Absolute Bioavailability of Microdosed Midazolam After Buccal Administration Is Dependent on Buccal Exposure Time

Jana Grass, Peter Rose, MD, Jürgen Burhenne, PhD, Antje Blank, MD, Walter Emil Haefeli, MD, and Gerd Mikus, MD, MSc

Abstract

Midazolam is an established probe drug to assess cytochrome P450 3A activity (phenotyping). Microdosed midazolam is increasingly used for this purpose; a buccal formulation might be of advantage, but buccal absorption might occur. We therefore tested in a single-center, open-label clinical trial with 12 healthy volunteers the absolute bioavailability of 10 μg of midazolam after buccal administration in relation to buccal exposure time. In relation to a drinking solution, there was an increase of midazolam exposure (area under the plasma concentration–time curve from time 0 to infinity) with increasing buccal exposure time with an apparent saturation at 100-second buccal exposure. Absolute bioavailability increased from 27.8% (95% confidence interval, 23.5-32.9) for the drinking solution (0 seconds) to 66.1% (95% confidence interval, 60.0-72.8) after 100-second buccal exposure with no further increase after 150 seconds. A Hill equation described the time dependency of midazolam bioavailability with maximal bioavailability as 64.5% and buccal exposure time resulting in half maximal bioavailability increase as 16 seconds. In conclusion, midazolam bioavailability is highly dependent on buccal exposure time, and even a few seconds of buccal exposure will increase bioavailability due to buccal absorption. This needs to be taken into account for any buccal administration of midazolam.

Keywords
bioavailability, buccal, CYP3A, midazolam

Children and adolescents with prolonged acute convulsive seizures occurring in the community setting can be managed by the buccal administration of midazolam, which is regarded as quicker and easier than getting intravenous access. Another, very different use of oral midazolam is the assessment of overall cytochrome P450 (CYP) 3A activity (phenotyping) by measuring the partial metabolic clearance to 1-OH-midazolam or specifically hepatic CYP3A activity using total clearance after intravenous midazolam administration. In recent times, the use of microdosed midazolam has been propagated for phenotyping, and analytical methods have been established and used in drug-drug interaction trials or even early clinical trials during drug development. Microdosing is defined as the administration of 1% of the dose of a therapeutic substance or 100 μg (whatever is less) to study the properties of this substance. Because these doses are far below the “no observed adverse effect” level, the probability of a pharmacological effect is close to zero. It is an effective approach to assess the propensity of pharmacokinetic drug interactions in early drug development or the pharmacokinetics of different application routes without measurable risk. No oral microdose formulation is commercially available, and therefore, for oral dosing, the intravenous solution is diluted to the required dose, thereby restricting the application of such a technique to dedicated clinical trial centers. To overcome this problem, a buccal film containing 30 μg was developed, which created a convenient ready-for-use dosage form that facilitates CYP3A phenotyping. Buccal films stick to the oral mucosa, cannot be spit out, and dissolve within minutes. However, in this trial, it was noted that buccal absorption occurs within 2 minutes and therefore might circumvent first-pass metabolism. Midazolam is almost exclusively metabolized by CYP3A; due to first-pass metabolism, the absolute bioavailability is low (20%-25%), there is no involvement of drug transporters, and it has a short

Department of Clinical Pharmacology and Pharmacoepidemiology, University of Heidelberg, Heidelberg, Germany

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Corresponding Author:
Gerd Mikus, MD, MSc, Department Clinical Pharmacology and Pharmacoepidemiology, University Hospital Heidelberg, Im Neuenheimer Feld 410, 69120 Heidelberg, Germany
Email: gerd.mikus@med.uni-heidelberg.de
half-life (3-4 hours).\textsuperscript{3,12,13} To have a reliable and reproducible phenotyping method with buccal microdosed midazolam, it is important to study the influence of midazolam exposure time in the oral cavity on first-pass metabolism of midazolam. The aim of the study was to assess the absolute bioavailability of microdosed midazolam after buccal administration in relation to the buccal exposure time and compare it with oral administration.

**Methods**

**Clinical Trial**

This phase 1, single-center, open-label clinical trial (EudraCT number: 2018-001409-10) was approved by the responsible Ethics Committee of the Medical Faculty of Heidelberg University and the competent authority (Federal Institute for Drugs and Medical Devices) responsible for Germany. The study was carried out in accordance with the standards of Good Clinical Practice and the specific legal requirements of Germany, the applicable version of the Declaration of Helsinki, and the International Council of Harmonization recommendations on good clinical practice. The trial was conducted at the Clinical Research Center (KliPS) of the Department of Clinical Pharmacology and Pharmacoepidemiology, Heidelberg University Hospital, which is certified according to DIN EN ISO 9001:2015. All participants gave written informed consent before any trial-specific activities.

**Study Population**

Healthy nonsmoking volunteers (men and women) in the age range 18 to 60 years were recruited. They underwent a medical history assessment, physical examination, electrocardiogram, and routine laboratory analyses to evaluate their mental and physical health.

**Study Design and Conduct**

We evaluated the impact of the buccal exposure time to midazolam on the absolute bioavailability of microdosed midazolam. The study was divided into 2 parts. Beginning with a pilot phase of 4 exposure days in which 2 volunteers received 10 \( \mu \)g of midazolam intravenously to calculate absolute bioavailability, a 30-second and 60-second buccal exposure with the same dose, and an oral reference (10 \( \mu \)g of drinking solution = 0 seconds) to plan the buccal exposure times for the main study. Using the data obtained in the pilot phase, 5 different buccal exposure times plus an intravenous and oral reference were chosen for the main study, resulting in a total of 7 treatment days to be conducted in 10 volunteers. Study days were separated by a washout period of at least 48 hours. For the duration of the entire clinical trial, preparations containing St. John’s wort (hypericin, hyperforin), alcohol, and any citrus fruit products including grapefruit juice were not allowed.

Each participant received the following treatment sequence: 10 \( \mu \)g of midazolam intravenous reference, 5 treatments of 10 \( \mu \)g of midazolam buccal exposure with ascending exposure times, and a last treatment with the oral 10-\( \mu \)g reference drinking solution. The participants were instructed not to swallow during the exposure times. Thereafter, they rinsed their mouth with 50 mL of water and swallowed all rinsing fluid.

We administered 10 \( \mu \)g (0.01 mL of midazolam as Doricum V) 5 mg/5 mL, Roche Pharma AG [Grenzach-Wyhlen, Germany]) directly on the buccal mucosa between gum and cheeks with a calibrated Eppendorf pipette. For the intravenous bolus administration, 1 mL of midazolam 5 mg/5 mL was diluted with 199 mL of 0.9% sodium chloride solution, and 2 mL of the final solution (0.005 mg/mL) was injected. The drinking solution as oral reference consisted of 100 mL of tap water with 0.01 mL of midazolam 5 mg/5 mL. On treatment days, participants were instructed to fast 10 hours before and 4 hours after a single microdosed midazolam dose.

Blood was obtained through an indwelling catheter placed at the forearm of the participants using Li-Hep tubes (S-Monovette 2.7 mL LH-Gel; Sarstedt AG & Co., Nümbrecht, Germany) before drug administration and at 2, 5, 10, 15, 20, 30, and 45 minutes and 1, 1.5, 2, 2.5, 3, 4, 6, 8, and 10 hours thereafter. The opposite arm was used to administer the intravenous bolus of midazolam. Volunteers were instructed to completely collect 24-hour urine after midazolam dosing. Total urine volume was documented and an 8.5-mL sample was kept (Urin-Monovette 8.5 mL; Sarstedt AG & Co.). Blood samples were centrifuged at 4°C at 2300 \( \times \) g for 10 minutes, and separated plasma and urine samples were stored at –20°C until analysis.

**Analytical Quantification**

For the quantification of midazolam and 1-OH-midazolam concentrations in plasma and urine samples in the analytical chemistry lab of the department, an established ultra-performance liquid chromatography–tandem mass spectrometry method was used.\textsuperscript{3} In brief, the plasma samples were processed by solid-phase extraction and the extracts subsequently analyzed by gradient reversed-phase chromatography and positive electrospray triple quadrupole mass spectrometry in the multiple reaction monitoring mode. The urine samples were hydrolyzed by glucuronidase in acetate buffer pH 5.0 (100 units per sample) at 37°C for 24 hours. Hydrolyzed urine samples (50-\( \mu \)L aliquots) were diluted with blank plasma (200 \( \mu \)L each). These samples were subsequently handled identically as common plasma samples and further
processed by solid-phase extraction and analysis by ultra-performance liquid chromatography–tandem mass spectrometry. The assays fulfilled the pertinent guidelines on bioanalytical method validation of the US Food and Drug Administration\textsuperscript{14} and the European Medicines Agency\textsuperscript{15} with accuracy and precision values of ±15% or less. The lower limits of quantification were 0.370 pg/mL for midazolam and 0.253 pg/mL for 1-OH-midazolam in both matrices.

Pharmacokinetics and Statistics

Standard pharmacokinetic parameters (maximum plasma concentration, time to reach the maximum plasma concentration–time curve from time 0 to infinity [AUC\textsubscript{0-∞}], total clearance after intravenous administration, apparent oral clearance [Cl/F], volume of distribution, and terminal elimination half-life) were calculated using a noncompartmental analysis within Kinetica 5.0 (Thermo Fisher Scientific, Waltham, Massachusetts). Bioavailability was determined by dividing AUC\textsubscript{0-∞} after oral or buccal administration by AUC\textsubscript{0-∞} after intravenous administration. Using the urine concentration of 1-OH-midazolam after deconjugation, the total amount excreted as metabolite was calculated.

Log-transformed maximum plasma concentration, AUC\textsubscript{0-∞}, Cl/F, and bioavailability of midazolam were analyzed with repeated-measures analysis of variance and Dunnett’s post hoc analysis for the oral reference using Prism 8.4.2 (GraphPad, La Jolla, California). All values are presented as geometric mean with 95% confidence intervals. A \( P \) value of <.05 was considered statistically significant. Time dependency of buccal bioavailability was analyzed using a 4-parameter logistic equation (Hill equation)\textsuperscript{16} with \( F_{\text{min}} \) and \( F_{\text{max}} \) being the bioavailability plateaus at the low (drinking solution) and high ends of the curve, \( ET_{50} \) the exposure time yielding a half-maximal bioavailability increase, and \( S \) the unitless Hill slope factor:

\[
F = F_{\text{min}} + \frac{t^S \times (F_{\text{max}} - F_{\text{min}})}{(t^S + ET_{50}^S)}
\]

This equation was chosen as a universal and versatile equation describing physiological processes.

Safety Assessments

Participants were asked each study day about adverse events (AEs) before midazolam administration and at the end of the day. All AEs were coded using the Medical Dictionary for Regulatory Activities version 20.0 and were evaluated based on the clinical judgment of the same 2 investigators. The AE intensity was categorized according to US National Cancer Institute’s Common Terminology Criteria for Adverse Events version 4.03. The causal relationship of an AE to the study medication was classified as definitive, probable, possible, and not related.

Results

Clinical Trial

Twelve nonsmoking healthy volunteers with a median age of 25.2 years (range, 20-29 years) and a median body mass index at screening of 23.3 kg/m\(^2\) (range, 18.7-27.9 kg/m\(^2\)) completed all 7 study parts (4 study parts in the pilot phase). Their demographic data are shown in Table 1.

Pilot Phase.

Pharmacokinetics of midazolam were obtained for the 2 participants (1 woman) in the pilot phase with 10 \( \mu \text{g} \) of midazolam as a bolus, as a drinking solution, and as buccal administration for 30 seconds and 60 seconds (Table 2). In both participants, absolute bioavailability increased more than 2-fold with longer exposure times (Figure 1). With these data, the following exposure times for the main study were defined: 0 (oral drinking solution), 15, 30, 70, 100, and 150 seconds.

Main Study.

Full pharmacokinetic profiles were obtained for each of the 10 participants (6 women) after 10 \( \mu \text{g} \) of midazolam administered on every occasion, intravenously, orally as drinking solution, and after 5 different buccal exposure times (Figure 2). Midazolam AUC\textsubscript{0-∞} after administration of the oral reference (drinking solution) was much lower than after intravenous bolus administration of the same dose (98.7 vs 355 pg \( \cdot \) h/mL) resulting in an absolute oral bioavailability of 27.8\% (Table 3). Compared to the drinking solution, the AUC\textsubscript{0-∞} after buccal administration increased significantly depending on the duration of the buccal exposure time of midazolam but not reaching AUC\textsubscript{0-∞} after intravenous bolus (Table 3). Consequently, the absolute bioavailability increased from 27.8\% after the drinking solution to 66.1\% after 100-second buccal exposure time, a more than 2-fold increase. The apparent oral clearance of midazolam decreased from 1689 mL/min after the drinking solution to 710 mL/min after 100 s buccal exposure time. A longer exposure time (150 seconds) did not further decrease Cl/F, and bioavailability was not further increased. Midazolam exposure time did not influence the amount excreted as 1-OH-midazolam and corresponding glucuronide into urine (Table 3). The time dependency of the midazolam bioavailability followed a sigmoidal Hill equation with minimum bioavailability being fixed to the bioavailability calculated for the drinking solution. The maximum bioavailability calculated by the model was 64.5\% (95% confidence interval, 60.0%-89.6\%), the estimated time
Table 1. Demographic Characteristics of the 12 Study Participants

| Subject Number | Sex   | Age (y) | Height (cm) | Weight (kg) | BMI (kg/m²) | Ethnic Group |
|----------------|-------|---------|-------------|-------------|-------------|--------------|
| Pilot phase    |       |         |             |             |             |              |
| 1              | Male  | 25      | 163         | 49.8        | 18.7        | White        |
| 2              | Female| 25      | 175         | 85.5        | 27.9        | Arabian      |
| Main study     |       |         |             |             |             |              |
| 3              | Male  | 23      | 172         | 73.9        | 24.9        | White        |
| 4              | Female| 26      | 171         | 59.9        | 20.5        | White        |
| 5              | Male  | 25      | 189         | 98.2        | 27.5        | White        |
| 6              | Female| 25      | 171         | 72.1        | 24.6        | White        |
| 7              | Male  | 23      | 182         | 82.4        | 24.9        | White        |
| 8              | Male  | 21      | 182         | 84.5        | 25.5        | White        |
| 9              | Female| 29      | 160         | 54.5        | 21.2        | White        |
| 10             | Female| 27      | 170         | 57.5        | 19.9        | White        |
| 11             | Female| 27      | 179         | 69.7        | 21.7        | White        |
| 12             | Female| 29      | 174         | 68.8        | 22.7        | White        |
| Mean           |       | 25.16   | 174         | 71.4        | 23.34       | 91.7% White  |
| SD             |       | 2.86    | 7.82        | 13.9        | 2.86        | 8.3% Arabian  |

BMI, body mass index; SD, standard deviation.

Table 2. Individual Pharmacokinetic Data of the Pilot Phase (2 Participants) of the Study After Intravenous, Buccal (30 and 60 s), and Oral (0 s) Administration of 10 μg Midazolam on Each Occasion

| Parameter                  | Pilot Participant 1 | Pilot Participant 2 |
|----------------------------|---------------------|---------------------|
|                           | IV 0-s PO 30-s BA   | 60-s BA             |
|                           | 1000 89.4 158 217   | 691 60.2 156 157    |
| Cmax, pg/mL                | 1.8 45 45 19.8      | 1.8 45 30 45        |
| tmax, min                  | 862 226 469 531     | 719 212 503 462     |
| AUC0-∞, pg * h/mL          | 191 146 168 143     | 194 194 209 192     |
| t1/2, min                  | 193 739 355 314     | 232 786 331 361     |
| Cl or Cl/F, mL/min         | 39.5 144 74.4 56.9  | 55.4 203 86.1 92.8  |
| Vd, L                      | (100) 26.2 54.4 61.6| (100) 29.5 69.9 64.3|

AUC0-∞, area under the plasma concentration–time curve from 0 to infinity; BA, buccal administration; Cl, total clearance after intravenous administration; Cl/F, apparent oral clearance after buccal or oral administration; Cmax, maximum plasma concentration; F, absolute bioavailability; IV, intravenous injection; PO, peroral drinking solution; tmax, time to reach the maximum plasma concentration; t1/2, terminal elimination half-life; Vd, volume of distribution.

to reach half maximum bioavailability was short at 16 seconds (95% confidence interval, 10-50 seconds). The Hill slope was determined to be 2.87 (Figure 3).

Safety. All volunteers finished the study. A total of 5 AEs occurred (Table 4); all were mild, none serious, and all were transient without treatment. Three AEs were not related, 2 AEs (mild nausea after intravenous administration) were possibly related to the study drug.

Discussion

Because regulatory agencies require drug-drug interaction studies covering the major drug-metabolizing enzymes, midazolam is often used during clinical drug development. In recent years, microdosed midazolam has become increasingly popular because of the absence of pharmacological effects with this low dose.8,17–19 A major obstacle is that no microdosed oral formulation is commercially available and therefore intravenous solutions are diluted to obtain the required dose, which is then orally administered as a drinking solution. Therefore, this technique is restricted to dedicated trial centers and is not a widely used methodology. To overcome this, an experimental buccal film containing 30 μg of midazolam was developed and tested.11 After 2 minutes in the oral cavity, midazolam plasma concentrations were about 3-fold higher compared to an oral drinking solution of 30 μg of midazolam5,20 suggesting buccal absorption and thus reduced first-pass metabolism. To have a reliable measure of CYP3A activity with a microdosed buccal midazolam administration, we investigated the time dependency of the midazolam buccal bioavailability. This is especially important when CYP3A activity is to be assessed on several occasions in the same study.
Figure 1. Individual phase plasma concentration-time profile of midazolam in the pilot phase for participant 1 (left) and participant 2 (right) after intravenous, 0-second oral (drinking solution) administration, 30-second buccal exposure time, and 60-second exposure time of a single dose of 10-μg midazolam each.

Table 3. Geometric Mean (95% Confidence Interval) of the Pharmacokinetic Parameters of Midazolam After Intravenous, Oral, and Buccal Administration of 10-μg Midazolam on Each Occasion

| Parameter | IV | 0-s PO | 15-s BA | 30-s BA | 70-s BA | 100-s BA | 150-s BA |
|-----------|----|--------|---------|---------|---------|---------|---------|
| $C_{\text{max}}, \text{pg/mL}$ | 329 (223-486) | 48.3 (40.1-58.2) | 59.8 (48.7-74.8) | 78.0$^*$ (58.7-104) | 76.6$^*$ (53.5-110) | 95.2$^*$ (79.5-114) | 94.1$^*$ (75.5-117) |
| $t_{\text{max}}, \text{min}$ | 2.94 (1.8-4.8) | 31.2 (21.1-46.3) | 37.4 (25.8-54.2) | 35.5 (24.1-52.2) | 45.2 (31.2-65.6) | 32.9 (25.8-42.0) | 30.0 (22.2-40.6) |
| AUC$_{0-\infty}, \text{pg} \cdot \text{h/mL}$ | 355 (293-430) | 98.7 (76.9-127) | 155$^*$ (123-194) | 209$^*$ (176-249) | 219$^*$ (173-277) | 235$^*$ (193-285) | 234$^*$ (178-307) |
| $t_{1/2}, \text{min}$ | 149 (135-164) | 148 (128-171) | 136 (123-152) | 145 (129-163) | 144 (128-162) | 141 (128-162) | 141 (128-162) |
| Cl or Cl/F, mL/min | 469 (387-568) | 1689 (1316-2168) | 1077$^*$ (858-1351) | 798$^*$ (670-951) | 761$^*$ (601-964) | 710$^*$ (584-863) | 712$^*$ (543-936) |
| $V_d, \text{L}$ | 74.5 (62.9-88.3) | 296 (236-373) | 186 (150-231) | 140 (126-156) | 133 (109-162) | 133 (103-144) | 122 (96.7-152) |
| F, % | 75.6 (62.9-88.3) | 66.4 (23.5-32.9) | 73.0 (37.5-50.6) | 76.9 (52.2-66.2) | 76.2 (52.3-72.5) | 77.0 (60.0-72.8) | 73.5 (52.9-82.1) |

Ae, amount excreted in urine as 1-OH-midazolam after deconjugation; AUC$_{0-\infty}$, area under the plasma concentration-time curve from 0 to infinity; BA, buccal administration; Cl, total clearance after intravenous administration; Cl/F, apparent oral clearance after buccal or oral administration; $C_{\text{max}}$, maximum plasma concentration; F, absolute bioavailability; IV, intravenous injection; PO, peroral drinking solution; $t_{\text{max}}$, time to reach the maximum plasma concentration; $t_{1/2}$, terminal elimination half-life; $V_d$, volume of distribution.

$^*$P < .05 repeated measures analysis of variance and Dunnett's post hoc analysis vs oral drinking solution (0-s PO).

participant as in crossover drug-drug interaction trials. It is important to define the conditions of buccal administration; otherwise, varying buccal exposure times and hence midazolam absorption changes will affect the precision of the CYP3A estimate.

With increasing exposure time, bioavailability increased very quickly, reaching its maximum after 100-second buccal exposure time, with the magnitude of bioavailability increase reaching 2.4-fold. This is comparable to an earlier study where 5 mg of buccal and intravenous midazolam was investigated, and a bioavailability of 74.5% resulted. The buccal cavity containing the mucosa of the buccal, sublingual, gingival, palatal, and labial areas (about 200 cm$^2$) is active.
Table 4. Adverse Events in the Pilot Phase \((n = 2)\) and the Main Study \((n = 10)\) of the Clinical Phase 1 Trial With 10-\(\mu\)g Midazolam Each Intravenous, Buccal, and Oral in Healthy Volunteers

| Subject Number | IV   | 0-s PO | 15-s Buccal | 30-s Buccal | 60-s Buccal | 70-s Buccal | 100-s Buccal | 150-s Buccal |
|----------------|------|--------|-------------|-------------|-------------|-------------|--------------|--------------|
| Pilot phase 1  | I mild nausea\(^a\) | 0 | ND | 0 | 0 | ND | ND | ND | ND |
| Pilot phase 2  | I mild nausea\(^a\) | 0 | 0 | ND | 0 | 0 | ND | ND | ND |
| Main study 12  | 0 | 0 | I (common cold, headache, sore throat) | ND | I (common cold, headache, sore throat) | ND | I (common cold, with some sore throat, mild headache) | 0 |

Overall: 2 0 0 0 0 0 0 0

Mild: 2 0 0 1 1 0 1 0

\(^a\)Possibly related to study drug.

AE, adverse event; ND, study day was not done in pilot phase.

All adverse events were of mild intensity (…), not related to study drug.

Extensively vascularized. Blood flow through the buccal mucosa is generally not considered to be rate limiting for drug absorption, but only limited pharmacokinetic data are available.\(^{22}\) These very similar results after doses of 10 \(\mu\)g and 5 mg suggest that buccal absorption of midazolam is not dose limited in the range studied.

Any buccally absorbed compound with direct access to the systemic circulation bypasses first-pass degradation of the gut wall and liver.\(^{23}\) To determine the total CYP3A activity in these organs, oral administration of a midazolam drinking solution should be favored, which results in low bioavailability between 20% and 25%.\(^{3}\) CYP3A activity of the liver can be assessed by midazolam intravenous bolus administration\(^1\) since only 1% of the dose is excreted unchanged by the kidneys. If midazolam is administered as a buccal film, first-pass elimination of midazolam is reduced depending on the time of buccal exposure,\(^{11}\) with a minimal first-pass metabolism within minutes, indicating that the clearance obtained after buccal administration does not reflect combined gut wall and liver CYP3A activity. It might be possible to use buccal administration to assess CYP3A activity of the liver only. Using the 100-second and the intravenous clearance data set for log-log nonlinear regression analysis, a slope of 0.9043 resulted with adjusted \(r^2\) of 0.7371 with 10 observations. This might only be carefully interpreted, but it remains to be proven by a larger study where midazolam clearance after buccal administration is compared to the clearance after intravenous bolus administration.

Microdose studies using midazolam are an easy and reliable way to address these important clinical pharmacology questions. Midazolam pharmacokinetics have been shown to be dose proportional over a 30 000-fold dose range,\(^{20}\) and bioavailability and hepatic and gut extraction ratios are not dose dependent.\(^1\) After midazolam doses below 100 \(\mu\)g, no pharmacological effects have been observed.\(^{3,20}\) In contrast, when buccal administration was investigated using 5 mg, all volunteers fell...
asleep within 15 minutes or felt sedated within 3 minutes when 5 mg were administered intravenously.\textsuperscript{21} In 1 volunteer, sedation continued for 240 minutes following buccal and intravenous midazolam administration,\textsuperscript{21} stressing the importance of advising participants in regular dose trials not to drive or operate potentially hazardous machinery. Moreover, the addiction potential of midazolam restricts multiple therapeutic drug administrations, which can be neglected using microdoses. Therefore, microdosing is preferred, especially when pharmacokinetics have to be studied on multiple occasions.

To describe the time-dependency of the bioavailability increase, the 4-parameter logistic equation (Hill equation; maximal efficacy \([E_{\text{max}}]\) model) was applied successfully. The \(E_{\text{max}}\) model is the most fundamental description of the concentration effect relationship with strong theoretical support from the physicochemical principles for binding of drugs to receptors.\textsuperscript{24} Assuming that bioavailability increases over exposure time in the oral cavity as a result of absorption and thereby avoiding first-pass metabolism made us choose this model. Absorption from the oral cavity is very quick, and most of the dose is absorbed within 100 seconds; thereafter, bioavailability shows saturation. Following the timelines during the study conduct is therefore highly critical for the quality of the data obtained, and emphasis must be put on these circumstances when the protocol is being developed.

For the further development of buccal administration of midazolam, the goal of this application must be defined. First, if buccally administered midazolam will be used therapeutically using doses in the low milligram range, one needs to be aware that bioavailability is highest if patients keep the formulation in the oral cavity 1 minute or longer without swallowing. Any swallowing before will increase first-pass metabolism and consequently reduce resulting concentrations and pharmacological effects.\textsuperscript{21} Second, if gut and liver CYP3A activity is to be evaluated, buccal administration is not suitable, and an oral drinking solution should be used instead, with least contact time to the oral mucosa. This can be done using a microdose since pharmacological effects are not desired. Third, if only liver CYP3A activity is to be quantified, a midazolam microdose might be administered via the buccal route, with 2 minutes in the oral cavity without swallowing, but this is subject to further investigations.

Limitations
We cannot exclude that bioavailability could even be larger than observed due to some saliva that might run down the throat during the 2.5 minutes where swallowing was not allowed. However, due to the very small amount (10 \(\mu\)g = 10 \(\mu\)L) and the good absorption properties of the buccal mucosa, the risk of dilution by saliva or swallowing is very low. Conversely, we do not know whether larger fluid amounts immediately wetting the whole buccal mucosa would further increase bioavailability. To ensure the compliance of the study participants, the dosing procedure was explained on each study day and checked by means of self-assessment as well as by trained personnel. Even if care was taken not to open the lower lip or mouth until just before the buccal exposure, a slightly dry mucous membrane may have reduced the absorption.

**Conclusions**
Our study in healthy participants revealed that the bioavailability of microdosed midazolam after buccal administration is highly dependent on the buccal exposure time, with bioavailability increasing 2.4-fold with 100-second buccal exposure. This needs to be taken into account when the buccal route is used when treating acute seizures but also when a reliable CYP3A phenotyping should be carried out.

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**Conflicts of Interest**
The authors declare no conflicts of interest.

**Data Sharing**
The midazolam data can be requested from the corresponding author (gerd.mikus@med.uni-heidelberg.de).

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