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DETERMINATION OF MIDAZOLAM AND TWO METABOLITES OF MIDAZOLAM IN HUMAN PLASMA BY GAS CHROMATOGRAPHY—NEGATIVE CHEMICAL-IONIZATION MASS SPECTROMETRY

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SUMMARY

A method is described for measuring midazolam, a new anesthesia induction agent and hypnotic, and its hydroxymethyl and desmethyl metabolites in human plasma. Deuterated analogues of each compound are added to plasma as internal standards. The compounds are extracted from plasma with benzene containing 20% 1,2-dichloroethane and after removal of the extracting solvent are dissolved in a solution of bis-(trimethylsilyl)acetamide and acetonitrile. An aliquot of this solution is analyzed by gas chromatography—mass spectrometry with the mass spectrometer set to monitor in the gas chromatographic effluent the M^- ions of drug, metabolites and internal standards generated by methane electron-capture negative chemical ionization. For all three compounds, the limit of quantitation is 1 ng ml^{-1}, and the precision (relative standard deviation) at a concentration of 5 ng ml^{-1} is less than 6%. Measurable amounts of the hydroxymethyl, but not the desmethyl, metabolite of midazolam could be found in the plasma of humans given either an intravenous or an oral dose of midazolam maleate.

INTRODUCTION

Midazolam (MDZ) maleate is a new water-soluble benzodiazepine salt which is currently undergoing testing as an intravenously administered anesthesia induction agent [1—3] and as an orally active hypnotic [4].

Electron-capture (EC) gas chromatographic (GC) [5, 6] and radioimmunoassay (RIA) [7] methods for MDZ have been published. This paper reports a sensitive, specific and relatively simple gas chromatographic—mass spectrometric (GC—MS) assay for MDZ, its principal metabolite in human urine [5], hydroxymethylmidazolam (HMMDZ) and a minor metabolite of midazolam in the dog [8], desmethylmidazolam (DMMDZ). If present in sufficient quantities, DMMDZ will interfere with the EC-GC and RIA assays for MDZ.

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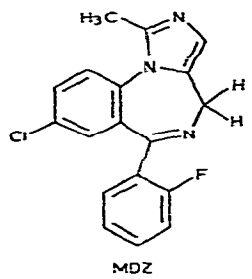
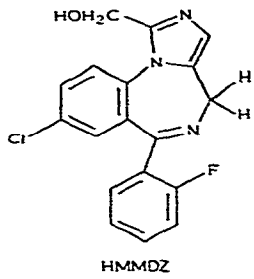
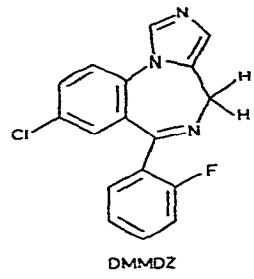


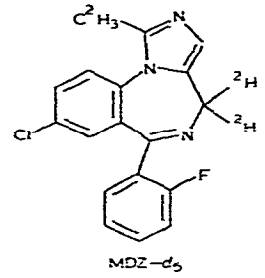
MDZ



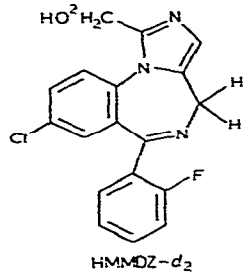
HMMDZ



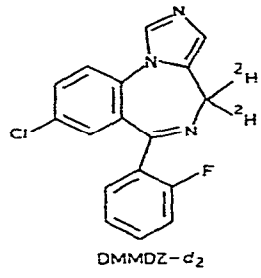
DMMDZ



MDZ–d_5



HMMDZ–d_2



DMMDZ–d_2

The reported assay features the use of MDZ-d_5, HMMDZ-d_2 and DMMDZ-d_2 as internal standards for MDZ, HMMDZ and DMMDZ, respectively, and the trimethylsilylation of the hydroxy group of HMMDZ to provide a compound with suitable GC properties.

EXPERIMENTAL

Equipment and operating conditions

Gas chromatograph. A Finnigan Model 9500 gas chromatograph was equipped with a 152 cm × 2 mm I.D. U-shaped borosilicate glass column packed with 3% Poly-S 176 on 80—100 mesh, high-performance Chromosorb W from Applied Science Labs., State College, PA, U.S.A. The column was conditioned at 320°C overnight with nitrogen as carrier gas. Methane (1.5 kg cm^{-2}) was used as GC carrier gas. The injector, column, interface oven and transfer line were operated at 310°C, 300°C, 250°C and 250°C, respectively. The retention times of MDZ, derivatized HMMDZ and DMMDZ under these conditions were 132, 72, and 156 sec, respectively. Prior to use, the GC column was conditioned daily with several injections of both Silyl-8® (Pierce Chemical Co., Rockford, IL, U.S.A.) and the reconstituted residue from an ethyl acetate extract of drug-free plasma.

Mass spectrometer. The ion source parameters of a Finnigan Model 3200 quadrupole mass spectrometer were set to give the maximum response consistent with reasonable ion peak shape and unit resolution. The modifications to the instrument to permit the detection of negative ions have been described [9]. The continuous dynode electron multiplier was set at −1.7 kV and the conversion dynode was set at +2.5 kV. Methane (Liquid Carbonic, 99%) at an ion source pressure of 68 Pa was used as negative chemical-ionization

reagent gas. To avoid "ghosting", the MS tuning and GC column conditioning were optimized using the response from the injection of microgram amounts of Ro 21-3547, the desfluoro analogue of MDZ.

Peak monitor. A Finnigan Promim® with a Rikadenki four-channel recorder was used to set the mass spectrometer to monitor *m/z* 325 (MDZ), *m/z* 330 (MDZ-d_5), *m/z* 311 (DMMDZ), *m/z* 313 (DMMDZ-d_2), *m/z* 413 (trimethylsilylated HMMDZ) and *m/z* 415 (trimethylsilylated HMMDZ-d_2). All channels were operated at a gain of 10^{-8} A/V, 100 msec dwell time and a filter setting of 0.5 Hz.

Glassware. Culture tubes (16 ml, Pyrex 9825) provided with Teflon®-lined screw caps were used for plasma extraction. Conical centrifuge tubes (5 ml, Pyrex 8061) were used for the evaporation of the benzene extract. All the tubes were purchased from SGA Scientific, Bloomfield, NJ, U.S.A. Prior to use, the glassware was treated with Siliclad® (Clay Adams, Parsippany, NJ, U.S.A.) and rinsed with methanol and dichloromethane.

Solvent evaporator. Solvents were removed at 60°C using a nitrogen evaporator (N-Evap®, Organomation Assoc.).

Shaker. Extractions were done by shaking (60 strokes min^{-1}) on a variable-speed reciprocating shaker (Eberbach Inc.).

Centrifuge. Centrifugation was carried out on a Damon/IEC Model CRU-500 refrigerated centrifuge operated at 1600 *g* and 10°C.

Chemicals

MDZ (maleate salt), HMMDZ, DMMDZ and Ro 21-3547 were prepared by Dr. A. Walser, and the deuterated compounds, MDZ-d_5, HMMDZ-d_2 and DMMDZ-d_2, were prepared by Dr. Yu-Ying Liu. Both chemists work for the Chemical Research Department, Hoffmann-La Roche Inc., Nutley, NJ, U.S.A. Nanograde methanol, benzene, 1,2-dichloroethane and acetonitrile were purchased from Burdick and Jackson Labs. (Muskegon, MI, U.S.A.). Bis-(trimethylsilyl)acetamide (BSA), boric acid (analytical reagent), potassium chloride and anhydrous sodium carbonate were purchased from Pierce Chemical Co., Mallinckrodt Chemical Co. (St. Louis, MO, U.S.A.), Fisher Scientific Co. (Pittsburgh, PA, U.S.A.) and J.T. Baker Chemical Co. (Phillipsburg, NJ, U.S.A.), respectively.

Solutions

Stock solutions. MDZ: 13.6 mg of MDZ maleate are dissolved in 10.0 ml of methanol to give solution A (1 mg ml^{-1} of MDZ free base). HMMDZ: 10.0 mg are dissolved in 10.0 ml of methanol to give solution B (1 mg ml^{-1}). DMMDZ: 10.0 mg are dissolved in 10.0 ml of methanol to give solution C (1 mg ml^{-1}). MDZ-d_5: 20.0 mg are dissolved in 10.0 ml of methanol to give solution D (2 mg ml^{-1}). HMMDZ-d_2: 2.0 mg are dissolved in 2.0 ml of methanol to give solution E (1 mg ml^{-1}). DMMDZ-d_2: 2.0 mg are dissolved in 2.0 ml of methanol to give solution F (1 mg ml^{-1}).

Tuning solution. 1.0 mg of Ro 21-3547 is dissolved in 10.0 ml of methanol (100 $\mu g\ ml^{-1}$).

Spiking solutions for MDZ, HMMDZ and DMMDZ. Aliquots (0.2 ml) of solutions A, B, and C are transferred into one 100-ml volumetric flask which

is brought to volume with methanol (2 μg ml^{-1}). Individual aliquots of this solution (either 3, 2, 1, 0.5 or 0.2 ml) are transferred to 10-ml volumetric flasks and brought to volume with methanol to give solutions containing 30, 20, 10, 5 or 2 ng of MDZ, HMMDZ and DMMDZ per 0.05 ml of solvent.

Spiking solutions for internal standards. A 0.1-ml volume of solution D and 0.2 ml of solutions E and F are transferred into one 100-ml volumetric flask which is brought to volume with methanol (2 μg ml^{-1}). Then 2 ml of this solution are transferred to a 10-ml volumetric flask and brought to volume with methanol to give a solution containing 20 ng of MDZ-d_5, HMMDZ-d_2 and DMMDZ-d_2 per 0.05 ml of solvent.

1 M pH 10 borate buffer. Boric acid (61.8 g) and potassium chloride (74.7 g) are dissolved in 1000 ml of distilled water. This solution is then titrated to pH 10 with a solution containing 106 g of sodium carbonate per 1000 ml of distilled water.

20% 1,2-dichloroethane in benzene. 200 ml of dichloroethane are added to 800 ml of benzene.

BSA. The contents of one BSA ampule (1 ml) are added to 4 ml of acetonitrile. This solution is prepared just prior to its use.

Procedure

Calibration curve samples (prepared in duplicate). Transfer 1 ml of drug-free control human plasma to each of twelve culture tubes containing either 30 ng, 20 ng, 10 ng, 5 ng, 2 ng or 0 ng of MDZ, together with equal amounts of HMMDZ and DMMDZ and 20 ng of each of the deuterated analogues.

Experimental ("unknown") samples. Transfer 1-ml aliquots of experimental plasma to culture tubes containing 20 ng of each of the deuterated analogues.

Extraction. To each plasma sample add 2 ml of 1 *M* pH 10 borate buffer followed by 6 ml of benzene—1,2-dichloroethane (4:1). Shake the tubes for 20 min, centrifuge the tubes and transfer 5 ml of each benzene extract to a centrifuge tube. Evaporate the solvent. Place tubes in a desiccator and further dry the residues under vacuum for 30 min. Dissolve the residues in 50 μl of BSA—acetonitrile (1:4) and allow the tubes to stand at room temperature for 20 min.

GC—MS analyses. The tuning solution containing Ro 21-3547 is injected and the mass offset value of this compound's M^- ion is determined. The same mass offset value is used to set the Promim to monitor the M^- ions of MDZ, DMMDZ and their respective deuterated analogues.

Aliquots (2—5 μl) from each sample extract are injected into the GC—MS system. Approximately 30 sec after an injection, the GC divert valve is closed and 15 sec later the ion source is turned on. After all the extracts have been analyzed, the mass spectrometer is tuned to monitor the M^- ions of silylated HMMDZ and silylated HMMDZ-d_2. Aliquots of the extracts are then injected using the same protocol just described above for the analysis of MDZ and DMMDZ.

Calculations. The peak heights in the ion chromatograms are measured and the ion ratios for *m/z* 325 vs. *m/z* 330, *m/z* 311 vs. *m/z* 313, and *m/z* 413 vs. *m/z* 415 are calculated. The calibration curve for MDZ is analyzed by linear regression. Calibration curves for HMMDZ and DMMDZ, however, are non-

linear and are analyzed using the computer program NONLIN [10]. Concentrations of MDZ in the experimental plasma samples are calculated using the equation x (ng) = $(R - b)/m$, where R is the experimental m/z ion ratio and b (intercept) and m (slope) are constants generated by the linear-regression analysis of the calibration curve data. Concentrations of DMMDZ and HMMDZ are calculated by using the rearranged isotope dilution equation x (ng) = $(RC - A)/(1 - RB)$, where R is the experimental ion ratio and A, B, and C are parameters generated by the NONLIN program.

Clinical samples

Two healthy male volunteers did not eat anything for 8 h before dosing. One volunteer received a 10-mg dose of MDZ maleate orally and the other volunteer received a 20-mg dose of MDZ maleate intravenously. Blood samples (10 ml) were obtained using heparinized Vacutainer® 6527 from Becton-Dickinson. Samples from the volunteer given the intravenous dose were obtained at —0.25, 0.025, 0.1, 0.25, 0.5, 0.75, 1, 1.5, 2, 3, 4, 5, 17 and 24 h post dosing. Samples from the volunteer given the oral dose were obtained at —0.25, 0.5, 1, 1.5, 2, 3, 4, 5, 6, 8, and 12 h post dosing. The blood was centrifuged for 0.5 h and the resulting plasma was isolated and stored at —20°C until analyzed.

RESULTS AND DISCUSSION

The negative chemical-ionization mass spectra of MDZ and DMMDZ consist only of M^- ions, while the spectrum of trimethylsilylated HMMDZ shows an $[M - (CH_3)_3SiOH]^-$ fragment ion at m/z 323 in addition to an M^- ion (Fig. 1). The negative CI mass spectra of the deuterated analogues used in the assay are identical to those of the protio compounds, except the masses are shifted by the appropriate number of daltons. As would be expected from GC—MS assays of benzodiazepines previously developed in our laboratory [11, 12], the ionization efficiencies of negative CI for these compounds are quite good. Only 25—50 pg of either MDZ, HMMDZ or DMMDZ are needed to generate a selected ion current profile for each M^- ion which has a signal-to-noise ratio of better than 5:1.

Fig. 2 shows typical selected ion current profiles at m/z 325 and 330 from the analyses of 1 ml of either control or experimental plasma spiked with 3.7 ng of MDZ (A) or plasma from a subject either 0.25 h before (B) or 12 h after (C) receiving a 10-mg oral dose of MDZ. Selected ion current profiles at m/z 413 and 415 from the analyses of 1 ml of either control plasma spiked with 5 ng of HMMDZ (A) or plasma from a subject either 0.25 h before (B) or 8 h after (C) receiving a 10-mg oral dose of MDZ are shown in Fig. 3. The limit of quantitation of this assay is 1 ng ml^{-1} for MDZ, HMMDZ and DMMDZ.

Assay precision and recovery were determined by spiking six separate 1-ml plasma samples with 5 ng ml^{-1} each of MDZ, HMMDZ and DMMDZ and analyzing the samples by the procedure described. The mean (± S.D.) concentrations found, 5.10 ± 0.10 ng ml^{-1} for MDZ, 5.00 ± 0.20 ng ml^{-1} for HMMDZ and 5.30 ± 0.30 ng ml^{-1} for DMMDZ, indicate a precision (relative standard deviation) of less than 6% at this concentration for all three compounds. The mean recoveries ± S.D. from these samples, based on a com-

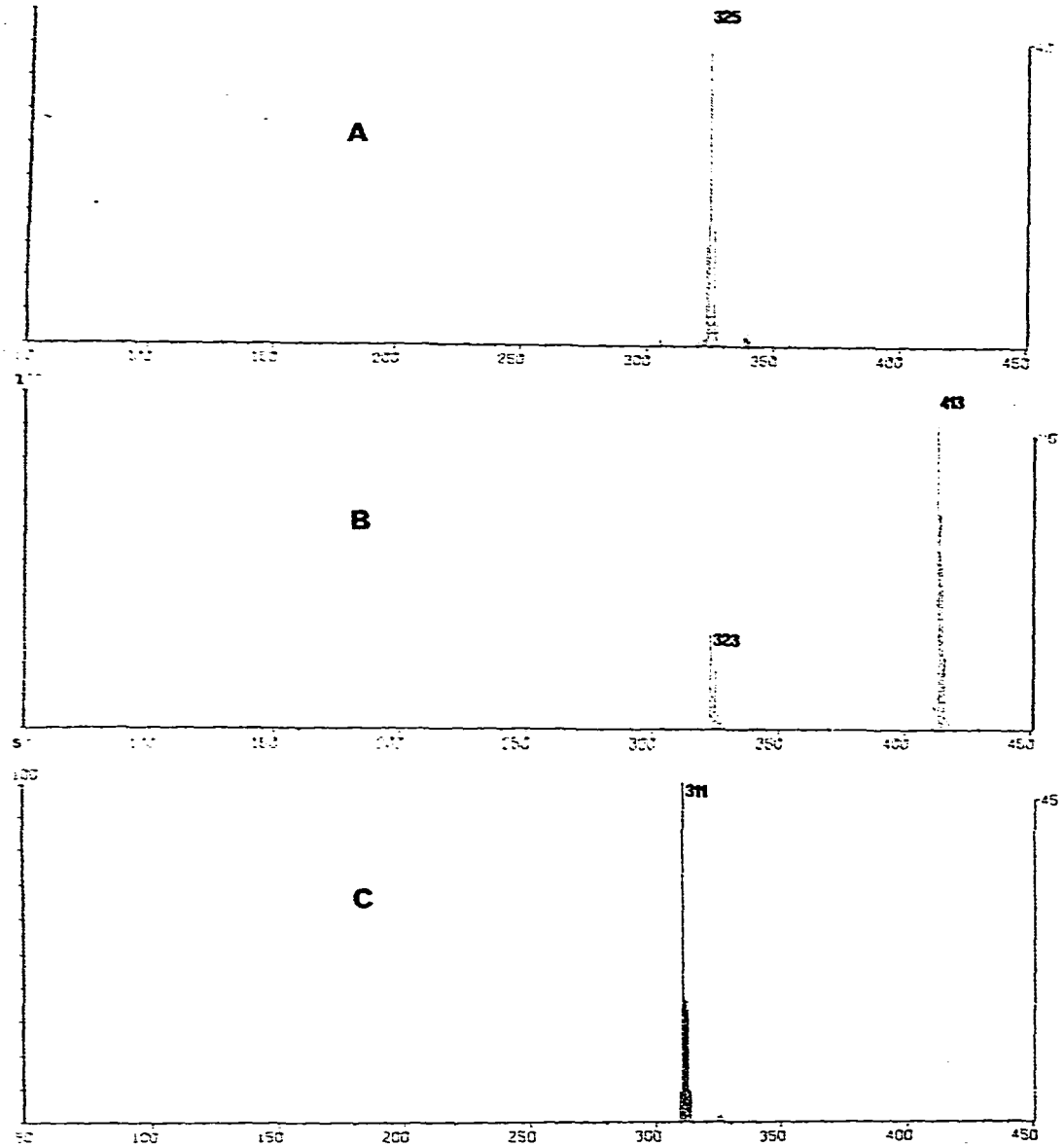


Fig. 1. Methane negative chemical-ionization mass spectra of MDZ (A), trimethylsilylated HMMDZ (B) and DMMDZ (C).

parison of the ion responses from the processed samples to the responses from the injection of known amounts of MDZ, trimethylsilylated HMMDZ and DMMDZ, are 105 ± 6% for MDZ and DMMDZ and 110 ± 3% for HMMDZ.

The plasma concentration—time curves for MDZ and HMMDZ in male volunteers following either an intravenous or an oral dose of MDZ are shown in Figs. 4 and 5, respectively. Assuming that distribution is complete after 2 h, the elimination half-lives of MDZ in the subjects given the intravenous

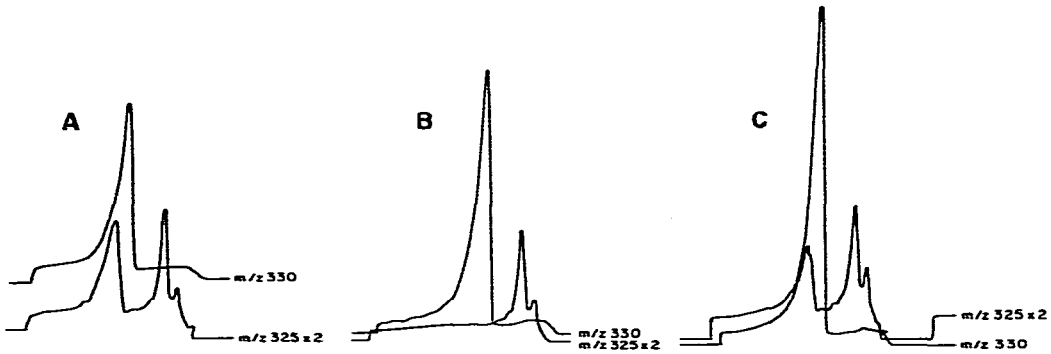


Fig. 2. Selected ion current profiles from the analysis of 1 ml of either control plasma spiked with 3.7 ng of MDZ (A) or plasma from a subject either 0.25 h before (B) or 12 h after (C) receiving a 10-mg oral dose of MDZ maleate. All plasma samples were spiked with 20 ng of MDZ-d_5. The measured concentration of MDZ in the 12-h post-dose sample was 1.3 ng ml^{-1}.

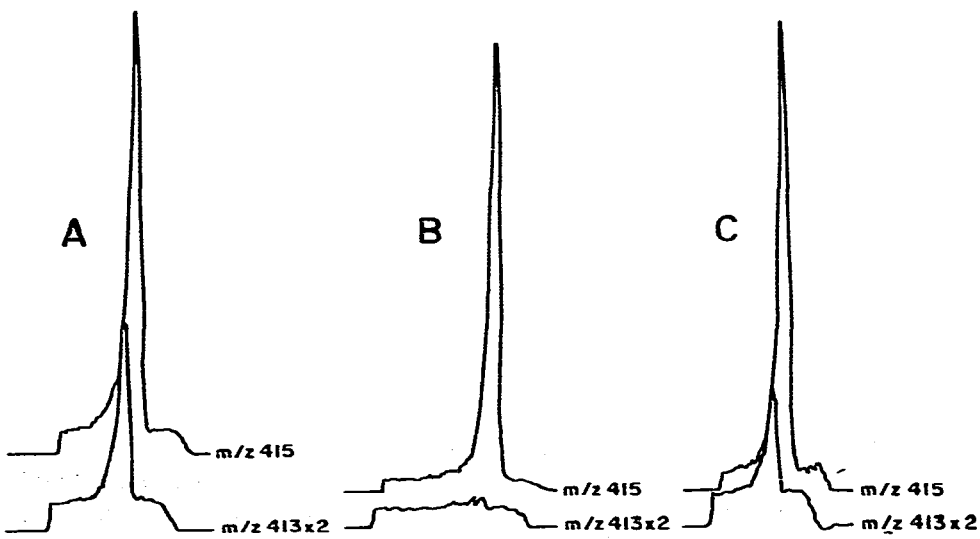


Fig. 3. Selected ion current profiles from the analysis of 1 ml of either control plasma spiked with 5 ng of HMMDZ (A) or plasma from a subject either 0.25 h before (B) or 8 h after (C) receiving a 10-mg oral dose of MDZ maleate. All plasma samples were spiked with 20 ng of HMMDZ-d_2. The measured concentration of HMMDZ in the 8-h post-dose sample was 2.5 ng ml^{-1}.

and oral doses of MDZ are 4 and 3 h, respectively. Following an intravenous dose of MDZ the area under the plasma concentration—time curve for HMMDZ is less than 10% of the area under the plasma concentration—time curve for MDZ. Following oral dosing, however, these areas are approximately equal. The half-times for HMMDZ following both routes of administration are similar, approximately 3 h, which suggests that HMMDZ will not accumulate following daily administration of MDZ.

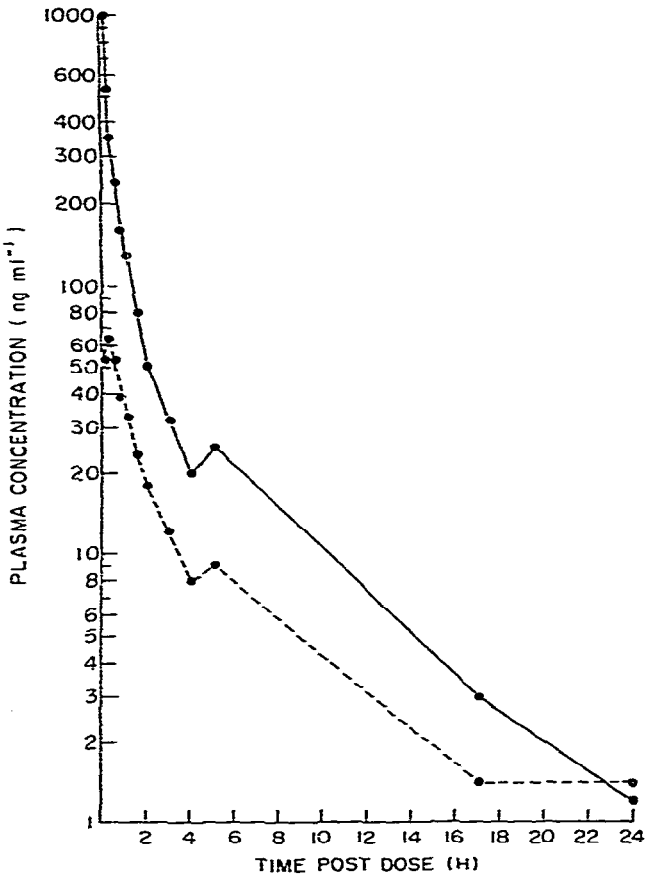


Fig. 4. Plasma concentration—time curve for a subject who had received a 20-mg intravenous dose of MDZ maleate. (——) MDZ; (— — —) HMMDZ.

In all of the plasma samples whose analyses are given in Figs. 4 and 5 and in all other plasma samples analyzed using this GC—MS method, no measurable levels of DMMDZ were found, although in a few of the samples a small, but non quantifiable, response for DMMDZ was observed. Eberts [13] has suggested that the desmethyl metabolite of triazolam, a benzodiazepine structurally similar to MDZ, results from the spontaneous loss of CO_2 from a carboxylic acid metabolite of triazolam which is generated from further oxidation of triazolam's hydroxymethyl metabolite. Experiments in our laboratory suggest that the corresponding carboxylic acid metabolite of MDZ will decompose to DMMDZ on standing at room temperature in plasma for many days. Nevertheless, our results suggest that DMMDZ is not present in sufficient amounts in normally preserved plasma from subjects given MDZ to interfere with the published EC-GC or RIA assays for MDZ at their quoted limits of quantitation.

CONCLUSIONS

A sensitive and specific GC—MS procedure has been described which can measure MDZ and HMMDZ in plasma following a therapeutic dose of the

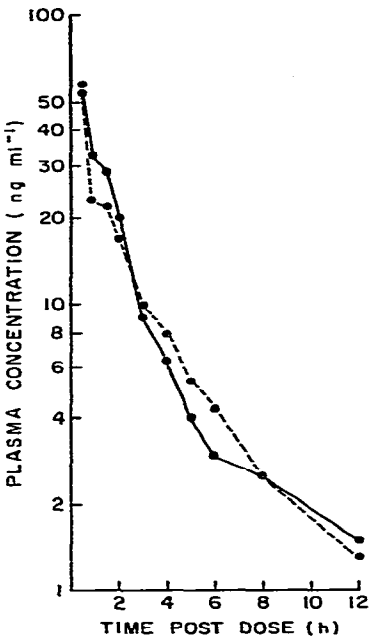


Fig. 5. Plasma concentration—time curve for a subject who had received a 10-mg oral dose of MDZ maleate. (——) MDZ; (— — —) HMMDZ.

drug. Another metabolite of MDZ, DMMDZ, could not be measured in plasma following a therapeutic dose of the drug.

ACKNOWLEDGEMENT

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REFERENCES

1 J.T. Conner, R.L. Katz, R.R. Pagano and C.W. Graham, Anesth. Analg., 57 (1978) 1.
2 R.J. Fragen, F. Gahl and N. Caldwell, Anesthesiology, 49 (1978) 41.
3 J.G. Reves, G. Corssen and C. Holcomb, Can. Anaesth. Soc. J., 25 (1978) 211.
4 M.T. Smith, M.J. Eadie and T. O'Rourke Brophy, Eur. J. Clin. Pharmacol., 19 (1981) 271.
5 C.V. Puglisi, J.C. Meyer, L. D'Arconte, M.A. Brooks and J.A.F. de Silva, J. Chromatogr., 145 (1978) 81.
6 D.J. Greenblatt, A. Locniskar, H.R. Ochs and P.M. Lauven, Anesthesiology, 55 (1981) 176.
7 R. Dixon, R. Lucek, D. Todd and A. Walser, Res. Commun. Chem. Pathol. Pharmacol., 37 (1982) 11.
8 S. Kolis, Hoffmann-La Roche Inc., Nutley, NJ, U.S.A., personal communication.
9 W.A. Garland and B.H. Min, J. Chromatogr., 172 (1979) 279.
10 W.A. Garland, R.R. Muccino, B.H. Min, J. Cupano and W.E. Fann, Clin. Pharmacol. Ther., 25 (1979) 844.
11 W.A. Garland and B.J. Miwa, Environ. Health Persp., 36 (1980) 69.
12 B.J. Miwa, W.A. Garland and P. Blumenthal, Anal. Chem., 53 (1981) 793.
13 F.S. Eberts, Drug Metab. Dispos., 5 (1977) 547.