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Note

Measurement of midazolam and α-hydroxymidazolam by gas chromatography with electron-capture detection

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Midazolam is a new benzodiazepine that has been suggested for use as a sedative prior to elective cesarean section [1]. It is thought to be suitable because of its pharmacological potency [2], elimination half-life, and minimal injection pain [3]. Midazolam has been studied as an anesthetic in pregnant ewes. Conklin et al. [4] monitored maternal and fetal hemodynamics and measured maternal and umbilical cord blood concentrations. Vree et al. [5] measured maternal and fetal plasma concentrations and maternal urinary excretion of midazolam and its metabolite 1-hydroxymethylmidazolam. However, there is no information on fetal tissue uptake.

The purpose of this study was to develop sensitive analytical methods to measure midazolam and its α-hydroxy metabolite in both tissue and plasma. This was necessary in order to study fetal tissue uptake of midazolam in guinea pigs.

Previous assays for midazolam and α-hydroxymidazolam have been developed for plasma and urine based on gas chromatography (GC) with electron-capture detection (ECD) [6, 7], a combination of GC—ECD and differential pulse polarography [8], gas chromatography—mass spectrometry [9] and high-performance liquid chromatography [10—12]. However, all of these procedures had drawbacks. These included: (1) lack of sensitivity; (2) lack of an available internal standard; (3) excessive time consumption owing to different extraction and/or detection steps for midazolam and α-hydroxy-

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midazolam; (4) lack of repeatability; (5) lack of the necessary instrumentation in our laboratory.

We would like to report a sensitive GC method that can be used in tissue and plasma for both midazolam and its α-hydroxy metabolite. A derivatization step allows analysis of midazolam in its underivatized form in the same chromatogram as the trimethylsilyl derivative of α-hydroxymidazolam.

EXPERIMENTAL

Reference compounds

Reference crystals of 8-chloro-6-(2-fluorophenyl)-1-methyl-4*H*-imidazo[1,5-*a*][1,4]-benzodiazepine (midazolam), 8-chloro-6-(2-fluorophenyl)-1-hydroxymethyl-4*H*-imidazo[1,5-*a*][1,4]-benzodiazepine (α-hydroxymidazolam) and the internal standard, 7-chloro-1,3-dihydro-1-methyl-5-phenyl-2*H*-1,4-benzodiazepin-2-one (diazepam) were provided by Hoffman-La Roche (Nutley, NJ, U.S.A.). The structures of the compounds are shown in Fig. 1. Stock solutions of midazolam and α-hydroxymidazolam were prepared in acetone—methanol (1:5) to yield concentrations equivalent to 10 μg/ml free base. Diazepam was made up to 1 μg/ml.

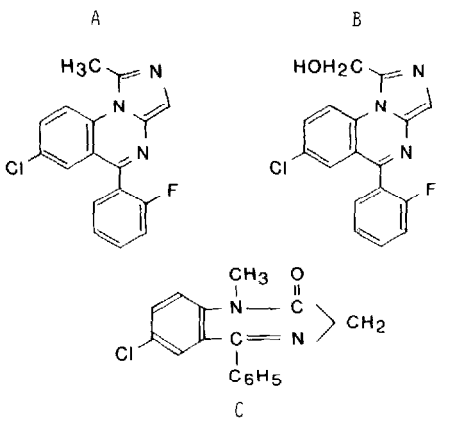

Fig. 1. Structures of midazolam (A), α-hydroxymidazolam (B) and diazepam, the internal standard (C).

Chemicals

Spectral-grade solvents from various manufacturers were used: SpectrAR ethyl acetate from Mallinckrodt (Paris, KY, U.S.A.), Photrex acetone from Baker (Phillipsburg, NJ, U.S.A.) and Omnisolv benzene from E.M. Science (Cherry Hill, NJ, U.S.A.). Methyl *tert.*-butyl ether was obtained from Burdick and Jackson Labs. (Muskegon, MI, U.S.A.). The silanizing agent, bis(trimethylsilyl)trifluoroacetamide (BSTFA), and silylation-grade acetonitrile were purchased from Pierce (Arlington, TX, U.S.A.). All other chemicals used were analytical grade.

Apparatus

A Hewlett-Packard 5840 gas chromatograph equipped with a ^{63}Ni electron-capture detector was used to analyze the compounds. The chromatographic

conditions are similar to those suggested previously for GC—ECD [6], with the major modification being a temperature program. The chromatograph was fitted with a 2 m × 4 mm I.D. AW DMCS-treated glass column packed with 5% OV-101 coated on 80—100 mesh Gas Chrom Q (Alltech Assoc., Deerfield, IL, U.S.A.). The carrier gas was argon—methane (95:5) (Matheson Gas Products, East Rutherford, NJ, U.S.A.). The instrument conditions were: carrier gas flow-rate, 20 ml/min; injection port temperature, 270°C; detector temperature, 325°C; oven temperature programmed from 270°C (5 min) at 15°C/min to 310°C (1 min). Total run time was 9 min.

Animals

Pregnant guinea pigs were given an intravenous injection of midazolam, 1 mg/kg, and sacrificed at various times after injection. Maternal blood and fetal blood, brain and carcass were obtained. Plasma was obtained by centrifugation. All plasma and tissue samples were frozen until analysis. Carcasses were homogenized using a Waring blender (Waring Products Division DCA, New Hartford, CT, U.S.A.) and brains were homogenized using a Tekmar Tissumiser (Tekmar Company, Cincinnati, OH, U.S.A.). Samples were frozen until analysis.

Plasma

Guinea pig plasma samples (0.5—1.0 ml) and spiked plasma were extracted using a procedure similar to that described by Lesko et al. [13]. A 50-μl volume of 1 μg/ml diazepam (internal standard) was added to the plasma. Next, the samples were made basic with 0.5 ml of 2 *M* sodium carbonate saturated with sodium chloride. The samples were then extracted with 5 ml of methyl *tert.*-butyl ether, centrifuged and the organic layer was transferred to a clean silanized tube. A derivatization step similar to that of Heizmann and Van Alten [6] was then used. The major modification was the use of a different ratio for the BSTFA mixture used for derivatization. The ether was dried over anhydrous sodium sulfate, the mixture centrifuged and the ether transferred to a 5-ml Reacti vial (Pierce, Rockford, IL, U.S.A.). The ether was evaporated under nitrogen. The samples sat overnight at this point. The residue was derivatized with 100 μl of a mixture of benzene–acetone–acetonitrile–BSTFA (4:1:1:1) for 10 min at room temperature. The reagent was evaporated under nitrogen at 38°C. Samples were immediately reconstituted with 30 μl of benzene—ethanol—acetone (8:1:1). A 2-μl aliquot was injected into the gas chromatograph. Standard curves ranged from 3.12 to 500 ng/ml for midazolam and from 7.8 to 500 ng/ml for α-hydroxymidazolam.

Tissue

Tissue samples were homogenized following dilution of each gram of tissue with 5 ml of water. A 5-ml portion of tissue homogenate was then transferred to a 30-ml silanized tube containing 100 μl of 10 μg/ml internal standard. Distilled water (2 ml), 0.1 ml of 6 *M* sodium hydroxide and 2 ml ammonium chloride—ammonium hydroxide buffer (pH 9.2) were added to the homogenate. After mixing, the solution was extracted with 10 ml ethyl acetate on a reciprocating shaker (25 min), centrifuged (10 min, 1000 *g*), and 8–9 ml of the organic layer were transferred to a clean, silanized centrifuge tube. Then,

2 ml of 0.25 *M* sulfuric acid were added. After vortexing for 1 min, the samples were again centrifuged for 3 min. The upper organic layer was removed by aspiration and discarded. Next, 0.27 ml of 6 *M* sodium hydroxide were added to the aqueous layer in order to make the solution basic. The excess remaining ethyl acetate was blown off and 5 ml methyl *tert.*-butyl ether were added. The sample was vortexed for 30 s and centrifuged for 10 min. The organic layer was transferred to a clean centrifuge tube and dried over anhydrous sodium sulfate for 20 min. The mixture was centrifuged, the organic layer transferred to a 5-ml Reacti vial and evaporated to dryness under nitrogen. The samples then sat overnight. The residue was derivatized with 100 μl of a mixture of benzene–acetone–acetonitrile–BSTFA (4:1:1:1) for 10 min. The samples were evaporated to dryness under nitrogen at 38°C and immediately reconstituted with 30 μl of benzene–ethanol–acetone (8:1:1). The samples were vortexed for 30 s, then a 2-μl aliquot was injected into the gas chromatograph.

RESULTS

The retention times for diazepam, midazolam and α-hydroxymidazolam were 5.05, 6.56, and 7.85 min, respectively. Typical plasma and tissue chromatograms are shown in Figs. 2 and 3, respectively. Samples and standards were quantitated by peak area.

Precision was determined for both high and low standards by repeat analyses of eight plasma standards. The resulting coefficients of variation for midazolam

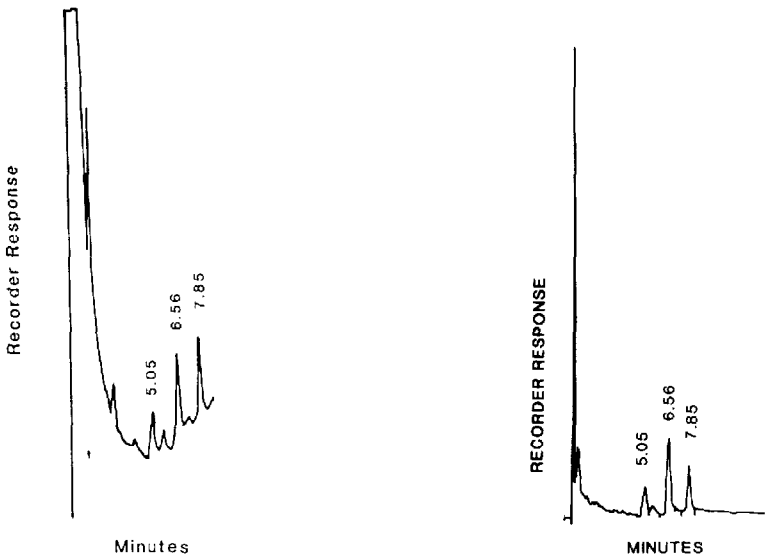

Fig. 2. Chromatogram of plasma extract containing 100 ng/ml midazolam (6.56 min), 100 ng/ml α-hydroxymidazolam (7.85 min) and 50 ng/ml diazepam, the internal standard (5.05 min).

Fig. 3. Chromatogram of guinea pig tissue homogenate extract containing 31.95 ng/ml midazolam (6.56 min), 41.00 ng/ml α-hydroxymidazolam (7.85 min) and 200 ng/ml diazepam, the internal standard (5.05 min).

were 8.4% (100 ng/ml) and 7.6% (25 ng/ml). The α-hydroxy metabolite yielded coefficients of variation of 9.0% (100 ng/ml) and 6.4% (25 ng/ml). Accuracy was determined by ten repeated injections of a 100 ng/ml midazolam and 100 ng/ml α-hydroxymidazolam spiked plasma sample. The resulting coefficients of variation were 7.7% (midazolam) and 4.4% (metabolite). Calibration curves were linear to 500 ng for midazolam and α-hydroxymidazolam following derivatization. Typical least-squares regression lines were $y = 0.010x - 0.054$ for midazolam and $y = 0.007x + 0.215$ for the trimethylsilyl derivative of α-hydroxymidazolam. In these equations, y is the dependent variable, peak area percentage (area midazolam or α-hydroxymidazolam divided by area of internal standard) and x is the concentration of midazolam or α-hydroxymidazolam. Sensitivity for midazolam was < 3 ng/ml and for the metabolite was < 7 ng/ml.

In tissue, the coefficients of varation of midazolam and α-hydroxymidazolam at 100 ng/ml were 8.2 and 9.6%, respectively ($n = 12$). Calibration curves were linear to 1000 ng/ml for both midazolam free base and its metabolite extracted from tissue. Typical least-squares regression lines were $y = 0.039x + 0.269$ for midazolam and $y = 0.041x + 0.262$ for the trimethylsilyl derivative of the metabolite. Sensitivity for midazolam and α-hydroxymidazolam from tissue was < 2 ng/ml following derivatization.

These methods have been used to quantitate guinea pig maternal and fetal plasma, guinea pig fetal carcass homogenate and guinea pig fetal brain homogenate from 1 to 60 min after injection. Table I lists mean values ± S.D.

TABLE I

MIDAZOLAM AND α-HYDROXYMIDAZOLAM IN GUINEA PIG PLASMA AND TISSUES

Compound	Concentration (mean ± S.D.)			
	Maternal plasma (ng/ml)	Fetal plasma (ng/ml)	Fetal brain (ng/g)	Fetal carcass (ng/g)
Midazolam	152 ± 137	91 ± 92	227 ± 150	220 ± 81
α-Hydroxymidazolam	22 ± 11	5 ± 13	37 ± 63	15 ± 17

DISCUSSION

Owing to the limited volume of guinea pig plasma available and the sensitivity required, a technique was needed that was reproducible and enabled analysis of midazolam and α-hydroxymidazolam by the same method. Sensitivity for the tissue did not prove to be a problem since each gram was diluted with 5 ml of water. This provided enough sample for 5 ml to be extracted.

We were unable to obtain reproducible results with the internal standard suggested by Heizmann and Van Alten [6]. Diazepam was tried giving excellent chromatographic properties. Results were reproducible, retention time was relatively short and no plasma peaks interfered. In a recently published paper, Arendt et al. [14] also utilized diazepam as the internal standard for analysis of midazolam and its 1- and 4-hydroxy metabolites. He did not derivatize the

metabolites for analysis. With our packing, α-hydroxymidazolam had poor response and peak shape without derivatization.

We originally attempted to use BSTFA with 1% trimethylchlorosilane (TMCS) as a catalyst since we have had favorable results with this combination in our other assays. However, the solvent system benzene–acetone–acetonitrile—BSTFA/TMCS (4:1:1:1) was not stable over a period of 0.5 h. Even though we dried the solvent system before addition of the BSTFA/TMCS, moisture in the air or the solvent system itself caused the inactivation of the silanizing agent. We then tried BSTFA alone, and the problem was eliminated. We were able to successfully quantitate midazolam and the α-hydroxy metabolite in guinea pig plasma and tissue.

This method is sensitive and quantitative. It utilizes inexpensive GC equipment which is common in many laboratories. The plasma extractions are relatively rapid. Twenty plasma samples and eight standards can be analyzed in two days. The extraction and analysis of twelve tissue samples and eight standards can be accomplished in two days.

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