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Short communication

# Determination of midazolam in human plasma by liquid chromatography with mass-spectrometric detection

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**Abstract**

A liquid chromatographic assay with mass-spectrometric detection was developed for the quantitative determination of the cytochrome P450 3A phenotyping probe midazolam in human plasma. Sample pretreatment involved a one-step extraction of 600 μl aliquots with ethyl acetate. Midazolam and the internal standard, lorazepam, were separated on a column (150 mm × 4.6 mm, i.d.) packed with 5 μm Zorbax Eclipse XDB-C8 material, using a mobile phase composed of methanol and 10 mM aqueous ammonium acetate (60:40, v/v). Column effluents were analyzed using mass-spectrometry with an atmospheric pressure chemical ionization source. Calibration curves were linear in the concentration range of 1.00–200 ng/ml. The accuracy and precision ranged from 92.8 to 112% and 0.056 to 13.4%, respectively, for four different concentrations of quality control samples analyzed in triplicate on eight separate occasions. The developed method was subsequently applied to study the pharmacokinetics of midazolam in a group of 35 human subjects at a single dose of 25 μg/kg.


*Keyword:* Midazolam

## 1. Introduction

Midazolam is an imidiazobenzodiazepine that is used clinically for sedative purposes prior to minor medical procedures and surgery [1]. Midazolam is almost completely metabolized to 1′-hydroxy-midazolam and, to a lesser extent, to 4′-hydroxy-midazolam via two enzymes in the cytochrome P450 (CYP) CYP3A subfamily, CYP3A4 and CYP3A5 [2]. Currently, most medications are dosed based upon the assumption that each individual in the general population metabolizes drugs at approximately the same rate. However, it has been shown that hepatic CYP3A microsomal activity may vary as much as 40-fold in vitro, and five-fold differences have been observed in vivo [3]. Such a wide range of metabolic activity present in the population may lead to unwanted therapeutic outcomes such as sub-therapeutic or toxic drug levels. Due to this wide variability in CYP3A expression, the need for the use of an in vivo probe such as midazolam to predict the CYP3A metabolic phenotype of patients treated with narrow therapeutic window agents is evident [3,4]. For the purpose of CYP3A phenotyping, doses of midazolam administered to humans are typically very low, which poses a significant challenge on analytical methods. In contrast to gas chromatographic methods with electron capture or mass spectrometric detection [5–9], most of the currently available validated liquid chromatographic methods used to determine midazolam in human plasma are hampered by a lack in sensitivity to allow for accurate estimation of complete pharmacokinetic profiles, necessitate the use of large sample volumes for extraction (≥1 ml) [10,11], and/or require triple–quadrupole mass spectrometry [12], which is not currently available in most laboratories (Table 1). Here, we describe a novel, sensitive analytical method for the determination of midazolam concentrations in human plasma based on liquid chromatography coupled with single-quadrupole mass-spectrometric detection.

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Table 1
Liquid chromatographic methods for the analysis of midazolam in human plasma

| Sample pretreatment | Internal standard | Detection (nm) | LLOQ (ng/ml) | Reference |
|---|---|---|---|---|
| LLE (cyclohexane) | Flurazepam | 200 | 10 | Portier et al. [16] |
| LLE (diethyl ether) | Diazepam | 254 | 7 | Carrillo et al. [17] |
| LLE (diethyl ether) | Flurazepam | 215 | 10 | Blackett et al. [18] |
| LLE (diethyl ether) | Prazepam | APCI-MS | 6.5 | Shiran et al. [15] |
| LLE (diethyl ether) | Flurazepam | 220/254 | 15 | Vasiliades et al. [19] |
| LLE (diethyl ether) | None | 215 | 30 | Vree et al. [20] |
| LLE (diethyl ether) | Ro 05-6669 | 245 | 5 | Ha et al. [21] |
| LLE (diethyl ether–cyclohexane) | Diazepam | 240/300 | 1 | Eeckhoudt et al. [11] |
| LLE (diethyl ether–methylene chloride) | None | 254 | 50 | Puglisi et al. [22] |
| LLE (diethyl ether–methylene chloride) | Climazolam | 220 | 30 | Vletter et al. [23] |
| LLE (diethyl ether–methylene chloride) | Flurazepam | 220 | 30 | Chan et al. [24] |
| LLE (diethyl ether–2-propanol) | Methylclonazepam | ESI-MS | 0.5 | Marquet et al. [10] |
| LLE (heptane–isoamylalcohol) | Desmethylclomipramine | 250 | 23.4 | ter Horst et al. [25] |
| LLE (methylene chloride) | Clonazepam | 232 | 25 | Hayball et al. [26] |
| LLE (methylene chloride) | Medazepam | 240 | 7.5 | Odou et al. [27] |
| LLE (*n*-butyl chloride) | Methylclonazepam | 220 | 10 | Manjoub et al. [28] |
| LLE (isopropanol–methylene chloride) | Climazolam | 220 | 12.5 | Lee et al. [29] |
| LLE (*t*-butylmethyl ether) | Diazepam | 240 | 7 | Johnson et al. [30] |
| LLE (toluene) | Flurazepam | 207 | 100 | Van Brandt et al. [31] |
| SPE (C18) | Clonazepam | 254 | 15 | Mastey et al. [32] |
| SPE (C18) | Climazolam | 254 | 50 | Sautou et al. [33] |
| SPE (C18) | Alprazolam | Nano ESI-MS–MS | 1.5 | Kapron et al. [34] |
| SPE (C18) | Alprazolam | ESI-MS–MS | 0.25 | Kashuba et al. [12] |
| Column switching | Flurazepam | 230 | 10 | Lauber et al. [35] |

*Abbreviations:* LLOQ, lower limit of quantitation; LLE, liquid–liquid extraction; SPE, solid phase extraction; APCI, atmospheric pressure chemical ionization; MS, mass spectrometry; ESI, electrospray ionization.

## 2. Experimental

### 2.1. Chemicals

Midazolam (lot # 081-122-1; purity, 99.5%) was obtained from UFC Ltd. (Manchester, United Kingdom). The internal standard lorazepam originated from Sigma (St. Louis, MO, USA). All other chemicals and solvents were of analytical grade or better. Deionized water was generated with a Hydro-Reverse Osmosis system (Durham, NC, USA) connected to a Milli-Q UV Plus purifying system (Malbourough, MA, USA). Drug-free heparinized human plasma was obtained from the National Institutes of Health Clinical Center Blood Bank (Bethesda, MD, USA).

### 2.2. Preparation of stock solutions and standards

Stock solutions of midazolam were prepared independently in triplicate by dissolving the appropriate amount of drug, corrected for impurity, in HPLC-grade methanol at a concentration of 0.500 mg/ml, and were then stored in glass at −20 °C for up to 4 weeks. The difference in drug concentration in each of the triplicate stock solutions, estimated from the mean peak area following repeat analysis of a dilution of the stock, was determined to be within 5%. Out of one of the midazolam stock solutions, a working solution containing 100 μg/ml was prepared in methanol, which was further used for the construction of calibration samples and quality control (QC) samples. An internal standard working solution of 4 μg/ml was prepared by dilution with HPLC-grade methanol, and was stored for later use at −20 °C.

### 2.3. Sample pretreatment

Samples were prepared by spiking 600 μl blank human plasma in 15 ml polypropylene tubes with midazolam at the indicated concentrations (below), and lorazepam at a final concentration of 100 ng/ml (25 μl of the methanolic working solution). Blank samples and samples containing only internal standard were corrected for methanol content, so that all samples had a final methanol content of 7.7%. After adding 600 μl of 0.1 M ammonium acetate, the samples were vortex-mixed and 4 ml of water-saturated ethyl acetate was added to each tube. The samples were vortex mixed for 1 min and then centrifuged at 2000 × *g* for 5 min (4 °C). The organic phase was transferred to a clean glass tube using a disposable plastic pipette, and evaporated to dryness under a continuous stream of air at 40 °C. The extracts were reconstituted with 125 μl of a mixture of methanol and 0.01 M ammonium acetate (60:40, v/v), and 75 μl were injected for chromatographic analysis.

### 2.4. Equipment

Chromatography was performed on a HP1100 system (Agilent Technology, Palo Alto, CA, USA), which

included a binary pump, a refrigerated autosampler, a degasser, a photodiode-array detector, and a single-quadrupole mass-spectrometric (MS) detector (Agilent 1100 MSD) equipped with an atmospheric pressure chemical ionization source. The autosampler was maintained at 4 °C and the column was at ambient temperature. The analytes were separated on a stainless steel column (150 mm × 4.6 mm, i.d.) packed with 5 μm particle size Zorbax Eclipse XDB-C8 material (Agilent), preceded by a Zorbax phenyl (4.6 mm × 12.5 mm, i.d., 5 μm particle size) guard column (Agilent). Samples were eluted isocratically using a mobile phase composed of methanol − 0.01 M ammonium acetate (60:40, v/v) at a flow rate of 1.0 ml/min. The MS conditions were as follows: fragmentor, 90 V; gain, 1; drying gas flow, 5 l/min; nebulizing gas pressure, 55 psi; drying gas temperature, 330 °C; and capillary voltage, 2200 V. Selected-ion monitoring was accomplished at *m/z* 326 for midazolam, and *m/z* 322 for the internal standard.

The chromatographic data were collected and analyzed using the software package Chemstation (Agilent). Calibration graphs were calculated by least-squares linear regression analysis of the peak area ratio of midazolam and the internal standard versus the drug concentration of the nominal standard. The zero concentration sample (blank) was used to visually verify the purity of the reagents and the lack of other potentially interfering (endogenous) substances, but was not considered for the regression analysis of standards. The goodness-of-fit of various calibration models was evaluated by visual inspection, the correlation coefficient and an ANOVA lack-of-fit test.

### 2.5. Validation procedures

Method validation with respect to accuracy and precision was performed according to procedures described in detail elsewhere [13]. Calibration standards in drug-free human heparinized plasma were prepared freshly by serial dilution at midazolam concentrations of 1.00, 2.00, 5.00, 10.0, 30.0, 100, and 200 ng/ml, such that the total amount of methanol added was identical in each sample. Pools of quality control (QC) samples of midazolam in plasma were prepared similarly at concentrations of 1.00, 3.00, 85.0, and 170 ng/ml, and stored in batch at −20 °C for the duration of the validation procedure. Validation runs included a calibration curve and QC samples analyzed in triplicate and were performed on eight separate occasions.

The lower limit of quantitation (LLOQ) of the assay was assessed by determining the concentration of midazolam at which the values for precision and accuracy were less than 20%, excluding outliers.

The accuracy and precision of the assay were assessed by the mean relative percentage deviation (DEV) from the nominal concentrations and the within-run and between-run precision, respectively. The accuracy for each tested concentration was calculated as:

$$\mathrm{DEV} = 100 \times \left\{ \frac{([\mathrm{analyte}]_{\mathrm{mean}} - [\mathrm{analyte}]_{\mathrm{nominal}})}{[\mathrm{analyte}]_{\mathrm{nominal}}} \right\}$$

Estimates of the between-run precision were obtained by one-way analysis of variance (ANOVA) using the run day as the classification variable. The between-groups mean square ($MS_{bet}$), the within-groups mean square ($MS_{wit}$), and the grand mean (GM) of the observed concentrations across runs were calculated using the software package NCSS 2001 (J. Hintze, Number Cruncher Statistical Systems, Kaysville, UT, USA). The between-run precision (BRP), expressed as a percentage relative standard deviation, was defined as:

$$\mathrm{BRP} = 100 \times \left( \frac{\sqrt{((\mathrm{MS}_{\mathrm{bet}} - \mathrm{MS}_{\mathrm{wit}})/n)}}{\mathrm{GM}} \right)$$

where $n$ represents the number of replicate observations within each run. For each concentration, the estimate of the within-run precision (WRP) was calculated as:

$$\mathrm{WRP} = 100 \times \left( \frac{\sqrt{(\mathrm{MS}_{\mathrm{wit}})}}{\mathrm{GM}} \right)$$

The extraction efficiency of the assay, expressed as a percentage, was measured by comparison of extracted plasma samples and aqueous samples injected without extraction in replicates of five at midazolam concentrations of 1.00, 3.00, 85.0, and 170 ng/ml.

Possible matrix effects were investigated by infusing a 15 μM solution of midazolam into the MS, post column, using a syringe pump via a tee. After a constant response was established, five blank plasma samples that had been extracted and reconstituted, as detailed above, were injected. A slight matrix effect was noted at a retention time of 2 min. No further matrix effects were noted, showing that there is no interference with midazolam and lorezepam, both of which have significantly longer retention times.

### 2.6. Pharmacokinetic analysis

Midazolam (Roche Laboratories, Nutley, NJ, USA) was injected intravenously over a 30–60 s period at a dose of 25 μg/kg to 35 human subjects. Blood samples (7 ml each) were collected prior to drug administration, and at 5 and 30 min, and 1, 2, 4, 5, and 6 h post-infusion. The samples were centrifuged at 2000 × *g* for 10 min (4 °C) immediately after collection, and were stored at −20 °C at the day of collection and at −80 °C afterwards, until the day of analysis. The current experiment was approved by the Institutional Review Board, and the patients signed informed consent before study entry for the blood sampling procedure. Plasma concentration–time data of midazolam were analyzed by a non-compartmental model using the software package WinNonlin v4.0 (Pharsight Corporation, Mountain View, CA, USA).

## 3. Results and discussion

### 3.1. Specificity

Fig. 1 displays typical chromatograms of an extract of a blank human plasma sample (A), an extract of a plasma sample spiked with midazolam at a concentration of 1 ng/ml (B), and an extract of a plasma sample taken at 5 h after the administration of midazolam (dose, 25 μg/kg) (C). The chromatographic peaks showed symmetrical resolution, with no interfering peaks present for all compounds in drug-free specimens, obtained from six individuals. Midazolam ($t_R = 11.36$ min) and the internal standard lorazepam ($t_R = 6.79$ min) were well separated, and the overall chromatographic run time was established at 15 min. The selectivity and resolution of the chromatographic system was also confirmed by co-injection of midazolam with one of its major metabolites, 1′-hydroxy-midazolam.

### 3.2. Validation characteristics

The measurement variance over the range of 1.00–200 ng/ml increased proportionally with the midazolam concentration, as detected by a one-sided *F*-test at an $\alpha$-value of 5%. Therefore, a weighting factor was applied inversely proportional to the variance at the given concentration level (1/[nominal midazolam concentration]$^2$). Using least-squares linear-regression, a mean (±standard deviation) correlation coefficient of 0.9974 ± 0.00176 (range, 0.9946–0.9988) was obtained (Table 2). The statistical evaluation of the coefficients of the mean ordinary least-squares line indicated an acceptable degree of bias in the slope (coefficient of variation, 8.20%) and in the intercept (coefficient of variation, 40.7%), suggesting minor matrix and blank effects [14]. In blank human plasma spiked with midazolam at a concentration of 1.00 ng/ml, only 2 out of 24 samples were outside the acceptable ±20% deviation limits for accuracy [13], while the remaining samples had a mean percentage deviation from the nominal concentration and within and between-run variability of ±7.23, 0.056, and 13.4%, respectively. Based on these results, the lower limit of quantitation for midazolam was established at 1.00 ng/ml. This represents a 7–100-fold increase in sensitivity as compared to assays based on HPLC with UV detection (Table 1).

Table 2
Back-calculated concentrations from calibration curves run in duplicate on four occassions

| Nominal (ng/ml) | Gm (ng/ml) | S.D. (ng/ml) | DEV (%) | R.S.D. (%) | *n* |
|---|---|---|---|---|---|
| 1.00 | 1.05 | 0.062 | 5.13 | 5.89 | 8 |
| 2.00 | 1.93 | 0.262 | −3.68 | 13.6 | 6 |
| 5.00 | 4.64 | 0.227 | −7.23 | 4.89 | 8 |
| 10.0 | 10.2 | 0.247 | 1.52 | 2.43 | 8 |
| 30.0 | 31.9 | 1.43 | 6.34 | 4.48 | 7 |
| 100 | 101 | 1.36 | 1.40 | 1.34 | 8 |
| 200 | 199 | 5.27 | −0.388 | 2.65 | 8 |

*Abbreviations*: GM, grand mean; S.D., standard deviation; DEV, percent deviation from nominal value; R.S.D., relative standard deviation; *n*, total number of replicate observations within the validation runs.

Validation data of the analytical method in terms of accuracy and precision are summarized in Table 3. Based upon analysis of QC samples analyzed on eight different occasions, the final method was shown to be accurate, with an average accuracy at the four tested concentrations within 12%,

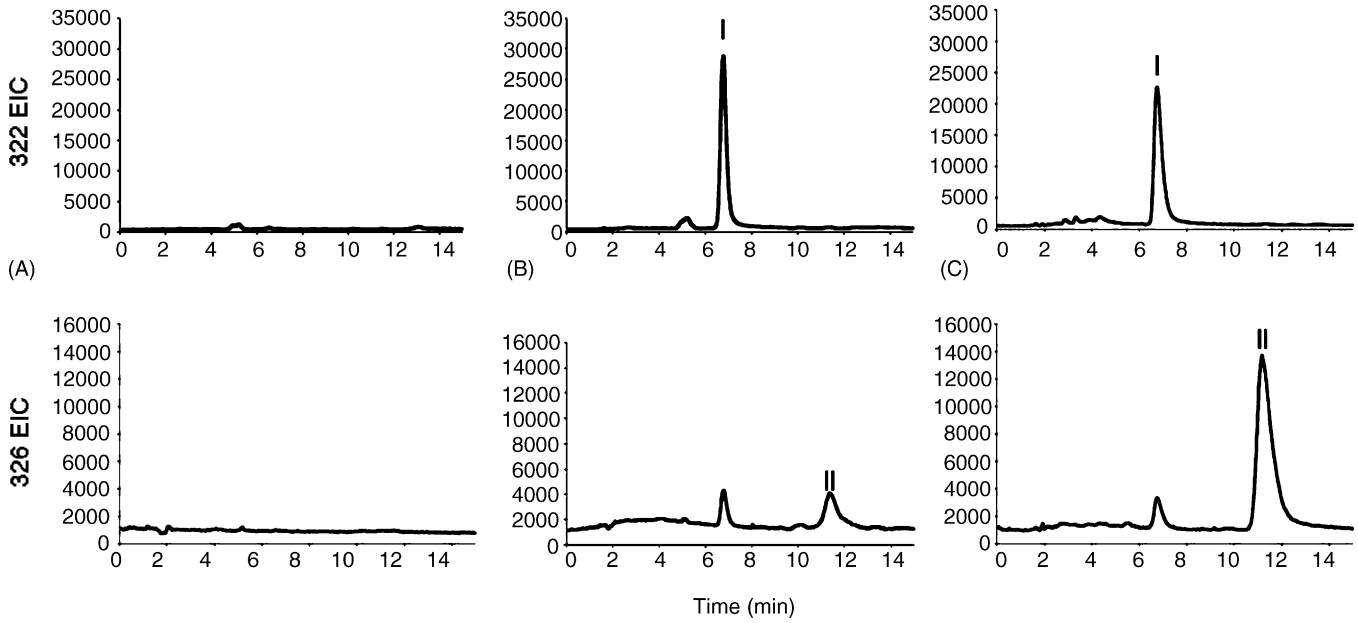


Fig. 1. Chromatograms from reversed-phase HPLC analysis of a blank human plasma sample (A), a human plasma sample spiked with midazolam at a concentration of 1 ng/ml (B), and a plasma sample obtained from a female patient with cancer at 5 h after intravenous administration of midazolam (dose, 25 μg/kg) (C). The labeled chromatographic peaks indicate midazolam (II) and the internal standard lorazepam (I).

Table 3
Assessment of accuracy and precision from quality control samples[a]

| Nominal (ng/ml) | GM (ng/ml) | S.D. (%) | DEV (%) | BRP (%) | WRP (%) | *n* |
|---|---|---|---|---|---|---|
| 1.00 | 0.928 | 0.176 | −7.23 | 13.4 | 0.056 | 22 |
| 3.00 | 3.13 | 0.312 | 4.48 | 12.1 | 0.112 | 22 |
| 85.0 | 94.9 | 8.42 | 11.6 | 2.58 | 0.865 | 24 |
| 170 | 160 | 8.89 | −5.60 | 4.00 | 0.608 | 24 |

*Abbreviations*: GM, grand mean; S.D., standard deviation; DEV, percent deviation from nominal value; WRP, within-run precision; BRP, between-run precision; *n*, total number of replicate observations during the validation runs.

[a] Three samples at each concentration were run on eight different occasions.

and precise, with a within-run and between-run precision always within 15%. The mean overall extraction efficiency for midazolam was independent of the spiked concentration, and not significantly different from 100%.

### 3.3. Plasma concentration–time profile

The mean observed concentration–time profile of midazolam in a group of 35 human subjects (median dose, 1.8 mg; range, 1.2–2.7 mg) is shown in Fig. 2. The mean (±standard deviation) observed peak concentration of midazolam in this group was 62.8 ± 22.0 ng/ml, with a mean area under curve extrapolated to infinity of 96.1 ± 42.7 ng h/ml and a mean systemic clearance of 360 ± 132 ml/min (range, 118–704 ml/min), which is similar to previously obtained values [2]. In 28 of 35 subjects, the observed midazolam concentration had already dropped below 5 ng/ml at 4 h after drug administration (overall mean concentration, 4.46 ± 2.50 ng/ml). This provides further evidence that for the purpose of CYP3A phenotyping with non-sedative doses of midazolam, an analytical method is required with a lower limit of quantitation at the low ng/ml level to avoid the generation of overestimated values for drug clearance.

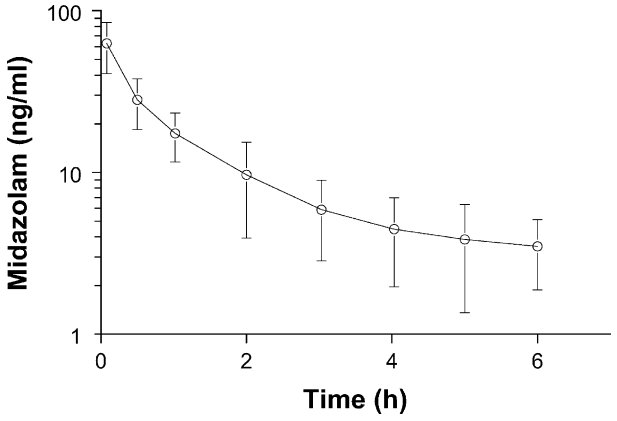


Fig. 2. Mean plasma concentration–time profile of midazolam following intravenous administration of midazolam at a dose of 25 μg/kg to a group of 35 human subjects (median total dose, 1.8 mg). Symbols represent mean values, and error bars represent standard deviations.

## 4. Conclusion

In conclusion, we have described a novel method for the quantitative determination of midazolam in human plasma, which is specific, accurate and precise, and can be easily implemented in routine practice. The sample pretreatment procedure is based on a simple and efficient solvent extraction, thereby eliminating the need of solid phase extraction, column switching procedures, and/or the use of large volumes of plasma for sample clean-up. Two previously described assays for the determination of midazolam in human plasma with single-quadrupole mass-spectrometric detection have reported lower limits of quantitation for midazolam of 0.5 ng/ml [10] and 6.5 ng/ml [15], respectively. It is of importance to indicate here that the lower limit of quantitation can be somewhat misleading when it is reported simply in terms of sample concentration rather then the innate sensitivity. This is because it does not give an indication of the sample size required or the proportion of the analyte in the sample actually injected for analysis. For example, the method reported by Marquet et al. [10] has a lower limit of quantitation of 0.5 ng/ml but requires 2 ml plasma, which might be problematic when serial analyses are being planned on the same material collected from patients. In addition, this method uses a microbore system, which may be less convenient for some laboratories. Our currently described assay provides an alternative to this method with a similar degree of sensitivity, at a lower limit of quantitation of 1.00 ng/ml, using only 600 μl sample aliquots. This method will be implemented in several current and future clinical pharmacokinetic investigations in which midazolam is used as a CYP3A phenotyping probe.

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