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Pharmacokinetics of midazolam, 1′-OH-midazolam, 4-OH-midazolam, 1′-OH-midazolam-β-D-glucuronide, and 4-OH-midazolam-β-D-glucuronide in serum and urine from patients undergoing cardiac surgery

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Abstract: Background: The benzodiazepine midazolam is widely used pre- and intraoperatively in intensive care units. 1′-OH-midazolam, one of the major metabolites, is pharmacologically active. Accumulation of 1′-OH-midazolam, e.g. due to hepatic dysfunction or renal insufficiency, may therefore enhance pharmacological activity. Growing evidence suggests that sex, age, drug interactions, and inflammation also have an impact on midazolam disposition and activity. Due to the complex interplay of these factors, finding the optimal midazolam dose for each critically ill patient is challenging.
Methods: We aimed to elucidate the factors that contribute significantly to pharmacokinetics of midazolam and its main metabolites in patients undergoing cardiac surgery. We collected serum and urine samples from 15 patients 1, 2, 3, 4, and 5 hours after the beginning of cardiac surgery and determined the concentrations of midazolam, 1′-OH-midazolam, 4-OH-midazolam, 1′-OH-midazolam-β-D-glucuronide, and 4-OH-midazolam-β-D-glucuronide by LC-MS/MS.
Results: Oxidation to 4-OH-midazolam and subsequent glucuronidation played a role in metabolism and elimination of midazolam in our patient cohort. Patients showed relatively variable concentrations of midazolam and its metabolites, due to differences in midazolam dose and administration routes, demographic and clinical parameters. Thus, we evaluated pharmacokinetic parameters for individual patients and not for the whole patient cohort. We established a logarithmic multiple regression model linking urinary concentrations of midazolam, 1′-OH-midazolam, and 1′-OH-midazolam-β-D-glucuronide with explanatory variables.
Conclusion: Our model linked urinary concentrations of midazolam, 4-OH-midazolam, and 1'-OH-midazolam-β-D-glucuronide to serum concentration, age, surgery infusion volume, creatinine concentration, and/or body temperature.

Keywords: midazolam, pharmacokinetics, 1'-OH-midazolam, 4-OM-midazolam, 1′-OH-midazolam-β-D-glucuronide, 4-OH-midazolam-β-D-glucuronide, cardiac surgery, LC-MS/MS.

Supplementary material: The supplementary material to this paper is available at https://data.mendeley.com/datasets/rfgvzy78p9. The tables and the figure contained in this material are referred in text by numbers prefixed by 'S'.

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Introduction

Administrating sedative drugs to critically ill patients has been a standard intervention in intensive care units (IUCs) for decades [1, 2]. The benzodiazepine midazolam is a widely used sedative in ICUs [3]. Midazolam acts on the central nervous system (CNS) by binding to gamma-aminobutyric acid (GABA) A receptors. Since the neurotransmitter GABA and midazolam bind to different binding sites, midazolam potentiates the inhibitory effect of GABA [4]. The main effect of midazolam is sedation, but it also shows anxiolytic, hypnotic, anticonvulsant, and muscle relaxant properties [5]. As sedative, midazolam is pre- or intra-operatively administered, its anxiolytic and hypnotic properties are exploited by applying midazolam during general anesthesia as part of the maintenance phase [6].

One of the advantages of midazolam is that it may be administered by multiple routes, i.e. orally, intranasally, buccally, intravenously, or intramuscularly. Preoperative administration is typically done intramuscularly or orally. After oral administration, midazolam is rapidly absorbed from the gastrointestinal tract [7]. During surgery, midazolam is frequently administered intravenously.

Another advantage of midazolam is its considerably short half-life compared to other benzodiazepines, e.g. diazepam. The elimination half-life of midazolam is generally 1.5 to 3.5 hours [8]. In healthy volunteers, no difference in the elimination half time was found between continuous infusion and single-dose bolus injection [8]. Biotransformation of midazolam involves hepatic microsomal oxidation and glucuronide conjugation [9]. Microsomal oxidation, catalyzed by cytochrome P450 (CYP) isoenzymes 3A4 and 3A5 [10–12], predominantly leads to 1′-hydroxymidazolam (1′-OH-M) and to a lesser amount of 4-hydroxymidazolam (4-OH-M). These metabolites are conjugated in the liver to 1′-hydroxymidazolam-β-D-glucuronide (1′-OH-MG) and 4-hydroxymidazolam-β-D-glucuronide (4-OH-MG), respectively. Glucuronidation of 1′-OH-M is catalyzed by uridine diphosphate glucuronosyltransferase (UGT) isoforms 2B4/2B7 and 1A4, glucuronidation of 4-hydroxymidazolam by UGT isoform 1A4 [13–15].

1′-OH-M is a pharmacologically active metabolite, although to a considerably lower extent than midazolam. Several papers even hint at low pharmacological activity of 1′-OH-MG [8, 16, 17]. In common settings, the pharmacological activity is mainly caused by midazolam. However, in case of accumulation of 1′-OH-M (and/or 1′-OH-MG), the metabolite(s) may significantly contribute to pharmacological activity. 1′-OH-M accumulation may result from altered drug metabolism, as frequently observed in critically ill patients, e.g. due to hepatic dysfunction or renal insufficiency [17–19]. In addition, sex and age of the patients, drug interactions due to co-medication and/or diminished activity of the enzymes CYP3A4 and CYP3A5 due to inflammation may affect disposition of midazolam [3, 8, 20, 21].

Due to interindividual variability and the complex interplay of numerous factors, finding the optimal midazolam dose for critically ill patients is challenging and critical [8, 19]. Too low doses result in too short sedation periods, while excess of midazolam causes adverse effects, including delayed awakening, respiratory depression, delirium, prolonged duration of intensive care stay, long-term cognitive impairment, and even increased risk of death [2].

In this study, we aimed at elucidating factors that contribute significantly to pharmacokinetics of midazolam and its main metabolites in patients undergoing cardiac surgery. We collected serum and urine samples from 15 patients at five time points (1, 2, 3, 4, and 5 hours after the beginning of surgery) and determined the concentrations of midazolam, 1´-OH-M, 4-OH-M, 1´-OH-MG, and 4-OH-MG in these samples by LC-MS/MS. From this data, we evaluated pharmacokinetic parameters and by multivariate regression analysis, we established a model linking urinary concentrations of midazolam and its metabolites to explanatory variables.

Materials and Methods

Standards and chemicals

Midazolam in the form of solid was provided by Roche Pharma AG (Grenzach-Wyhlen, Germany). Midazolam metabolites 1´-OH-M, 4-OH-M, 1´-OH-MG, and 4-OH-MG in the form of solid were from Toronto Research Chemicals (North York, Ontario, Canada). All substances delivered by Toronto Research Chemicals followed permission from Austrian customs and the Austrian Ministry of Health in accordance with regulations on psychoactive substances. Deuterated internal standards (IS) of morphine-3-β-D-glucuronide-d_3 (M3G-d_3), midazolam d_4 (M-d_4), and α-hydroxy-alprazolam-d_5 (α-OH-A-d_5) at a concentration of 0.1 mg/mL in methanol were purchased from LGC Standards (Kielpin, Poland). Ammonium carbonate buffer (0.01 M, pH 9.3) for extraction was prepared as followed: to 900 mL of ammonium carbonate solution (0.96 g/L), ammonium hydroxide was added to pH 9.3 and the solution was made up to 1000 mL with water (AC buffer).

Patients and sample collection

The study was approved by the Ethics Commission of the Medical University of Vienna (Ethics vote number ESKt uNdr: i1e028/2015). The study included 15 patients from the Division Cardiac, Thoracic, Vascular Anesthesia and Intensive Care, Medical University of Vienna, Vienna, Austria. Patients under 18 years of age were excluded from the study. Patients that obtained more than five erythrocyte concentrates per hour were also excluded. Patients were informed about the purpose of the study and that sampling would be painless. All patients signed a written informed consent form. Data obtained during analysis was anonymized in accordance with the protection of patient privacy under the code of medical ethics. Demographic (sex, age, weight) and clinical data (body temperature, C-reactive protein (CRP) concentration, creatinine concentration, urine volume, infusion volume) and midazolam doses administered are summarized in Table 1. Information on co-medication is given in Table S1.

Blood and urine samples were collected from each patient 1, 2, 3, 4, and 5 hours after surgery start time, resulting in 150 samples in total. Blood samples (1 mL each) and urine samples (1 mL each) were collected with sterile 2 mL syringes, blood samples from an arterial catheter, urine samples from a urinary catheter. Blood and urine samples were centrifuged at 14,000 g and

Table 1. Demographic and clinical data and midazolam doses administered.

					Midazolam dose [mg]					
Patient	Sex	Age [y]	Weight [kg]	Body temperature [°C]	Bolus (i.v.)	Premedication (p.o.)	CRP [mg/dL]	Creatinine [mg/dL]	Urine [mL]	Infusion [mL]
1	m	80	83	35	3.0	7.5	0.01	0.9	700	3400
2	m	68	72	18	—	—[1]	5.8	0.34	2100	8700
3	m	80	88	28	3.0	—	0.03	1.5	1680	4700
4	m	48	78	36	3.0	—	0.3	1.04	380	3400
5	f	73	71	33	3.0	—	3.5	1.18	660	3600
6	m	72	89	34	5.0	7.5	0.9	1.06	750	4200
7	f	71	55	35	3.5	—	0.1	0.7	700	2300
8	m	65	76	33	5.0	7.5	0.2	1.1	1600	4100
9	f	37	55	36	3.0	7.5	0.4	0.62	350	3150
10	m	74	77	36	2.0	3.75	0.07	0.81	1400	6800
11	m	84	83	33	—	—[2]	0.08	1.38	200	6300
12	f	82	98	34	2.0	—	0.35	0.96	450	5060
13	f	67	71	36	5.0	7.5	0.2	0.7	850	5200
14	f	51	73	35	3.0	7.5	0.3	0.8	1000	2200
15	m	56	77	30	5.0	3.75	0.22	0.74	600	3800

[1]: i.v. continuous infusion 10 mg/h/24h (before surgery), [2]: i.v. continuous infusion 5 mg/h/24h (before surgery)

supernatants transferred to Eppendorf tubes. Pools of drug-free (blank) samples were used to optimize and calibrate the LC-MS/MS method. All samples were stored at 4°C.

Sample preparation

1.0 ml serum and urine samples were vortexed and centrifuged (14,000 g) twice and the supernatants were used for solid phase extraction (SPE). Bond Elut C18, 500 mg, 6 mL cartridges (Agilent) were rinsed with 1 mL methanol, 1 mL of AC buffer, and 2 mL water (LC-MS grade). 200 µL aliquots of supernatants were vortexed and mixed with 5 mL of AC buffer and the mix of IS at a concentration of 200 ng/mL. Then, the samples were loaded onto the SPE cartridge and passed through slowly. The SPE cartridge was washed with 2 mL of AC buffer and vacuum dried for 5 min. The analytes were eluted with 2 mL of 1 M acetic acid/methanol (1:9, v/v) under gravitational force into silanized glass vials. The eluates were evaporated under a nitrogen stream, reconstituted in 100 µL mobile phase and injected into the HPLC-MS/MS system.

LC-MS/MS method

An Agilent 1200 liquid chromatograph (Agilent) equipped with a binary pump (G1312 A) and an autosampler (G1329 A) was used. Chromatographic separation was achieved on a Poroshell 120 EC-C18, 3.0 × 100 mm, 2.7 µm column (Agilent). A Poroshell 120, EC-C18, 3.0 × 5 mm (Agilent)

column was used as guard column. The column oven temperature was set at 40°C. Solvent A was 2 mM ammonium formate, 0.2% (v/v) formic acid in water, solvent B 2 mM ammonium formate, 0.2% (v/v) formic acid in acetonitrile. The gradient was as follows: 0 min: flow rate 0.5 mL/min, 95% A, 5% B; 10 min: flow rate 1.0 mL/min, 10% A, 90% B; 15 min: flow rate 1.0 mL/min, 10% A, 90% B.

A 6410 triple quadrupole mass spectrometer (Agilent) with an ESI source, operated under a positive mode was used. Multiple reaction monitoring (MRM) detection was employed. The operational parameters of the ESI source were as follows: vaporizing temperature 350°C; pressure of the nebulizing gas 40 psi; flow of the drying gas 9 L/min; capillary potential 3.5 kV.

The following MRM transitions were monitored *m/z*: 326.1/291.1 and 326.1/249.1 for midazolam, 342.1/324.1 and 342.1/203 for 1´-OH-M, 342.1/325 and 342.1/297 for 4-OH-M, 518.1/324 and 518.1/342.1 for 1´-OH-MG, 518.1/342.1 and 518.1/234 for 4-OH-MG, 330.1/295.2 and 330.1/253.2 for M-d_4 (IS for midazolam), 330.1/302.1 and 330.1/210.1 for α-OH-A-d_5 (IS for 1´-OH-M and 4-OH-M), and 465.2/289.3 for M3G-d_3 (IS for 1´-OH-MG, and 4-OH-MG).

Calibration curves were established by analyzing drug-free pools of serum and urine samples, spiked with midazolam and its metabolites in the range from 1.0 to 500 ng/mL and mix of IS at a concentration of 200 ng/mL. Limit of detection (LOD) and limit of quantification (LOQ) of the LC-MS/MS method were as follows: midazolam, 1´-OH-M, and 4-OH-M: LOD 0.05 ng/mL, LOQ 0.5 ng/mL; 1´-OH-MG and 4-OH-MG: LOD 0.1 ng/mL, LOQ 1 ng/mL. The given LODs and LOQs applied for serum and urine samples.

Pharmacokinetic analysis

Pharmacokinetic parameters were evaluated using Phoenix WinNonlin version 8.4 Software (Certara, Princeton, NJ 08540, USA). Pharmacokinetic parameters were calculated by a noncompartmental approach. The parameters included HL_λ_z, AUC_{last}, MRT_{last}, AUC_{all}, AUC_{INF_obs}, and MRT_{INF_obs}. HL_λ_z is the terminal half-life, calculated by ln 2 /λ_z, with λ_z being the elimination rate constant [h^{-1}]. AUC_{last}, the area under the concentration-time curve from time 0 to the time of the last measurable concentration (t_{last}), was calculated using the trapezoidal linear interpolation rule. MRT_{last} is the mean residence time calculated on a basis of concentration-time curve from time 0 to t_{last}. AUC_{all} describes the area under the curve, including all time-points. AUC_{INF_obs}, the AUC from time of dosing extrapolated to infinity, was calculated as $AUC_{last} + C_{last}/\lambda_z$, with C_{last} being the last concentration ≥LOQ. MRT_{INF_obs} is the mean residence time based on the concentration-time curve extrapolated to infinity. For metabolites, the respective mean exit time, MET, was calculated. MRT/MET could not be calculated for patients with multiple administration of midazolam.

Data evaluation and statistical analysis

Numerical values were reported as a mean ± SD. Statistical analyses were performed with the SAS/STAT Software, Version 9.4 (SAS Institute, Cary, NC, USA). For correlation analyses, Pearson correlation coefficient and Spearman's rank correlation coefficient rho (ρ) were evaluated. In addition, multiple linear regression analysis with an automatic procedure for backward elimination of covariates was carried out. The Akaike criterion with a correction for small sample sizes (AICc) was chosen to select the optimal model. For all statistical tests, $p < 0.05$ was considered to be statistically significant.

Results

Characteristics of the patient population

Demographic and clinical data and midazolam doses administered are summarized in Table 1 for each of the patients. 9 (60%) patients were males, 6 (40%) females. The average age of the patients was 67.2 ± 13.7 years. The patients had an average weight of 76.4 ± 11.5 kg. Hypothermia during surgery, defined as a decrease in body temperature to <35°C, occurred in 8 (53%) patients. 5 (33%) patients received midazolam during surgery, 8 (53%) obtained midazolam pre- and intraoperatively, and 2 (13%) obtained midazolam i.v. in form of a continuous infusion before surgery. In 2 (13%) patients, the C-reactive protein (CRP) concentration was >0.5 mg/dL, indicating inflammation. In 3 (20%) patients, the creatinine concentration was above and in 1 (7%) below the normal range (adult males: 0.74 to 1.35 mg/dL, adult women, 0.59 to 1.04 mg/dL). The average urine volume was 895 ± 555 mL, the average infusion volume 4461 ± 1751 mL. Patients obtained in average 11 drugs in addition to midazolam.

Concentration of midazolam and its metabolites in serum and urine samples

The 75 serum and 75 urine samples were analyzed by LC-MS/MS to determine the concentrations of midazolam, 1´-OH-M, 4-OH-M, 1´-OH-MG, and 4-OH-MG. The concentration-time profiles for midazolam and its metabolites are shown in Fig. S1. Midazolam could be detected in all serum samples, except samples from four patients (patients 7, 8, 10, 15) collected 4 and 5 hours after surgery start time, respectively. In serum samples collected after 1 h, the maximal midazolam concentration was 853.8 ng/mL and with ongoing surgery, the midazolam concentration decreased drastically. After 5 h, midazolam concentrations were <1.5 ng/mL, indicating that midazolam was rapidly metabolized. Midazolam was also found in 95% of the urine samples, however, the midazolam concentration was <5 ng/mL. The metabolite 1´-OH-M was detected in all serum samples collected within 3 h after surgery start time and in 73% of the serum samples collected later on. 1´-OH-M was also found in all urine samples, except one sample collected after 5 hours. The maximal 1´-OH-M concentrations in serum and urine samples were 79.3 ng/mL and 113.7 ng/mL, respectively. In 59% of the serum samples, the 4-OH-M concentration was <LOD, in 36% between LOD and LOQ. 4-OH-M was not detected in any of the urine samples. 1´-OH-MG was found in 85% of the serum samples, but the concentrations were rather low (<3.5 ng/mL). In all urine samples except three, the 1´-OH-MG concentration was >LOD. Maximal concentrations were 448.7 ng/ml, 447.9 ng/ml, 351.1 ng/mL, 217.3 ng/mL, and 88.1 ng/mL in samples collected after 1 to 5 h, respectively. 4-OH-MG was detected in 47% of the serum samples, in 39% the concentration was <LOQ. 4-OH-MG was detected in 84% of the urine samples, in 75% the concentration was >LOQ. In the urine samples collected 1, 2, 3, 4, or 5 hours after surgery start time, the highest concentrations were 143.3 ng/mL, 66.2 ng/mL, 114.0 ng/mL, 51.0 ng/mL, and 20.0 ng/mL, respectively.

Pharmacokinetic parameters

Next, we evaluated the pharmacokinetic parameters of midazolam, 1´-OH-M, 4-OH-M, 1´-OH-MG, and 4-OH-MG in serum samples for each individual patient. The parameters included

HL_λ_z, AUC_{last}, MRT_{last}, AUC_{INF_obs}, and MRT_{INF_obs} for midazolam, and HL_λ_z, AUC_{last}, MET_{last}, AUC_{INF_obs}, and MET_{INF_obs} for its metabolites.

We observed big interindividual differences in pharmacokinetic parameters of midazolam, in particular in AUC_{last}, and AUC_{INF_obs} (Table S2). Differences in these parameters were even observed for patients who were given the same midazolam doses, e.g. patients 1, 9, and 14; patients 3, 4, and 5; and patients 6, 8, and 13. AUC values were considerably high (>1000 ng h/mL) in patients 8, 10, 11, 12, 13, and 15. Particularly high HL_λ_z and MRT_{INF_obs} values were observed for patient 2.

In most patients, AUC_{last} of 1′-OH-M was similar to AUC_{INF_obs} (Table S3), hinting at rapid glucuronidation of 1′-OH-M.

Pharmacokinetic parameters of 4-OH-M were similar between patients (Table S4), because in most samples, the concentration was <LOD or between LOD and LOQ. HL_λz and AUC_{INF_obs} could only be calculated for 5 patients. For these patients, AUC_{last} reflected quite well AUC_{INF_obs}.

Pharmacokinetic parameters for 1′-OH-MG are summarized in Table S5. HL_λ_z and AUC_{INF_obs} could be calculated for serum samples from 9 patients. With the exception of patient 13, AUC_{last} was almost as high as AUC_{INF_obs}.

For 4-OH-MG (Table S6), HL_λ_z and AUC could be calculated for 9 and 13 patients, respectively. AUC_{last} almost reached AUC_{INF_obs}. Considerably high AUC values of ~2826 were obtained for patient 12 in serum.

Correlation analyses

Since only five corresponding serum and urine samples were available from each patient, we could not draw reliable conclusions about correlations between the examined variates in each given patient. When we searched for linear correlation between concentrations of midazolam and its metabolites in joint samples pooled from all patients, we only observed significant linear correlation between midazolam concentration in serum (p = 0.011) and 1′-OH-MG concentration in serum (p = 0.042) with 4-OH-MG concentration in urine; midazolam concentration in urine and 1′-OH-MG concentration in serum (p = 0.003) and 1′-OH-MG concentration in urine (p <0.0001) (Table S7).

However, significant Spearman's correlation, assessing monotonic relationships, were found between midazolam in serum and midazolam in urine (p = 0.022); midazolam in serum and 4-OH-MG in urine (p <0.0001); midazolam in urine and 1-OH-MG in serum (p = 0.003); midazolam in urine and 1′-OH-MG in urine (p <0.0001); 1′-OH-M in urine and 1′-OH-MG in serum (p = 0.025) and urine (p = 0.0002) (Table S8).

Analysis of covariates

We assumed that the lack of expected linear correlation between serum and urine concentrations might originate from highly variable renal function in patients under the study. For any compound its speed of renal elimination may be expressed by the following equation:

$$\frac{dA}{dt} = CL_r \times C_s(t)$$

where A is the amount of the substance eliminated by kidneys until the time point t, dA/dt is the speed of renal elimination at t, $C_s(t)$ is the concentration of the substance in serum at t, and CL_r is the renal clearance of that substance.

The following equation also holds:

$$\frac{dA}{dt} = Q_u \times C_u(t)$$

where Q_u is the speed of urine secretion, and C_u (t) denotes urine concentration of the given compound.

Combining the two equations above yields:

$$C_u = \frac{CL_r}{Q_u} \times C_s$$

Q_u was determined based on the volume of urine secreted during 300 minutes interval (constant speed of urine production was assumed in a given patient). Due to differences in renal clearance between patients, we assumed, that:

$$CL_r = k \times GFR$$

where k is a proportionality constant common to the entire population of patients and GFR (glomerular filtration rate) is an indicator of renal function. GFR was calculated based on creatinine concentration with the aid of the Cockroft-Gault equation [22].

Finally, we assumed that:

$$C_u = \frac{k \times GFR}{Q_u} \times C_s$$

i.e. linear correlation between and C_u was expected for all measured concentrations of a given compound. Table 2 contains estimates of Pearson and Spearman correlation coefficients for the above expressions computed for the selected compounds.

Due to the high variability in $\frac{GFR}{Q_u} \times C_s$, which ranged from 5.4 to 25326, linear correlation analysis was also performed for the logarithmically transformed variates (Table 3).

In order to determine factors that were not included in the considered model we attempted to link the observations with potential covariates using multiple linear regression.

Table 2. Estimates of Pearson and Spearman correlation coefficients between $\frac{GFR}{Q_u} \times C_s$ and C_u for midazolam, 1′-OH-M, and 1′-OH-MG.

Analyte	n	Pearson		Spearman	
		coefficient	p	coefficient	p
Midazolam	59	–0.022	0.87	0.277	**0.03***
1′-OH-M	38	–0.135	0.42	–0.155	0.35
1′-OH-MG	21	0.415	0.06	0.375	0.09

Table 3. Estimates of Pearson correlation coefficients between log-transformed $\frac{GFR}{Q_u} \times C_s$ and C_u for investigated compounds.

Analyte	*n*	Pearson	
		coefficient	*p*
Midazolam	59	0.221	0.09
1′-OH-M	38	–0.187	0.26
1′-OH-MG	21	0.351	0.12

For each substance, we initially considered the following potential covariates: serum concentration of the substance [ng/mL]; patient's age [y]; weight [kg]; sex; body temperature [°C]; creatinine concentration [mg/dL]; CRP [mg/dL]; volume of collected urine [mL]; and total volume of infused fluids [mL].

The general form of the equation is as follows and regression coefficients (β) are compiled in Table 4.

$$mz_{urine} = \beta_0 + \beta_{mz_{serum}} \cdot mz_{serum} + \beta_{age} \cdot age + \beta_{sex} + \beta_{surg\_inf} \cdot surg_{inf} +$$
$$+ \beta_{vol\,urine} \cdot vol_{urine} + \beta_{creat} \cdot creatinine + \varepsilon$$

where ε is a randomly distributed error with zero mean.

Table 4. Optimal linear multiple regression models linking the urine concentrations of investigated compounds with explanatory variables. The models were selected as the results of the automatic backward elimination procedure.

Substance	Selected covariates	Regression coefficients ± SE	Pearson correlation	*p*
Midazolam	Midazolam in serum	0.00191 ± 0.00132	0.63	**<0.0001*****
	age	–0.00360 ± 0.00168		
	sex (if m)	–0.488 ± 0.408		
	surgery infusion	–0.000466 ± 0.000166		
	collected urine	0.00111 ± 0.00045		
	creatinine	–1.12 ± 0.88		
1′-OH-M	sex (if m)	–9.47 ± 7.63	0.29	**0.046***
	surgery infusion	0.00412 ± 0.00253		
	CRP	–3.61 ± 2.83		
1′-OH-MG	1′-OH-MG in serum	74.1 ± 34.1	0.74	**<0.0001*****
	age	–10.1 ± 2.8		
	weight	7.98 ± 3.78		
	temperature	24.7 ± 13.2		

The final equation has the following form:

$$mz_{urine} = 6.564 + 0.00191 \cdot mz_{serum} - 0.00360 \cdot age + \begin{cases} -0.488 & \text{m} \\ 0 & \text{f} \end{cases} -$$
$$- 0.000466 \cdot surg_{inf} + 0.00111 \cdot vol_{urine} - 1.12 \cdot creatinine + \varepsilon$$

Multivariate analysis revealed that the urinary midazolam concentration was significantly associated with the midazolam concentration in serum, age, sex, infusion volume, urine volume, and the creatinine concentration. We found a significant association between the urinary concentration of 1′-OH-M and sex of the patient, infusion volume and CRP. The 1′-OH-MG concentration in urine was significantly associated with 1′-OH-MG concentration in serum, age, weight, and body core temperature of the patients.

We also established an analogous regression model on a logarithmic scale, i.e. for log-transformed concentrations as well as so transformed interval covariates.

In the case of logarithmic transformation, the form of regression equation is as follows and the regression coefficients are presented in Table 5.

$$\log mz_{urine} = \beta_0 + \beta_{mz\_serum} \cdot \log mz_{serum} + \beta_{age} \cdot \log age + \beta_{sex} + \beta_{surg\_inf} \cdot \log surg_{inf} + \beta_{vol\ urine} \cdot \log vol_{urine} + \beta_{creat} \cdot \log creatinine + \varepsilon$$

The performance of the logarithmic models is illustrated in Fig. 1. The logarithms of measured concentrations are plotted vs predicted logarithms of concentrations. The (partially shown) ellipses indicate regions into which 95% of experimental points are expected to hit.

Table 5. Optimal logarithmic multiple regression models linking the urine concentrations of investigated compounds with explanatory variables. The models were selected as the results of the automatic backward elimination procedure.

Substance	Selected covariates (logarithms)	Regression coefficients ± SE	Pearson correlation	*p*
Midazolam	midazolam in serum	0.147 ± 0.039	0.683	**<0.0001*****
	surgery infusion	–1.40 ± 0.23		
	urine volume	0.364 ± 0.146		
	CRP	0.054 ± 0.048		
	creatinine	–0.749 ± 0.237		
1′-OH-M	1′-OHM in serum	–0.150 ± 0.121	0.364	**0.025***
	temperature	2.25 ± 1.91		
	surgery infusion	1.05 ± 0.61		
	urine volume	0.363 ± 0.346		
1′-OH-MG	1′-OH-MG in serum	1.60 ± 0.48	0.900	**<0.0001*****
	age	–8.48 ± 1.23		
	weight	12.1 ± 2.0		
	temperature	8.38 ± 3.20		
	surgery infusion	1.02 ± 0.72		

Discussion

Most studies investigating midazolam pharmacokinetics so far either focused only on midazolam [3, 23–26], determined midazolam and 1′-OH-M [7, 19, 24, 27, 28], or targeted midazolam, 1′-OH-M, and 1′-OH-MG [16, 17, 29, 30]. The number of papers including 4-OH-M and/or

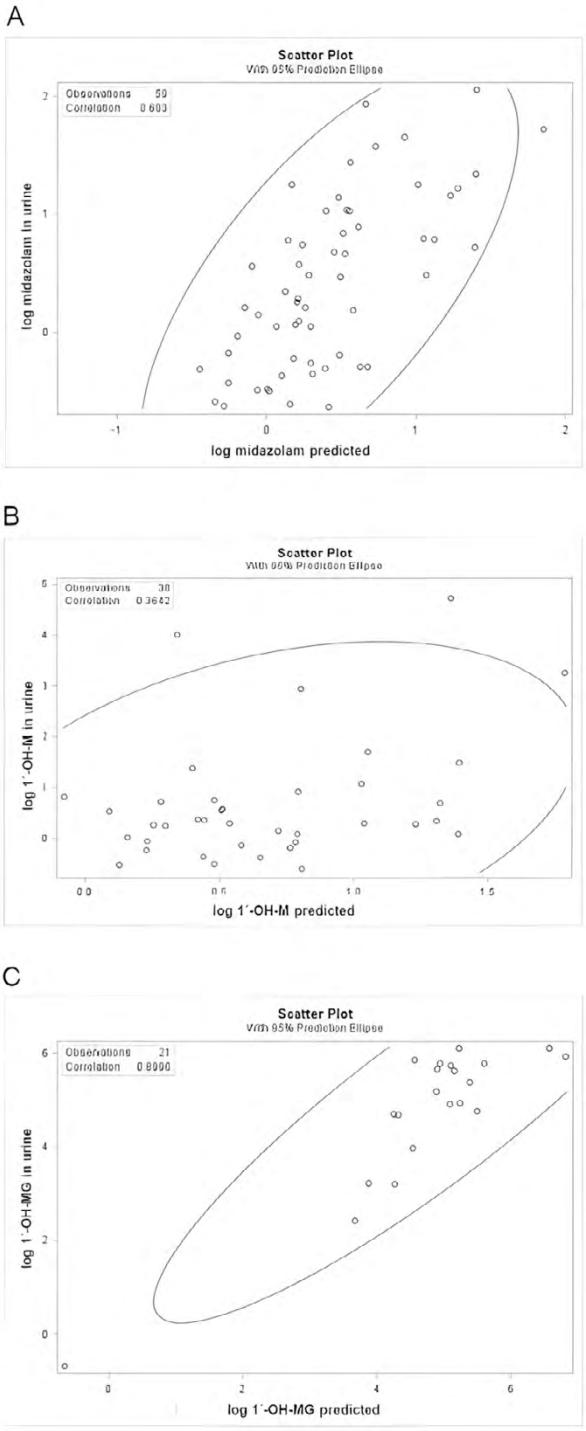

Fig. 1. Measured vs predicted (by the regression model) logarithms of concentrations in urine. **A**) midazolam, **B**) 1´-OH-M, **C**) 1´-OH-MG.

4-OH-MG is, however, scarce [31–33]. In this study, we simultaneously determined the concentration of midazolam, 1′-OH-M, 4-OH-M, 1′-OH-MG, and 4-OH-MG in serum and urine samples collected from patients under cardiac surgery 1, 2, 3, 4, and 5 h after surgery start time.

The concentration of glucuronides is commonly determined by either an indirect [17, 34] or a direct [12, 32, 35] approach. The indirect approach is based on cleaving the glucuronides by adding glucuronidase, converting 1′-OH-MG and 4-OH-MG to 1′-OH-M and 4-OH-M, respectively. In our study, we selected the direct approach since it lacks time-consuming enzymatic deconjugation. Midazolam, 1′-OH-M, 4-OH-M, 1′-OH-MG, and 4-OH-MG were directly separated on the chromatographic column and subsequently detected by MS/MS.

We observed rather high variability in the concentrations of midazolam and its metabolites in serum and urine. The variability observed can be explained in part by differences in midazolam doses and/or administration routes. However, we observed variability also for patients that were subjected to identical treatment schemes and midazolam doses, e.g. patients 1, 9, and 14; patients 3, 4, and 5; and patients 6, 8, and 13.

Unfortunately, our patient cohort was rather small and heterogeneous with respect to sex, age, weight, body core temperature, creatinine concentration, CRP, urine volume, and infusion volume. However, in spite of the patient cohort heterogeneity, we could observe several trends. Midazolam, 1′-OH-M, and 1′-OH-MG were detected in almost all serum and urine samples collected from the 15 patients. In general, in all serum samples the midazolam concentration was higher than that of its active metabolite 1'-OH-M. Only in two patients (patients 5 and 6), the concentration of 1′-OH-M in serum exceeded that of midazolam. Excess of 1′-OH-M over midazolam concentrations in serum has already been reported for critically ill patients with organ dysfunction [36]. In general, 1′-OH-M concentrations exceeded 1′-OH-MG concentrations in serum. As medically desirable, high concentrations of midazolam in the first sample rapidly decreased in samples 2 to 5 and, along with 1′-OH-M, reached low concentrations at the end of surgery. In patients that obtained midazolam pre-operatively, higher concentrations of midazolam metabolites, in particular 1′-OH-MG, were already found in urine samples collected 1 h after beginning of surgery. Concentrations of 4-OH-M and 4-OH-MG in serum samples were either <LOD or between LOD and LOQ.

In urine samples, 1′-OH-MG concentrations exceeded those of midazolam and 1′-OH-M. In contrast to 4-OH-M, which could not be detected in any of the urine samples, 4-OH-MG was found in almost all urine samples. In most urine samples, 1′-OH-MG concentration exceeded concentration of 4-OH-MG. As mentioned above, data on 4-OH-MG concentrations in urine is limited. In our patient cohort, oxidation of midazolam to 4-OH-M and subsequent glucuronidation played a role in metabolism and elimination of midazolam.

Due to the heterogeneity of the patient cohort, we refrained from evaluating pharmacokinetic parameters for the whole patient cohort but evaluated them for individual patients. Particularly high HL_λ_z and MRT_{INF_obs} values were observed for midazolam in serum from patient 2, most probably resulting from the extremely low body core temperature of 18°C. Hypothermia also occurred in patients 3, 5, 6, 8, 11, 12, and 15. Considerably high AUC values of ~28000 ng h/mL were obtained for midazolam in serum from patient 12, which may be a consequence of altered distribution of the drug in the patient's body as a result of accumulation in adipose tissue (the patient with the highest weight), perhaps impaired tissue perfusion processes, impaired elimination of the drug by the kidneys or inhibition of biotransformation processes.

By searching for linear relationship between urinary and serum concentrations of midazolam and its metabolites, we found significant linear correlation between midazolam concentration in

serum and urinary 4-OH-MG concentration; urinary midazolam concentration and 1′-OH-MG concentration in serum; urinary midazolam and urinary 1′-OH-MG concentration; and 1′-OH-M concentration in serum and urinary 4-OH-MG concentration. It is well known that in critically ill patients, a number of variables may have an impact on drug pharmacokinetics [3, 8, 17, 21].

In previous studies, one-compartment [37] or multi-compartment [38–41] models have been established to describe the pharmacokinetic behavior of midazolam. In a study involving stable critically ill children (median age 9.8 weeks) who were administered a single dose of ^{14}C- midazolam as an oral microtracer, a one-compartment model was selected to describe midazolam pharmacokinetics [37]. A two-compartment model was used to model midazolam pharmacokinetics in pediatric intensive care patients [39] and obese and non-obese adolescents aged 11–18 years [41]. Albrecht *et al.* who investigated the effect of age on the pharmacokinetics of midazolam in male volunteers found an open-three compartment model applicable for describing plasma concentration–time courses [38]. In a study on morbidly obese patients, a three-compartment model with two equalized peripheral volumes of distribution showed an improved fit over a two-compartment model, whereas a full three-compartment model could not be estimated with adequate precision [40].

Unfortunately, in our study focusing on patients undergoing cardiac surgery, the patient cohort was relatively heterogeneous. We tried to build a compartmental model within the population pharmacokinetics framework, but these attempts were unsuccessful, probably due to too wide a distribution of pharmacokinetic parameters and small number of subjects. Thus, a meaningful compartmental model could not be established. However, by multivariate regression analysis, we could establish models linking urinary concentrations of midazolam, 1′-OH-M, and 1′-OH-MG to explanatory variables. According to the final logarithmic model, urinary concentration of midazolam was positively associated with midazolam concentration in serum, urine volume, and CRP and negatively associated with surgery infusion volume and creatinine concentration. Urinary 1′-OH-M concentration was positively associated with body core temperature, surgery infusion volume and urine volume and negatively associated with 1′-OH-M concentration in serum. Among the covariates, body temperature had the strongest impact. Urinary 1′-OH-MG concentration was positively associated with 1′-OH-MG concentration in serum, body weight, body core temperature, and surgery infusion volume, and negatively associated with age. Body weight, patient age, and body temperature had the strongest impact on midazolam pharmacokinetics.

In general, advanced age may have a variety of effects on drug disposition and metabolism, e.g. due to impairments in homeostasis and organ functions and/or the occurrence of comorbidities [42]. Age was identified as important factor contributing to high interindividual variability of midazolam pharmacodynamics [19]. With increasing age, both midazolam infusion rate and plasma concentration decreased almost constantly [19]. Elderly patients were found to show slower midazolam clearance and prolonged midazolam half-life compared to younger patients [21]. To achieve target levels of sedation, elderly patients require lower midazolam infusion rates and lower midazolam plasma concentrations [19].

It is well known that the body temperature has an impact on drug metabolism and elimination. Therapeutic hypothermia alters disposition, metabolism, and response of a variety of drugs commonly used in IUCs, including those metabolized by CYP 450 [43]. The temperature range for therapeutic hypothermia has been categorized into mild hypothermia (rectal temperature 35–36.5°C), moderate hypothermia (rectal temperature 32–35°C), and severe hypothermia (rectal temperature <32°C) [44]. According to this classification, two patients

(patients 2 and 15) of our cohort showed severe hypothermia. Particularly high HL_λ_z and MRT_{INF_obs} values of midazolam in serum were observed for patient 2. In a study on traumatic brain injured patients, lowering the core temperature to <35°C resulted in a five-fold increase in midazolam plasma concentrations compared to persons with normal temperature [26]. Midazolam metabolism was also reduced by mild short duration of hypothermia in normal healthy volunteers [28]. However, in a study by Bjelland *et al.* including fourteen patients treated with therapeutic hypothermia following cardiac arrest (33–34°C), no significant effect on elimination half-life of midazolam was seen compared to eight matched critically ill patients (36–38°C) without hypothermia [24].

Our logarithmic multivariate regression model suggests urinary midazolam concentration to be negatively associated with creatinine concentration. Creatinine concentration in serum, the most widely used marker for renal function [45], has already been reported to be a covariate, influencing midazolam pharmacokinetic parameters [8]. With deteriorating renal function, increased concentrations of 1´-OH-MG were found in serum [3].

CRP, a widely used marker of inflammation, was positively associated with urinary concentration of midazolam. Inflammation is known to affect drug metabolism, mainly by downregulation of hepatic and extrahepatic cytochrome P450s as well as other drug-metabolizing enzymes and transporters [20].

Our logarithmic multiple regression model hints at positive association between body weight and urinary concentration of 1´-OH-MG. Obesity increased the distribution volume of midazolam compared to the distribution volume in individuals with normal body weight. The elimination half-life of midazolam was significantly prolonged in obese individuals compared to normal-weight persons [40].

Conclusions

We determined the concentrations of midazolam and its metabolites in serum and urine samples collected from 15 patients at five time points during cardiac surgery by LC-MS/MS. In contrast to most previous studies investigating pharmacokinetics of midazolam, we did not only focus on the metabolites 1´-OH-M and 1´-OH-MG, but also included 4-OH-M and 4-OH-MG. Data indicates that in our patient cohort, oxidation to 4-OH-M and subsequent glucuronidation played a role in metabolism and elimination of midazolam.

In general, we observed rather high interindividual variability in the concentrations of midazolam and its metabolites, caused by differences in midazolam dose and administration routes, but also by differences in demographic and clinical parameters. In spite of these differences, we could establish a multiple variable regression analysis, linking urinary concentrations of midazolam, 1´-OH-M, and 1´-OH-MG to co-variables including serum concentration, age, surgery infusion volume, creatinine concentration, and/or body temperature.

We are aware of the fact that the present study involved a limited number of patients. Further studies are required to confirm our findings and to assess the predictability of our model.

Institutional review board statement

The study was conducted in accordance with the Declaration of Helsinki and approved by the Ethics Commission.

Informed consent statement

Informed consent was obtained from all subjects involved in the study.

Data availability statement

The dataset generated during the current study is available from the corresponding author on reasonable request. Supplementary material is also available as Mendeley Dataset (https://data.mendeley.com/datasets/rfgvzy78p9).

Conflict of interest

The authors declare no conflict of interest.

Authors' contribution

M.P. — collecting samples, chemical analytics, writing, editing; S.R. — consulting, chemical analytics; W.J. — theoretical considerations in the field of pharmacokinetics, statistical modelling, corresponding author; M.W. — pharmacokinetic analysis in the framework of non-compartmental pharmacokinetics, discussion; M.C.-M. — supervising, writing, editing, discussion; M.H. — clinical supervising, consulting.

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