Midazolam in Plasma from Hospitalized Patients as Measured by Gas–Liquid Chromatography with Electron-Capture Detection

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The present assay was developed for quantifying midazolam in plasma of patients hospitalized in intensive-care units or undergoing anesthesia and receiving many other drugs as well. Plasma samples are alkalized with NaOH and midazolam is extracted into n-hexane. The organic phase is evaporated and reconstituted in n-butyl acetate, and the midazolam is quantified by gas–liquid chromatography with electron-capture detection. The calibration graph for midazolam was linear in the ranges 5–200 and 200–800 μg/L. The CVs for precision and reproducibility of the assay were <5%. The method was very specific for midazolam; most of the drugs commonly used in anesthesia and in the intensive-care unit did not interfere with the assay. The lowest detectable concentration was 1 μg/L. The method is adaptable for use with an automated chromatographic system.

Additional Keyphrases: anesthesia • benzodiazepines • extraction with n-hexane

Midazolam is a water-soluble benzodiazepine widely used for induction of anesthesia and for sedation of artificially ventilated patients in intensive-care units (ICU). Its elimination half-life is relatively short (1.5 to 3.0 h) in young, healthy patients (1–3), longer (4 to 8 h) in elderly or obese patients (4). In humans, it is eliminated rapidly by the liver and, unlike most benzodiazepines, its major metabolite is less potent than the parent compound (5).

Now that carphbine (for long-term infusion) and alfalone have been withdrawn from the market, midazolam is one of the few short-acting drugs remaining for long-term sedation of ICU patients. However, Byatt et al. (6) and Byrne et al. (7) report prolonged elimination half-lives of 20 and even 53 h in very sick patients receiving midazolam for sedation in ICU. Therefore, one must be cautious in dosing midazolam in this category of patients, and measuring the midazolam concentration in plasma might be very helpful in certain circumstances, especially when assessment of the neurological status is of importance. A potential difficulty in measuring midazolam concentration in plasma from these patients is that they also receive many other drugs, which may interfere with the midazolam assay; a very specific assay is mandatory.

Today the technique most extensively used for analyzing midazolam is gas–liquid chromatography with electron-capture detection (GLC-ECD) (2–4, 8, 9), although "high-performance" liquid chromatography (10, 11), gas chromatography–mass spectrometry (10, 12), and radioimmunoassay (13) have also been used. Most of these assays were developed for measuring midazolam in plasma or serum obtained from animals or healthy volunteers. However, we needed a sensitive and specific assay both for drug monitoring and for pharmacokinetic determinations of midazolam in ICU and anesthetized patients. From the literature, we decided to try GLC-ECD; however, the method of Greenblatt et al. (4) involves the use of benzene, a potent carcinogen, and the method of Rutherford (14), modified by Heizmann (personal communication) is not specific enough. Therefore, we decided to develop a more specific GLC-ECD assay for quantifying midazolam in hospitalized patients.

Materials and Methods

Chemicals and Reagents

Midazolam hydrochloride, the metabolite α-hydroxymidazolam, and the internal standard Ro 21-6296 [8-fluoro-6-(2-fluorophenyl)-1-methyl-3-bromo-4H-imidazo-(1,5-a) (1,4) benzodiazepine] were obtained from Hoffmann-La Roche, Basel, Switzerland. The analytical-grade organic solvents n-hexane and n-butyl acetate, and sodium hydroxide were provided by Merck, Darmstadt, F.R.G., and used without further purification.

Apparatus

We used a gas chromatograph (Hewlett Packard, Palo Alto, CA 94304; Model 5880A) equipped with a 60Ni electron-capture detector and fitted with a 183 cm × 0.2 mm (i.d.) glass column packed with SP-2250 (methyl and phenyl silicone (50:50)) coated on Supelcoport (particle size 80–100 mesh; Supelco, Bellefonte, PA 16823). The carrier gas was nitrogen. The instrument conditions were: carrier-gas flow rate 33 mL/min, injection-port temperature 290 °C, detector temperature 320 °C, and oven temperature 270 °C. The sample was injected with a Model HP 7672A autosampler.

Calibration Standards

Dissolve 4 mg of midazolam hydrochloride in 100 mL of water, and dilute aliquots of this solution with water to make working standards with concentrations in the range 250 to 40 000 μg/L. Prepare plasma standards by reconstituting drug-free plasma with these aqueous solutions, to give final concentrations of midazolam (base form) of 5–800 μg/L. Stored in aliquots of 0.5 mL at –20 °C, the plasma standards are stable for at least six months. Prepare internal standard solutions by dissolving 2 mg of Ro 21-6296 in 10 mL of methanol, then diluting with water to the appropriate range between 25 and 50 μg/L.

Procedure

Transfer 0.2 to 0.4 mL of standard plasma or patient’s plasma to a 15-mL glass tube, then add 0.1 mL each of the internal standard and of 2 mol/L NaOH solution. (For all pipetting steps we used a Microlab 1000 pipette (Hamilton Bonaduz, 7402 Bonaduz, Switzerland)). To extract midazolam and the internal standard, add 4 mL of n-hexane and mix on a reciprocating shaker (Infors, Basel, Switzerland) at

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Clinical Samples

Five milliliters of blood was collected from artificially ventilated patients with unstable hemodynamics, who were being treated in the surgical ICU and were receiving various kinds of drugs: opiates, other benzodiazepines, cardiovascular medication, and other drugs commonly used in anesthesia and ICU. Midazolam, for sedation, was given as a continuous infusion. The blood samples (6 mL each) were collected at the following times: 0.5, 1, 2, 3, and 4 h during the infusion; then at 5, 10, 30, 60 min, and at 2.5, 4, 6, 8, 12, 24, 36, and 48 h after stopping the infusion. The blood was drawn into a "Venoject" collecting tube containing lithium heparin (Terumo, 3030 Leuven, Belgium). The plasma, separated by centrifugation for 10 min at room temperature, was stored at -20 °C until assay. Because the midazolam concentrations in the plasma samples from these patients are unpredictable, we rapidly screen samples and classify them into three categories of midazolam concentrations: low (<200 µg/L), middle (200–800 µg/L), and high (800–1200 µg/L). Each category of samples is then measured with appropriate concentrations of standards and controls. Samples with midazolam concentrations exceeding 800 µg/L are diluted with blank plasma into the middle range.

Specificity Tests

To evaluate potential interference of other drugs, we also obtained plasma samples from patients receiving commonly used medication in the ICU, extracting and quantifying midazolam as described above.

Stability Tests

We dissolved midazolam hydrochloride in human plasma. The final concentration of midazolam base was 178 µg/L. The sample was divided into small portions of 0.5 mL. We tested the stability of midazolam by measuring its concentrations in samples that were stored at 22 °C for one day and at -20 °C for six months. We injected the reconstituted extracted sample five times within 24 h to ascertain the stability of midazolam in n-butyl acetate.

Results

Figure 1 depicts chromatograms of patients' plasma samples containing low (<200 µg/L) and middle-range (200–800 µg/L) concentrations of midazolam. The retention times of midazolam and internal standard were 4.67 and 10.0 min, respectively. The major metabolite of midazolam is α-hydroxymidazolam ([15]). A mixture of midazolam and α-hydroxymidazolam, dissolved in n-butyl acetate and injected directly onto the column, was found to be resolvable, because the metabolite appeared approximately 0.6 min
after the parent compound. In the procedure involving extraction with n-hexane, the metabolite was not co-extracted. Total chromatography time between successive samples was 15 min.

In the low range (5–200 µg/L) the equation of the calibration graph is \( y = 0.012x - 0.002 \) (r > 0.999) and for the middle range (200–800 µg/L) that equation is \( y = 0.004x - 0.007 \) (r > 0.999). The precision of the assay was evaluated by analyzing a sample 10 times on the same day; the CV for these analyses was <6%. The reproducibility of the method was determined by analyzing four drug-supplemented plasma samples on various days; the CV for this assay was <8%.

The results are summarized on Table 1. The extraction recoveries (n = 5) of midazolam and internal standard were 90.3% (±2.0 SD) and 93.0% (±3.7 SD), respectively, for a midazolam concentration range of 90–800 µg/L. The limit of detection was 1 µg/L with the ratio of peak height to base noise >10. Midazolam is stable in plasma samples; we observed no significant change of midazolam concentrations after storing for one day at room temperature and at -20 °C for up to six months. The midazolam extracted from plasma was stable for at least 24 h in n-butyl acetate. The specificity of the assay was excellent; none of the following drugs interfered: aminophylline, amoxicillin, atracurium, atropine, buprenorphine, cefotiam, dexamethasone, diclofenac, dicumarol, digoxin, diltiazem, disopyramide, dobutamine, emepropiam, enflurane, epinephrine, etomidate, fentanyl, flunoxacillin, flunitrazepam, halothane, heparin, hydralazine, levodopa, lidocaine, mannitol, maprotiline, mephenesin, morphine, neostigmine bromide, nifedipine, nitroglycerine, norepinephrine, ornidazole, oxazepam, pentoxyfylline, phenolamine, phenylephrine, propranolol, promethazine, propranolol, propranolol, quindine, scopolamine, streptomycin, succinylcholine, thiopental, triazolam, and verapamil.

**Discussion**

The assay described here is sensitive, selective for midazolam, and more rapid than the currently used method (9). Its selectivity was established by showing that the major metabolite, α-hydroxymidazolam, as well as other drugs commonly used in the clinic were not detected. The preparation time for a set of 10 samples is about 2 h. The chromatogram has a stable baseline, so the assay has been used extensively with an automated injection, thereby allowing overnight run of a large number of samples. Under optimal conditions one technician can evaluate 20 duplicate samples per working day. Only 0.2 to 0.4 mL of plasma is required for each measurement. It should be noted that extraction with n-hexane of midazolam and internal standard at a concentration <1000 µg/L had an efficiency of 90% and was independent of midazolam concentration (unpublished data).

Compared with published methods, the present assay demonstrated almost the same CVs for precision and reproducibility. Furthermore, the current method is very sensitive, detecting as little as 1 µg/L compared with 4 µg/L (8), 5–10 µg/L (2), and 2 µg/L for derivatization methods (9), or with 50 µg/L for the liquid-chromatographic method (10). It was comparable in sensitivity with the assay proposed by Smith et al. (3) as well as the more complex gas–liquid chromatography/mass spectrometry technique of Rubio et al. (12).

We have used the present assay to quantify midazolam in more than 1200 samples during six months. Column stability and performance was retained for at least 2000 injections.

As an illustration of the utility of the current assay, Figure 2 depicts the midazolam plasma concentration time course of a 66-year-old patient intubated and ventilated postoperatively in an ICU after coronary bypass surgery. The patient was sedated with midazolam, receiving an infusion at a rate of 232 µg/min during a 4-h period. Figure 1 illustrates the chromatograms of plasma from a hospitalized patient before midazolam (A) and 1.5 h after midazolam administration. The midazolam concentrations were <1, 321, and 9.35 µg/L, respectively. To determine the pharmacokinetic parameters for this patient, the plasma concentration data were fitted to a multi-compartment model with the nonlinear regression program **nlme** (16). The three-compartment model described the data better than the two-compartment model (difference in -2 log likelihood = 15.5, two degrees of freedom: \( P < 0.005 \), chi square distribution (16)). The elimination half-life was 5.54 h, the elimination clearance was 280 mL/min, and the volume of distribution at steady state \( (V_{dss}) \) was 1.17 L/kg. These values are similar to those reported by Harper et al. (17) in this category of patients.

In conclusion: the present assay is shown to be specific for measurement of midazolam in hospitalized patients who are concomitantly receiving many other drugs. The method is simple inasmuch as no derivatization is necessary, and it is precise, accurate, and sufficiently sensitive. The limit of

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**Table 1. Within- and Between-Day Precision of the**

**Midazolam Assay**

<table>
<thead>
<tr>
<th>Added</th>
<th>Measured</th>
<th>CV, %</th>
<th>n</th>
</tr>
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<tbody>
<tr>
<td><strong>Within-day</strong></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>891b</td>
<td>912.5</td>
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<td>10</td>
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<td>17.5</td>
<td>5.15</td>
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<tr>
<td><strong>Between-day</strong></td>
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<tr>
<td>540b</td>
<td>533.1</td>
<td>7.08</td>
<td>12</td>
</tr>
<tr>
<td>17b</td>
<td>179.3</td>
<td>6.74</td>
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</tr>
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<tr>
<td>22.7c</td>
<td>24.5</td>
<td>7.72</td>
<td>5</td>
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*Midazolam base. b0.2 mL plasma was extracted. c0.4 mL was extracted.*
detection compares well with that of the most sophisticated 
gas–liquid chromatographic/mass spectrometric technique.

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