Midazolam is an ultra short acting benzodiazepine drug, which is readily metabolized by CYP 3A4 into active hydroxy metabolites. The half life of midazolam is about 1 to 4 hours. Like other benzodiazepines it has low toxicity, but serious acute poisonings and fatal cases have been reported. We have described high performance liquid chromatographic method with photodiode array (HPLC-PDA) and mass spectrometric (LC-MS) detection for determination and confirmation of midazolam in biological samples in therapeutic or toxic concentration.

The described methods involved a simple solid phase extraction. The chromatographic separation was performed on a Symmetry C8 column, using a gradient of acetonitrile and phosphate buffer pH 3.6 as the mobile phase. The limit of detection and limit of quantification were 0.0115 and 0.0383 mg/L, respectively. Midazolam was determined using an photodiode array detector operated at 200.5 nm, and confirmed by LC-MS method. Described HPLC-PDA method could be used as a screening technique thanks to library of drugs and their metabolites which has. The method was applied for identification and quantification of midazolam in two forensic cases.

INTRODUCTION

Midazolam (8-chloro-6-(2-fluorophenyl)-1-methyl-4H-imidazo [1,5-a] [1,4] benzodiazepine) is a short-acting drug in the benzodiazepine class that is used for treatment of acute seizures and for inducing sedation and amnesia before medical procedures. It has potent anxiolytic, amnestic, hypnotic, anticonvulsant, skeletal muscle relaxant and sedative properties. Midazolam has a fast recovery time and is the most commonly used benzodiazepine as a premedication for sedation. Less commonly it is used for induction and maintenance of anesthesia (1-3).

Midazolam, like many other benzodiazepines, has a rapid onset of action, high effectiveness and low toxicity level (4).

It is metabolised by cytochrome P450 (CYP3A4) enzymes into an active metabolite alpha-1-hydroxy and 4-hydroxymidazolam and by glucuronide conjugation. Age related deficits, renal and liver status effect the pharmacokinetic factors of midazolam as well as its active metabolite (5).

However, the active metabolite of midazolam is minor and contributes to only 10 percent of biological activity of midazolam.

Midazolam is poorly absorbed orally with only 50 percent of the drug reaching the bloodstream (4). The half-life is one to four hours in adults. In the elderly, as well as young children and adolescents, the elimination half life is longer (2).

The therapeutic as well as adverse effects of midazolam are due to its effects on the GABAA receptors. It does not activate GABAA receptors directly but, as with other benzodiazepines, it enhances the effect of the neurotransmitter GABA on the GABAA receptors resulting in neural inhibi-
tion. Those results in the following pharmacological properties being produced: sedation, hypnotic, anxiolytic, anterograde amnesia, muscle relaxation and anti-convulsant (2).

The sedative effect of intravenous midazolam is accentuated by any concomitantly administered medication, which depresses the central nervous system, such as alcohol, opioids, or tricyclic antidepressants. Caution is advised when midazolam is administered concomitantly with drugs that are known to inhibit the P450 3A4 enzyme system. These drug interactions may result in prolonged sedation due to a decrease in plasma clearance of midazolam (2).

A midazolam overdose is considered a medical emergency and generally requires the immediate attention of medical personnel. Treatment is supportive. The antidote for an overdose of midazolam (or any other benzodiazepine) is flumazenil (2). While effective in reversing the effects of benzodiazepines it is not used in most cases as it may trigger seizures in mixed overdoses and benzodiazepine dependent individuals (3).

Symptoms of midazolam overdose can include: ataxia, dysarthria, nystagmus, slurred speech, somnolence, mental confusion, hypotension, impaired motor function, reflexes, coordination and balance, dizziness, respiratory arrest, vaso-motor collapse, coma and death (3).

The concentrations of midazolam and/or its major metabolite, 1-hydroxymidazolam glucuronide, may be quantified in plasma, serum or whole blood in order to monitor for safety in those receiving the drug therapeutically, to confirm a diagnosis of poisoning in hospitalized patients or to assist in a forensic investigation of a case of fatal overdose (6). They could be determined using chromatographic technique such as high performance liquid chromatography with ultraviolet or mass spectrometric detection (7-14).

The aim of this work to describe liquid chromatographic methods for toxicological and forensic cases.

**METHODS**

*Materials*

Analytical standard of midazolam (99.5 % s.s.), was obtained by Toronto Research.

Acetonitril, sodium hydrogen phosphate, methanol, ammonium acetate, phosphoric acid, and acetic acid were of HPLC, MS or p.a. purity, obtained from MERCK. Water was purified by Millipore Milli-Q system.

Oasis HLB cartridges for solid phase extraction were obtained from Waters.

Serum sample of patient acute poisoned by midazolam and urine, serum and tissue samples after autopsy were analyzed.

*Chromatography*

The method used high performance liquid chromatograph Waters Alliance 2695XE Separations Module pump with Waters 2696 Photodiode Array Detector and Empower Login Software.

The mobile phase was mixture of acetonitrile (A) and phosphate buffer pH 3.6 (B).

Ratio of mobile phases A and B and flow are in gradient Table 1.

| Table 1. Ratios of mobile phases A and B for HPLC-PDA method |
|---|---|---|---|---|
| time (min.) | flow (mL/min) | A % | B % | curve |
| 3.0 | 1.0 | 85 | 15 | 6 |
| 9.0 | 1.0 | 20 | 80 | 6 |
| 28.0 | 1.5 | 20 | 80 | 6 |
| 31.0 | 1.5 | 20 | 80 | 6 |
| 31.5 | 1.5 | 85 | 15 | 6 |
| 35.0 | 0.3 | 85 | 15 | 6 |

The method was used column Symmetry® C8 (wat 054270) 4.6 x 250mm (Waters) with guard column Sentry Guard Symmetry® C18, at the temperature of 30°C, with injector loop volume of 50 µL. Detection of midazolam was performed on 200.5 nm. Retention time of midazolam was about 14.8 minutes.

The LC-MS system consisted of a Waters 2695 separation module interfaced to a ZQ mass spectrometer equipped with an electrospray ionisation source. The apparatus was managed with a Masslynx software.

Analyses were run in positive mode (electrospray) with capillary and cone voltages set to 3.0 KV and 30V. Temperature of the source and desolvatation temperature were 125°C and 430°C, respectively, and nitrogen desolvatation gas flow was 400 L/h and . The mobile phase consisted of a mixture of acetoniitride/acetic acid 1% (A) and acetate buffer pH 3.5 with a flow rate of 0.2 mL/min with gradient shown in table 2.

| Table 2. Ratio of mobile phases A and B for LC-MS method |
|---|---|---|---|---|
| Time (min) | %A | %B | Flow (mL) | Curve |
| init. | 95 | 5 | 0.1 | 1 |
| 2 | 95 | 5 | 0.2 | 6 |
| 16 | 10 | 90 | 0.2 | 6 |
| 20 | 95 | 5 | 0.2 | 5 |
| 26 | 95 | 5 | 0.2 | 5 |

Run time was 26 min. Identification were performed after separation on XTerra® RP18 column in full scan mode (m/z= 100 – 500) by selecting the characteristic m/z = 326 for midazolam. The retention time of midazolam was 14.9 min. Compounds of matrix did not interfere midazolam analysis after extraction.

**Sample preparation**

Stock standard solution of midazolam was prepared by dissolving 10 mg of analytical standard in 10 ml methanol and stored at -20°C. Other concentrations of midazolam were made by diluting stock standard solutions with mobile phase to achieve calibration concentrations expected to meet in serum of poisoned patients.

Serum and urine samples were prepared by solid-phase extraction on Oasis HLB cartridges. Cartridges were conditioned with 1 mL of methanol and 1 mL of water, and then serum samples were loaded. After washing the cartridges with 1 mL of 5 % methanol, midazolam was eluted with 1 mL of methanol. Eluat was analyzed by HPLC-PDA method.
Tissue samples prepared by Stas’ method. Calibration and quality control samples were prepared by adding midazolam solution in blank (“drug-free”) human serum. The amounts of midazolam in spiked serum ranging from 0.1 to 1.5 mg/L. Concentrations of quality control samples were 0.5, 1.0 and 1.5 mg/L.

RESULTS

Midazolam concentrations were determined using weighed linear regression function. Calibration curve for midazolam in serum has shown in Table 3.

<table>
<thead>
<tr>
<th>conc. (mg/L)</th>
<th>AUC for serum spiked with midazolam</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.10</td>
<td>20692</td>
</tr>
<tr>
<td>0.25</td>
<td>51908</td>
</tr>
<tr>
<td>0.50</td>
<td>144325</td>
</tr>
<tr>
<td>1.00</td>
<td>247261</td>
</tr>
<tr>
<td>1.50</td>
<td>407842</td>
</tr>
</tbody>
</table>

The correlation coefficient for serum spiked by midazolam was 0.9942.

Picture 1 shows calibration curve for spiked serum.

Picture 1. Calibration curve for serum spiked by midazolam

Unknown concentrations of midazolam in biological samples were calculated using the corresponding factor from calibration curve. The factor is calculated from the mean value peak-area of midazolam for each mentioned concentration. Recovery was determined, for each concentration, as the mean of three samples by comparing the peak areas of the extracted and non-extracted samples.

Picture 2 shows chromatogram of serum spiked by midazolam solution concentration of 1 mg/L and midazolam UV spectrum.

Picture 2. HPLC chromatogram of spiked serum and midazolam UV spectrum

Analytical recovery for serum spiked by midazolam was 73.31 % (ranged from 58.02% to 83.55%).

Table 4. shows limit of detection, limit of quantitation, standard deviation and coefficient of correlation for serum spiked by midazolam.

<table>
<thead>
<tr>
<th>LoD</th>
<th>LoQ</th>
<th>SD</th>
<th>CV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0115</td>
<td>0.0383</td>
<td>0.00383</td>
<td>4.22</td>
</tr>
</tbody>
</table>

Calculation of midazolam concentration has done on the basis of calibration curve which has gotten after analysis of serum spiked by midazolam standard solution ranged from 0.1 to 1.5 mg/L. Linear regression for spiked serum were $Y = 272571 \times X - 8216.8$.

The method was applied to determine the concentration of midazolam in biological samples. Table 5. shows results of toxicological screening of post mortem material.

Table 5. Concentration of midazolam, clozapine and metabolite and lorazepam

<table>
<thead>
<tr>
<th>Sample</th>
<th>midazolam</th>
<th>clozapine</th>
<th>clozapine metabolite</th>
<th>lorazepam</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood</td>
<td>0.329</td>
<td>0.05 mg/L</td>
<td>0.08 mg/L</td>
<td>0.02 mg/L</td>
</tr>
<tr>
<td>Urine</td>
<td>0.190</td>
<td>0.89 mg/L</td>
<td>1.95 mg/L</td>
<td>0.97 mg/L</td>
</tr>
<tr>
<td>Gastric content</td>
<td>12.95</td>
<td>0.14 mg/L</td>
<td>-</td>
<td>0.15 mg/L</td>
</tr>
<tr>
<td>Liver</td>
<td>688.84</td>
<td>8569 µg/kg</td>
<td>355.37 µg/kg</td>
<td>17.23 µg/kg</td>
</tr>
<tr>
<td>Kidney</td>
<td>301.27</td>
<td>45.66 µg/kg</td>
<td>178.12 µg/kg</td>
<td>20.71 µg/kg</td>
</tr>
<tr>
<td>Brain</td>
<td>342.14</td>
<td>24.62 µg/kg</td>
<td>159.18 µg/kg</td>
<td>12.10 µg/kg</td>
</tr>
</tbody>
</table>

DISSCUSSION

Methods for determination of midazolam in biological samples use liquid chromatography with ultraviolet or mass spectrometric detection (6-14).

The mostly used method for isolation of midazolam from biological samples is alkaline liquid-liquid extraction (6-7,11-12). Many authors recommended diethyl ether as an extraction solvent (6,11). However, Bugey et al used n-butyl chloride for extraction (12).

We have used solid-phase extraction because it is simpler and faster then liquid-liquid extraction. Described solid-phase extraction make possible to extract drugs and their metabolites from different pharmacological group which is important for toxicological screening.

Different literature data reported separation of midazolam and its metabolite compound on C18 column (6-7,11-12). We used C8 column and also achieved very god separation of midazolam from matrix. Separation was performed with mobile phase which consisted acetonitrile and phosphate buffer pH 3.6. Applying of this mobile phase reached good sensitivity. Compounds of matrix did not interfere with analyte. The composition of mobile phase is similar to other described in literature (6-7,11-12).

Identification of midazolam was performed on the basis of derived UV spectrum. Thanks to computer library of UV spectrum we identified not only midazolam, but lorazepam,
clozapine and its metabolite 7-aminoclozapine, too. Quantification of identified drugs was made at wavelength of 200.5 nm.

Midazolam, also, could be quantified by high performance liquid chromatographic methods at 220 nm. (6,13).

We choose wavelength of 200.5 nm because it is the midazolam maximum of absorption.

The mean analytical recovery of HPLC method for determination of midazolam in serum after solid-phase extraction on Oasis HLB cartridges was satisfactory (73.31%). Calibration curve was linear over the range of 0.1 to 1.5 mg/L, and limits of detection and quantification were 0.0115 and 0.0383 mg/L, respectively.

The presented HPLC-PDA method was applied to the assay of midazolam. The therapeutic and toxic concentrations of this drug could be measured.

Like other authors (14), we also used liquid chromatography with mass spectrometry for confirmation of positive results. The combination of both detection types can complement each other and provides extensive case relevant data.

Acute poisonings with benzodiazepines alone not devoid of serious toxicity, but cases of severe coma or fatality have been reported.

Nishiyama et al. reported two cases of overdoses of intramuscular midazolam used as a premedication. Both cases had no reedation or complications, but the accidents happened as a result of a resident and nurse’s lack of experience with midazolam. The intramuscular doses, given at four times the normal quantity, fortunately caused no harm in their cases (15).

The postmortem tissue midazolam concentration reported in a death caused by self-injection of midazolam and sufentanil were 0.05mg/L in blood, 0.3 mg/L in urine, 930 µg/kg in liver and 290 µg/kg in kidney (16).

Michalodimitrakis et al. reported midazolam-related death of a 63 year old man that occurred during endoscopic retrograde cholangiopancreatography, after receiving 10 mg midazolam. The acute intoxication due to midazolam overdose was confirmed by high-pressure liquid chromatography (HPLC) analysis. Blood and urine levels of 2.8 and 0.18 mg/L were reported approx 40 min. after the dose, and postmortem after 2 days was 2.4 mg/L. The case strongly emphasizes the necessity of the precautions that should be taken when midazolam is intravenously administered (17).

Our results for midazolam concentration in postmortem blood are shown in Table 5. Blood concentration was 0.329 mg/L. It was not in the fatal range, but midazolam in combination with clozapine and lorazepam probably caused death.

Our data shows that concentration of midazolam in serum sample of patient who received in Emergency Unit in coma with fatal consequence was 1.12 mg/L, which was in the high toxic level.

**CONCLUSION**

Described HPLC-PDA method is simple, precise, accurate and sensitive. Confirmation of midazolam positive results for forensic cases by mass spectrometric method is necessary. Although less sensitive than LC-MS, HPLC-PDA method has advantage because it could be used as a screening technique thanks to library of high number of drugs and their metabolites UV spectrum. The presented HPLC-PDA method successfully has been applied to two real cases.
Apstrakt

Midazolam je lek iz grupe benzodiazepina sa ultrakratkim delovanjem, koji se brzo metabolizuje posredstvom CYP3A4 enzima u aktivne hidroksi metabolite. Poluvreme eliminacije midazolama je oko 1 do 4 h. Kao i drugi benzodiazepine ima malu toksičnost, mada su zabeleženi i slučajevi ozbiljnih akutnih trovanja, kao i slučajevi sa letalnim ishodom. U radu su opisane metode visoko efikasne tečne hromatografije sa UV skenirajućim (HPLC-PDA) i maseno spektrometrijskim detektorom za određivanje i potvrdu prisustva midazolama u biološkim uzorcima u terapijskim i toksičnim koncentracijama. U metodama je primenjena jednostavna čvrsto-faza ekstrakcija. Hromatografsko razdvajanje je izvršeno na Symmetry C8 koloni, uz korišćenje mobilne faze acetoniitril-fosfatni puffer pH=3,6 uz korišćenje gradijenta. Limit detekcije iznosio je 0,0115 mg/L, a limit kvantifikacije 0,0383 mg/L. Midazolam je određen uz korišćenje UV skenirajućeg detektor za talasnu dužinu od 200,5 nm u potvrden LC-MS metodom. Opisana HPLC-PDA metoda se može primeniti kao “screening” tehnika zahvaljujući biblioteci lekova i metabolite koje poseduje. Metoda je primenjena za identifikaciju i kvantifikaciju midazolama u dva sudsko-medicalna slučaja.

LITERATURA

2. Olkkola, KT.; Ahonen, J, Midazolam and other benzodiazepines, Handb Exp Pharmacol, 2008;182(182): 335–60
10. Marin SJ, McMillin GA, LC-MS/MS analysis of 13 benzodiazepines and metabolites in urine, serum, plasma and meconium, Methods Mol Biol. 2010;603:89-105

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