up to 11 h and urine samples up to 72 h after intake of one Myolastan tablet.

Materials and methods

Subjects and conditions

A total of ten healthy volunteers, five men and five women, 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. Following data are given as mean±SD, if not indicated otherwise. Participants were aged from 21 to 39 years (29.7±4.9), with a 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. No other medication was taken during the experiments, which was confirmed by urine analyses. Food and (nonalcoholic) beverage intake was not regulated to obtain practical conditions within the experiments. After 0, 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 5, 8, and 11 h, 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). In addition, urine samples were collected 2, 4, and 6 h after the intake, and further urine samples were then taken regularly with each micturition up to 72 h.

Determination of benzodiazepines

Blood samples were centrifuged immediately. The plasma was separated and stored at −18°C until analysis.

Benzodiazepine standards (1 mg/ml) were obtained from Cerilliant (Round Rock, TX). One millilitre of plasma was diluted with 3 ml of distilled water, spiked with 15 µl flurazepam solution (40 µg/ml) as internal standard, and vortexed. After centrifugation (5 min, 4500 turns/min) the supernatant was used for solid-phase extraction (SPE-ED Scan ABN, Applied Separations, Allentown, PA). SPE columns were conditioned with 2 ml methanol and 2 ml 0.1 M phosphate buffer, pH 6.0. Samples were rinsed through the columns; which 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 under nitrogen. Elution was performed with 2 ml of an anhydrous solvent mixture of dichloromethane, i-propanol, and ammonia. The eluate was evaporated to dryness at 60°C under nitrogen and reconstituted in 500 µl of ethyl acetate. Quantitative analyses were performed using gas chromatography combined with electron capture detection (GC–ECD) on an Autosystem XL device (Perkin Elmer, Vienna, Austria). An HP-5 MS column (30 m×0.25 mm, i.d., ×0.25 µm film thickness; J&W Scientific, Folsom, CA) was used. Operation conditions were as follows: carrier gas was 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.

Calibration for benzodiazepine quantification was performed by spiking benzodiazepine-free samples with the different analytes, resulting in calibration levels of 0, 100, 250, 500, 750, and 1000 µg/l for tetrazepam and 0, 20, 50, 100, 150, and 200 µg/l for diazepam and nordazepam. A linear calibration was achieved for all analytes. Limit of detection was calculated with the software B.E.N. 2.01 (provided by Herbold and Schmitt, Heidelberg, Germany)

with 20 µg/l for tetrazepam, 5 µg/l for diazepam, and 10 µg/l for nordazepam. In all measurement series, quality controls in two concentration levels and blank matrix as respective negative control were prepared and analyzed.

Urine samples (1.5 ml) were treated with 20 µl β-glucuronidase (140 U/ml, Roche, Mannheim, Germany) for 16 h at 38°C. For analysis, 0.5 ml urine was diluted with 3.5 ml distilled water and further prepared as described.

Confirmatory GC–MS analysis of urine samples

Screening analyses of urine samples were performed using a HP6890 GC device combined with a HP5973 mass selective detector (Agilent Technologies, Vienna, Austria). Operating conditions were as follow: DB-XLB column (30 m×0.25 mm, i.d., ×0.25 µm film thickness, J&W Scientific); carrier gas: helium at 1.0 ml/min; injection temperature 250°C, injection volume 1 µl; oven: 50°C, 25°C/min to 150°C, 10°C/min to 320°C, hold for 5 min, 20°C/min to 330°C, hold for 5 min; MS: ionization: electron impact at 70 eV; data collection: MSD Chemstation D.01.02.16 including Agilent Technologies mass spectral libraries Rev. D02.00 (Pfleger-Maurer-Weber).

Determination of creatinine

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

Results and discussion

In all of the plasma samples taken from the volunteers tetrazepam was present, meaning that a person can be influenced by tetrazepam up to at least 11 h after intake of one tablet. This was expected and is in good accordance with previously published data where tetrazepam was still detectable in plasma after 72 h [13]. Calculated from the analyzed plasma samples, after an average of 1.37 h (± 0.6) a plasma peak level was reached, which varied between 0.5 and 2.5 h. This is similar to previous studies that found the time to reach a plasma peak concentration varying, on average, between 0.94 [13] and 1.92 h [9], but in isolated cases took up to 4 h [14]. In our study, the individual peak levels, which were achieved at different measurement points in times, varied between 460 and 1180 µg/l (812±251 µg/l), lowering to a mean concentration of 20 µg/l after 11 h. The therapeutic range of tetrazepam is given as approximately 50 to 1000 µg/l in maximum [15]. All but two volunteers, therefore, had peak concentrations within the therapeutic range if one tablet of Myolastan was taken. The curve of mean tetrazepam plasma concentrations per measurement point in time, calculated from all volunteers, is given in Fig. 2. Mean plasma concentrations showed a large variation in the absorption phase due to the individual differences in absorption velocity. These data confirm the pharmacokinetic characteristics of tetrazepam given by the manufacturer and are similar to other experimental results [9, 16]. However, it seems that tetrazepam was eliminated somewhat faster from the plasma than was described for example by Bun et al. [13], who found tetrazepam concentrations of more than 50 µg/l even after 24 h.

Nordazepam was not detected in any of the plasma samples, and diazepam could be found in a concentration of 60 µg/l in only one sample. This sample showed the maximum individual concentration of tetrazepam (1030 µg/l), although this was not the highest tetrazepam concentration measured among all volunteers. Obviously the diazepam concentrations in the other plasma samples and the nordazepam concentration in all samples were too low to be detected. As the therapeutic concentration of

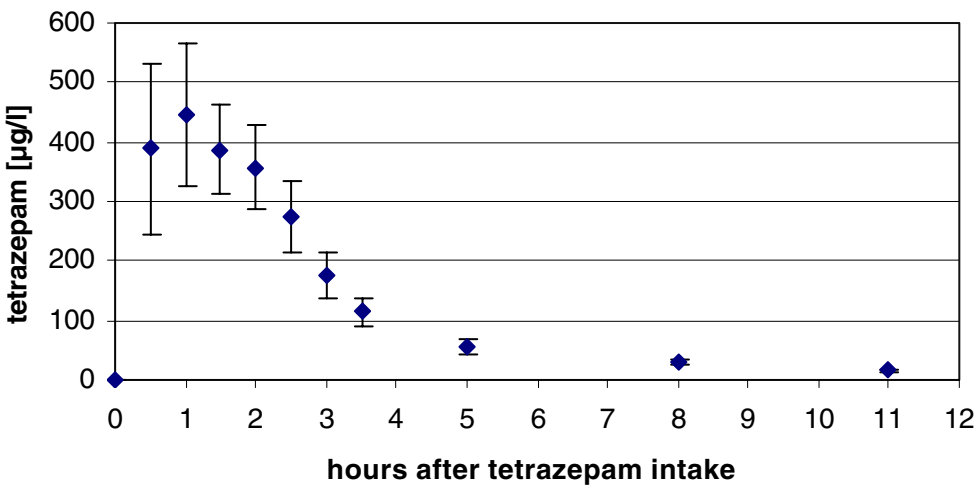

Fig. 2 Mean tetrazepam plasma concentration per measurement point in time calculated from all volunteers. Variance is given as standard error

diazepam is given as 200–2000 µg/l plasma [15], no relevant therapeutic effect can be assigned to diazepam after the intake of one Myolastan tablet.

Benzodiazepine concentrations of all urine samples were interpreted with respect to the creatinine content to compensate for the diuretical state. All urine samples were analyzed twice: GC–MS was used for identification of the benzodiazepines and the following quantitation was performed using GC–ECD. Tetrazepam could be detected in all urine samples, i.e., even in the first sample, meaning that already 2 h after the tablet intake, tetrazepam is excreted in the urine. In relation to the creatinine value, the highest concentration could be measured on average after 3.75 h (± 1.18). The time for reaching the peak concentration did not necessarily correlate between plasma and urine, and ranged from 15 min up to 5 h after the highest measured tetrazepam concentration in plasma. The peak concentrations in urine differed greatly and lay between 310 and 4570 µg/l. The detection of tetrazepam in the last urine samples taken from each volunteer had to be expected according to the known elimination half-life of tetrazepam of about 15–20 h. Tetrazepam was even reported to be found in urine up to 240 h after the intake of a 50-mg dose [17].

In all urine samples, diazepam was also detected even in the first sample. Furthermore, nordazepam was additionally detected between 4 and 26 h (mean 8.8 h) after the tetrazepam intake. Nordazepam is one of the earliest formed metabolites of diazepam and is regularly found after diazepam consumption. Further metabolites of diazepam, such as temazepam or oxazepam, could not be detected. The presence of diazepam and nordazepam in urine samples was confirmed by GC–MS screening, whereas blank urine samples spiked with 1 and 10 mg/l tetrazepam did not show diazepam or nordazepam in both methods. The presence of nordazepam additionally confirms the further metabolism of diazepam itself. A typical elimination curve of tetrazepam and its relation to diazepam and nordazepam is depicted in Fig. 3a. Tetrazepam and diazepam show a nearly parallel curve. Nordazepam appears a little later and seems then to be eliminated as a constant fraction. Diazepam shows a longer elimination half-life than tetrazepam, so a relative increase of diazepam could have been expected. It is likely that the sampling time was too short to observe such an effect. All urine samples together, on average 33% (± 14%) of the tetrazepam concentration was eliminated as diazepam, varying between 13 and 49%. Typical measured concentrations of diazepam and nordazepam set in relation to the respective tetrazepam concentrations are depicted in Fig. 3b.

The results of our study clearly show that tetrazepam is apparently metabolized to diazepam in the human body. To the best of our knowledge, this fact has not been described in the literature before. One fatal case of intoxication was reported where tetrazepam, diazepam, and nordazepam were detected in the blood, but here the ingested benzodiazepine mixture contained both diazepam and tetrazepam [18]. Investigations of possible autoxidation of tetrazepam preparations found different derivatives, but diazepam was not reported [19, 20], which also confirms our finding that diazepam is rather a metabolite and not a degradation product of tetrazepam. Metabolism of benzodiazepines mainly takes place in the liver, involving enzymes of the cytochrome P-450 family (CYP) that catalyze, for example, oxidation and reduction reactions [21]. These enzymes act as oxygenase rather than simple electron carriers, although most of the reactions begin with the transfer of electrons. Thus, the possibility of complex interactions during the metabolism processes can be explained, including, for example, hydroxylation reactions [22]. It is not known to which subtype of CYP enzymes tetrazepam is subject

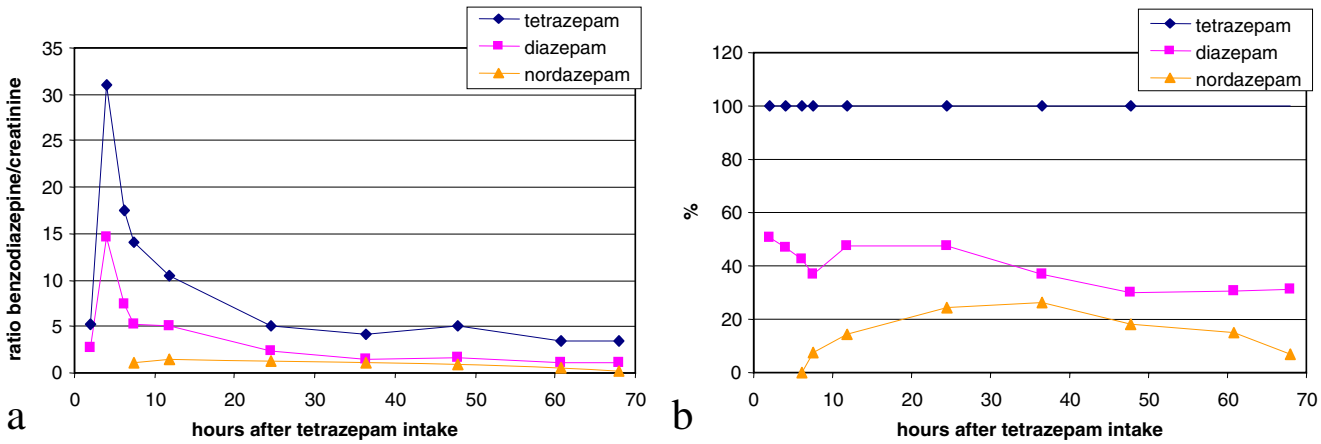

Fig. 3 Typical elimination curve of tetrazepam, diazepam, and nordazepam in urine. Values were creatinine corrected and, therefore, do not represent the absolute measured benzodiazepine concentration (**a**). Typical elimination kinetics of diazepam and nordazepam in relation to tetrazepam (**b**)

during metabolism, but it has been suggested that tetrazepam is transformed in the body similarly to diazepam [23], the latter showing a high affinity to CYP 3A4 [24]. It is reasonable that, first, CYP enzymes may catalyze the hydroxylation steps of the cyclohexenyl group, resulting in hydroxy metabolites that were already described in earlier reports [11]. Next, dehydration, i.e., the loss of two molecules H_2O, may lead to the formation of the phenyl group of diazepam (Fig. 1). Nevertheless, further research would be necessary to clear up the exact mechanisms involved in tetrazepam metabolism in detail.

The main hypothesis of this study that diazepam can be detected in urine after the intake of tetrazepam could be confirmed with all study participants. Therefore, the claim of the three detainees that the benzodiazepine results in their urine samples were due to the intake of prescribed Myolastan tablets could not be disproved. Consequently, an additional consumption of other benzodiazepines was not stated in the commissioned forensic expert opinions in two of the three cases. In one case, however, only nordazepam was detected. Here, the intake of tetrazepam 3 days before the urine sample had been taken, as was claimed by the accused, was considered unlikely. According to our study results, tetrazepam and diazepam should have been found additionally in the urine. Without this knowledge, it would have had to be stated that the detection of diazepam in the urine confirms the consumption of another benzodiazepine in addition to the prescribed Myolastan. This would have lead to unjustified stricter conditions of confinement for the detainees.

Tetrazepam is a medication with primarily muscle relaxant effects and is therefore commonly prescribed within patients. On the contrary, other benzodiazepines acting on the central nervous system with mainly sedating effects like diazepam are often “illegally” abused [25, 26], besides a number of other illicit substances [27]. When toxicological analyses detect diazepam beside tetrazepam in the urine, a consumption of further “illegal” benzodiazepines in addition to the officially prescribed tetrazepam must not coercively be assumed. This may concern offender or victim in criminal offences, as well as drug addicts in opiate substitution programs or, simply, motorists in road traffic. Our results are therefore important for a wide field of forensic applications.

Conclusions

The muscle relaxing benzodiazepine tetrazepam is a commonly prescribed drug for the treatment of muscle spasms of different origins including whiplash injury. It has not yet been reported in the literature that, beside the known demethylation and hydroxylation, tetrazepam is also metabolized to diazepam and further to nordazepam in the human body. Our study confirmed that both metabolites can be detected in urine after the intake of one Myolastan tablet, containing 50 mg tetrazepam, using two independent analytical methods. These results are of important medico-legal interests and can help to prevent false accusations and negative consequences for the examined persons.

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