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SIMULTANEOUS DETERMINATION OF MIDAZOLAM AND ITS THREE HYDROXY METABOLITES IN HUMAN PLASMA BY ELECTRON-CAPTURE GAS CHROMATOGRAPHY WITHOUT DERIVATIZATION

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SUMMARY

Electron-capture gas chromatography was carried out to determine midazolam and its three hydroxy metabolites (1-hydroxymethylmidazolam, 4-hydroxymidazolam and 1-hydroxymethyl-4-hydroxymidazolam) in human plasma. The assay involves extraction from plasma, buffered to pH 9.3, into cyclohexane–dichloromethane (6.4) and analysis by gas chromatography. The use of an HP-17 cross-linked, capillary column makes derivatization unnecessary The sensitivity of the method was 2–3 ng/ml for midazolam, 1-hydroxymethylmidazolam and 4-hydroxymidazolam, and 20 ng/ml for 1-hydroxymethyl-4-hydroxymidazolam. The extraction recovery of midazolam, 1-hydroxymethylmidazolam, 4-hydroxymidazolam and 1-hydroxymethyl-4-hydroxymidazolam was 99.3 ± 2.4, 67.0 ± 4.6, 92 7 ± 4.7 and 28.7 ± 6.3%, respectively. This gas chromatographic assay was used to assess the concentration-time profiles of midazolam and its metabolites in human plasma after rectal and intravenous administration of midazolam

INTRODUCTION

Midazolam is a 1,4-benzodiazepine with strong hypnotic properties, a suitable drug for use as premedication and as an induction agent for anaesthesia [1,2]. Midazolam possesses a high potency and short half-life. A high potency means a low therapeutic dose and thus low plasma concentrations after single administration. The metabolic pathway of midazolam is shown in Fig. 1. The (partly active) metabolites that have already been identified are 1-hydroxymethylmidazolam (1-OH), 4-hydroxymidazolam (4-OH) and 1-hydroxy-

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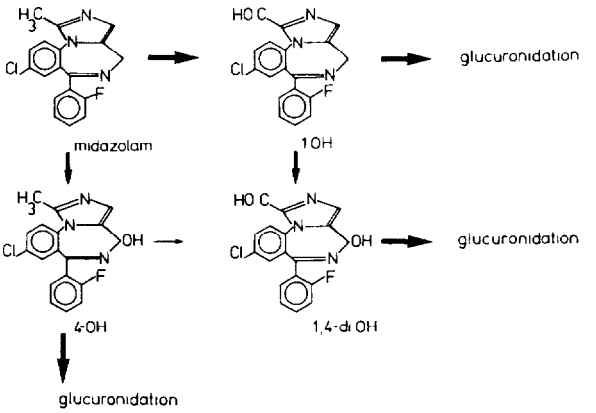

Fig. 1. Structures of midazolam and its main metabolites 1-hydroxymethylmidazolam (1-OH), 4-hydroxymidazolam (4-OH) and 1-hydroxymethyl-4-hydroxymidazolam (1,4-diOH). The thickness of the arrows represents the relative importance of the metabolic reactions.

methyl-4-hydroxymidazolam (1,4-diOH) [3]. The main metabolic route of midazolam in humans is the formation of the active metabolite 1-OH. This main metabolite is further conjugated to form the inactive 1-OH-glucuronide. The plasma concentrations of unconjugated 4-OH and 1,4-diOH are low, because they constitute a minor pathway, and as soon as they are formed they undergo further glucuronidation [3,4].

The aim of this investigation was to determine midazolam and its major metabolites simultaneously in plasma, obtained from volunteers after single intravenous (i.v.) and rectal administration of midazolam in a dose of 0.1 and 0.2 mg/kg body weight, respectively.

In order to perform an assay of both polar and apolar compounds after relatively low doses in one system, a combination of high selectivity and relatively low detection limits (± 5 ng/ml) for midazolam and its three metabolites had to be obtained. These two elements have been reported before, but not combined in one system. For example, the use of high-performance liquid chromatography (HPLC) gives a very high selectivity for midazolam and its three metabolites but a detection limit of only 30–40 ng/ml [4,5]. Two more sensitive HPLC methods with a detection limit of 15–25 ng/ml determine only midazolam and its major metabolite, 1-OH [6,7]. Gas chromatography (GC) gives selectivity and sensitivity problems without derivatization [7–14], except for the method developed by Arendt et al. [15], which determines midazolam and *two* hydroxy metabolites with a detection limit of 1–3 ng/ml for midazolam and 4-OH and 5–10 ng/ml for 1-OH. However, the use of benzene as an extraction solvent in this method is undesirable.

We report here a simple and rapid GC method for the simultaneous determination of midazolam and its three hydroxy metabolites in human plasma.

EXPERIMENTAL

Materials and reagents

Midazolam and its hydroxy metabolites were kindly provided by Hoffmann-La Roche (Mijdrecht, The Netherlands). The structures of midazolam and its metabolites are given in Fig. 1. The internal standard, diazepam, was obtained from Bergel (Hoofddorp, The Netherlands). Methanol and acetone were obtained from Baker Chemicals (Deventer, The Netherlands). Dichloromethane, cyclohexane (> 99.8% pure), toluene and borax ($Na_2B_4O_7 \cdot 10H_2O$) were obtained from Merck (Darmstadt, F.R.G.). All chemicals were of analytical grade and used without further purification. Blank plasma for the preparation of standard samples was obtained from the hospital's blood bank.

Apparatus

Chromatography was performed on a Hewlett-Packard Model 5710 A gas chromatograph, equipped with a 15-mCi ^{63}Ni electron-capture detector. The detector was connected to a CR-6A integrator from Chrompack (Middelburg, The Netherlands).

The column was an HP-17 cross-linked capillary wide-bore column (50% Ph-Me-silicone; 10 m × 0.53 mm I.D., 2.0 μm film thickness) from Hewlett-Packard (Amstelveen, The Netherlands).

The injection system consisted of a disposable glass insert, which was used without any pretreatment. The injection inlet was closed by a septum (Chromsep red 10 mm × 11.1 mm) from Chrompack.

The instrumental parameters for the GC system were: detector/injector temperature, 300°C; column temperature, 267°C; column flow-rate, 6.7 ml/min; total flow-rate (column plus make-up), 10 ml/min; column and make-up gas, argon–methane (9:1).

The concentrations of midazolam and its metabolites were calculated with an internal standard method using peak-height ratios.

Preparation of analytical standards

Stock solutions of midazolam, 1-OH and 4-OH were prepared in methanol at a concentration of 1 mg/ml. Stock solutions of 1,4-diOH and diazepam in methanol had concentrations of 0.5 and 0.6 mg/ml, respectively. The concentration of the standard solutions, made from the stock solutions, were 1000 ng/ml for midazolam and its metabolites and 600 ng/ml for diazepam. Stock and standard solutions were stored in amber bottles at 4°C.

The standard samples were prepared from the standard solutions. The recoveries were determined for two concentrations in plasma for all four compounds in six-fold. Standard solutions of appropriate concentrations were spiked into blank plasma to give concentrations of 30 and 300 ng/ml. These were extracted as described below, under *Extraction procedure*, except that the

internal standard was left out and the residue was reconstituted in 200 μl of toluene–methanol–acetone (80:15:5), to which diazepam was added to give a concentration of 240 ng/ml. In this way diazepam was used as an external standard in the same concentration as when used as an internal standard. Peak-height ratios of these extracted samples were compared with those of unextracted samples prepared identically, except for the addition of blank plasma and the extraction step.

The calibration range was based on the expected concentration of midazolam and its metabolites in plasma, obtained from volunteers, and established in the range 5–600 ng/ml for midazolam, 1-OH and 4-OH and 20–600 ng/ml for 1,4-diOH. Eight-point calibration curves were made in quadruplicate.

For the inter- and intra-day variation tests ($n=6$ and $n=5$, respectively), the concentrations were 20 and 200 ng/ml.

For the preparation of standard samples, clean glass-stoppered extraction tubes were spiked with the appropriate amount of the (internal) standard solutions and evaporated to dryness. The residue was redissolved in 1.0 ml of blank plasma and treated as described under *Extraction procedure*. The amount of internal standard for all the standard samples was 80 μl × 600 ng/ml = 48 ng.

For the inter-day variation test, two fresh standards were made daily, which were used to calibrate the integrator.

Extraction procedure

To clean glass-stoppered extraction tubes, 80 μl of internal standard solution (600 ng/ml diazepam) were added and evaporated to dryness. Then 1.0 ml of plasma, 0.75 ml of borax buffer ($Na_2B_4O_7 \cdot 10H_2O$ at a concentration of 0.05 g/ml, pH 9.3) and 7 ml of cyclohexane–dichloromethane (6:4) were introduced consecutively into the tubes, mixed on a Vortex mixer for 1 min and centrifuged for 5 min at 4000 *g*. After centrifugation most of the organic phase (upper layer) was transferred into 12-ml conical glass tubes and evaporated under a steam of nitrogen at 37°C. The residue was reconstituted in 200 μl of toluene–methanol–acetone (85:15:5), and 1 μl was injected onto the gas chromatograph.

RESULTS

Fig. 2A shows the chromatogram of an unextracted standard sample of midazolam, 1-OH, 4-OH and 1,4-diOH at a concentration of 20 ng/ml and the internal standard diazepam at 48 ng/ml. The retention times of the five compounds are 6.4, 7.5, 5.2, 17.0 and 4.3 min, respectively. The chromatogram of an extract of the same concentrations from spiked plasma is shown in Fig. 2B. Fig. 2C shows the chromatogram of a blank plasma sample, obtained from a

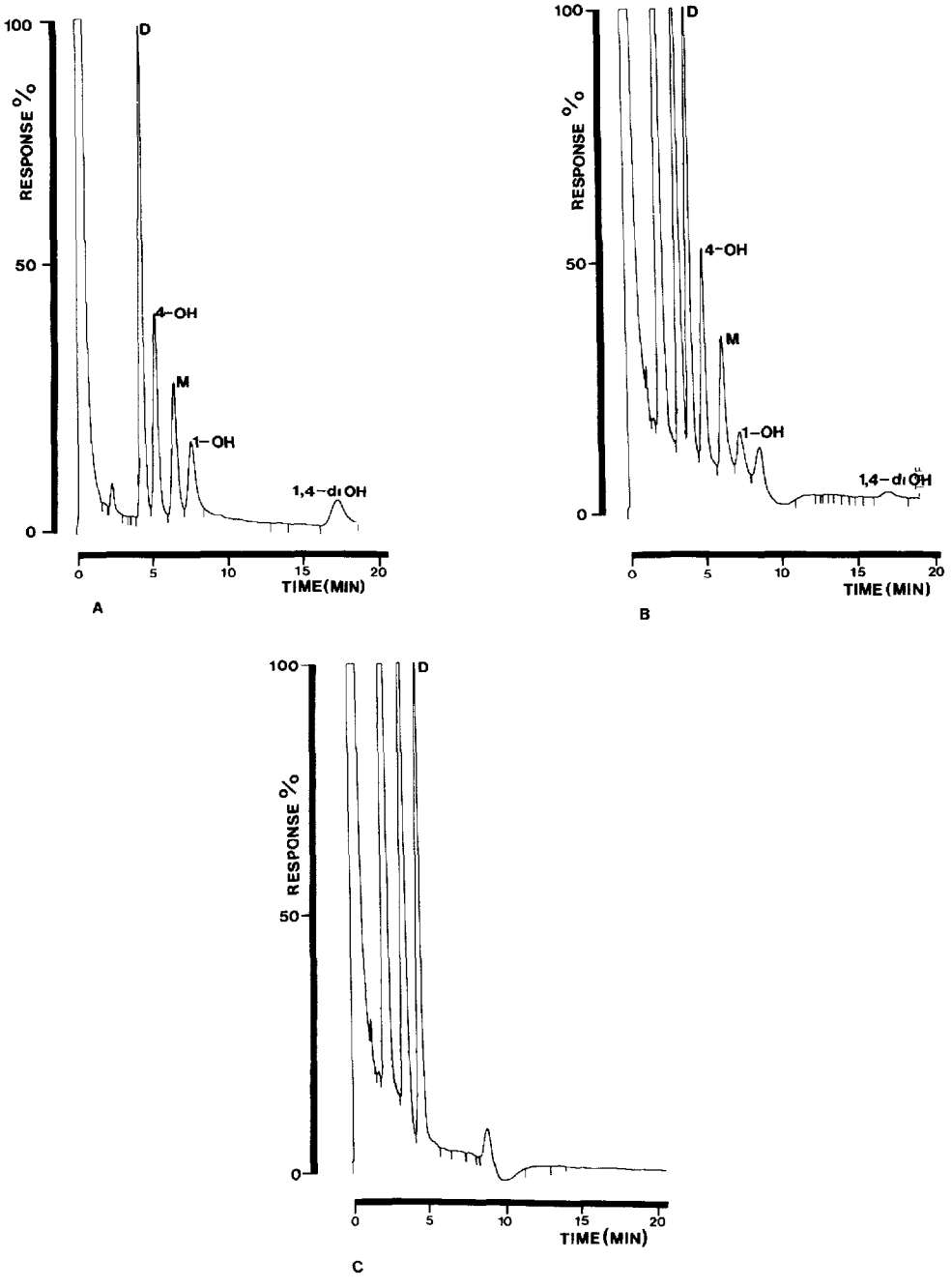

Fig. 2. Chromatograms of (A) unextracted standard sample containing 20 ng/ml midazolam and its metabolites, (B) extracted standard sample containing 20 ng/ml midazolam and its metabolites and (C) blank plasma sample. Peaks: D = diazepam; 4-OH = 4-hydroxymidazolam; M = midazolam; 1-OH = 1-hydroxymethylmidazolam; 1,4-diOH = 1-hydroxymethyl-4-hydroxymidazolam.

TABLE I

PRECISION DATA FOR MIDAZOLAM AND ITS THREE HYDROXY METABOLITES AT CONCENTRATIONS OF 20 AND 200 ng/ml

Compound	Coefficient of variation (%)			
	Intra-day variation ($n=6$)		Inter-day variation ($n=5$)	
	20 ng/ml	200 ng/ml	20 ng/ml	200 ng/ml
Midazolam	1.9	0.8	3.4	4.5
1-OH	7.2	3.5	9.9	5.3
4-OH	1.5	1.2	6.5	2.8
1,4-diOH	8.2	4.9	4.2	6.5

[a]The relative limit of detection is equal to the concentration where the signal is three times the relative standard deviation of the noise. The noise is the response from a blank plasma measured at the retention time of the respective compounds.

volunteer shortly before midazolam was administered and reveals the absence of interfering peaks at the retention times of the measured compounds.

The recoveries of midazolam, 1-OH, 4-OH and 1,4-diOH from plasma are 99.3 ± 2.4, 67.0 ± 4.6, 92.7 ± 4.7 and $28.7 \pm 6.3\%$, respectively (mean $\pm$ S.D.).

The eight-point calibration curves have an excellent linearity for midazolam, 1-OH and 4-OH in the concentration range 5–600 ng/ml and for 1,4-diOH in the range 20–600 ng/ml. The correlation coefficients average 0.998, 0.989, 0.998 and 0.995 for midazolam, 1-OH, 4-OH and 1,4-diOH, respectively ($n=4$).

The relative detection limit[a] of the method is 2–3 ng/ml for midazolam and its metabolites 1-OH and 4-OH and 20 ng/ml for 1,4-diOH.

The results of the inter- and intra-day variation tests are presented in Table I. The precision data show an intra-day variation of less than 5% for a plasma concentration of 200 ng/ml and less than 9% for 20 ng/ml for all compounds. The inter-day variation is less than 7% for 200 ng/ml and less than 10% for 20 ng/ml for all compounds.

DISCUSSION

We used electron-capture detection for midazolam and its metabolites, because of its good sensitivity for these halogenated compounds [9–15]. Flurazepam, prazepam, methylnitrazepam and desalkylflurazepam were unsuitable as internal standards, because of interference with midazolam and its metabolites or because of a poorer reproducibility than diazepam.

Derivatization of midazolam or its metabolites with bis(trimethylsilyl)trifluoroacetamide (BSTFA) [9,10,12,14], a reagent com-

monly used to enhance the detection and selectivity of polar compounds, was not necessary with our method. The detection limit was 2–3 ng/ml for midazolam, 1-OH and 4-OH and 20 ng/ml for 1,4-diOH without derivatization. The selectivity was excellent on the HP-17 column.

A low recovery percentage of 1,4-diOH was found ($28.7 \pm 6.3\%$), presumably because it is more polar than the other compounds and as a consequence of the single extraction procedure, which was deliberately chosen for convenience and simplicity. The use of an automatic sampler for convenience with these kinds of batch sample was not possible. Adsorption of especially 1-OH on the glass syringe was observed, for which the rinsing program of the autosampler was insufficient.

To obtain a good injection reproducibility the following recommendations are of paramount importance: (a) thorough rinsing of the injection syringe with the reconstitution solvent (toluene–methanol–acetone, 80:15:5); (b) replacement of the septum and glass insert after ca. thirty injections; (c) injection of an extracted blank plasma to verify the absence of adsorbed compounds (especially 1-OH) prior to every new sample injection; (d) injection of several standard samples prior to analysis.

The described method was used to assess plasma concentration–time profiles and the pharmacokinetics of midazolam and its metabolites in human plasma after a single i.v. administration of midazolam in a dose of 0.1 mg/kg and after a single rectal administration of 0.2 mg/kg body weight.

Fig. 3 shows the chromatogram of an extracted plasma sample taken from

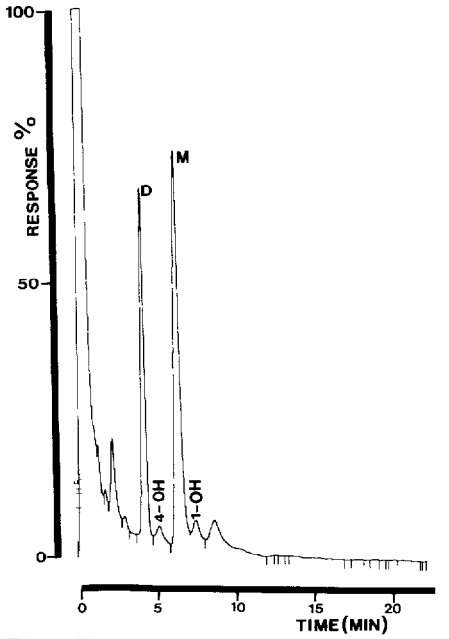

Fig. 3. Chromatogram of extracted plasma sample collected 60 min after administration of 0.1 mg/kg midazolam by i.v. bolus injection.

one volunteer 60 min after i.v. administration. The figure indicates that midazolam is detectable at a concentration of 57.5 ng/ml, whereas only minute amounts of 1-OH and 4-OH are detectable (8.5 and ca. 5 ng/ml, respectively). 1,4-diOH was not detectable.

Figs. 4 and 5 show the mean plasma concentration–time profiles of midazolam and its main metabolite 1-OH after rectal and i.v. administration to six healthy male volunteers. The i.v. pharmacokinetics of midazolam derived from Fig. 5 are in good agreement with previously reported profiles [4,9.11]. The pharmacokinetic parameters of midazolam and 1-OH after rectal administration can easily be derived from Fig. 4 using standard methods.

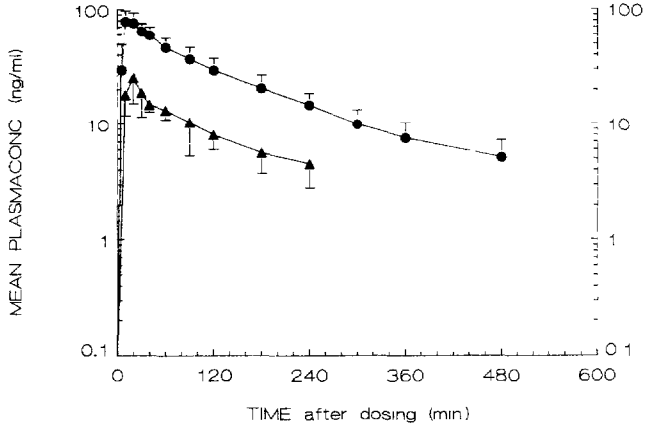

Fig. 4. Mean plasma concentration-time profiles of midazolam (●) and 1-hydroxymethylmidazolam (▲) after rectal administration of 0.2 mg/kg midazolam to six healthy male volunteers.

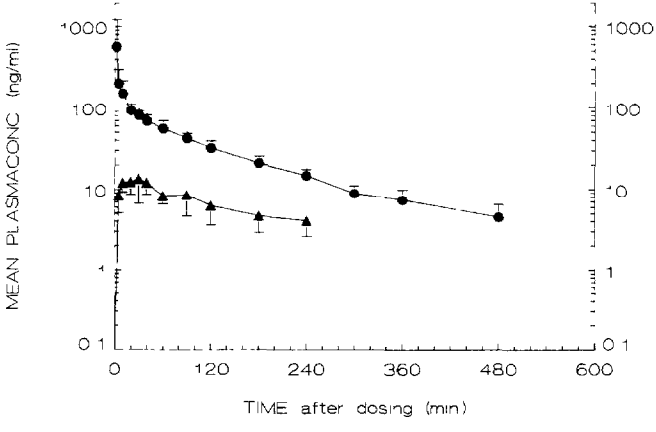

Fig. 5. Mean plasma concentration-time profiles of midazolam (●) and 1-hydroxymethylmidazolam (▲) after i.v. bolus administration of 0.1 mg/kg midazolam to six healthy male volunteers.

The plasma concentration–time profile of 4-OH is not plotted in these figures. The maximum plasma concentration of 4-OH is low (ca. 5 ng/ml) and is reached 40–60 min after dosing. After 4–6 h 4-OH is no longer detectable.

The metabolite 1,4-diOH was not detected in this midazolam study, probably owing to its minor formation, its fast glucuronidation and/or its low extraction recovery. The latter (ca. 29%) is substantially lower than that of midazolam, 1-OH or 4-OH (67–99%). This is a consequence of 1,4-diOH being the most polar of the three metabolites, and the use of a single extraction procedure.

CONCLUSION

We have developed and performed a simple and rapid GC method for the simultaneous determination of midazolam and its three hydroxy metabolites in human plasma without complicated derivatization or extraction techniques.

Our method has two important aspects. It combines a high selectivity for underivatized compounds by the use of a 50% Ph-Me-silicone capillary wide-bore column and a low detection limit of 2–3 ng/ml for midazolam, 1-OH and 4-OH and 20 ng/ml for 1,4-diOH in plasma samples. This combination of high selectivity and low detection limit has been published before only for the combined assay of midazolam and two of its three active metabolites [15]. Our method enabled us to perform a clinical study with midazolam that measured plasma concentration–time profiles of midazolam and its major metabolites after a single i.v. or rectal administration for a sufficient time period of 6–8 h.

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