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Short Communication

Solid-phase extraction of midazolam and two of its metabolites from plasma for high-performance liquid chromatographic analysis

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ABSTRACT

A rapid, sensitive and selective assay of midazolam and two of its metabolites in plasma, based on high-performance liquid chromatography, has been developed. The compounds are subjected to solid-phase extraction, using C_{18} cartridges (Bond-Elut). Recoveries are in excess of 90% for midazolam and its metabolites. The limit of quantitation of the assay is 50 ng/ml of plasma for each compound.

INTRODUCTION

Midazolam is a benzodiazepine, 8-chloro-6-(2-fluorophenyl)-1-methyl-4*H*-imidazo[1,5-*a*][1,4]-benzodiazepine, which has different indications according to its mode of administration [1]. Parenterally administered, it is a short-acting anaesthesia-inducing agent [2]. Given orally, it exhibits hypnotic properties [3]. Midazolam is being increasingly used in children for premedication before anaesthesia, as it is reported to have a short half-life and rapid action [4], especially by the rectal route [5].

For many years, midazolam and most of its metabolites have been assayed by specific and sensitive methods. These include gas chromatography [6–13] and high-performance liquid chromatography (HPLC) [5,8,12,14–17].

The method presented here is for the assay of midazolam (I) and two of its metabolites, 1-hydroxymethylmidazolam (II) and 4-hydroxymidazolam (III) by reversed-phase HPLC using a UV detector (operating at 254 nm). The feature of this method is the way in which the compounds are extracted from plasma. Solid-phase extraction is used, unlike the other methods which always involve liquid–liquid extraction. This method of extraction has various advantages compared with liquid–liquid extraction, particularly convenience and rapidity [18,19].

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EXPERIMENTAL

Materials

The solid-phase extraction was performed using the Vac-Elut system (Analytichem International, Harbor City, CA, USA) and C_{18} 100-mg Bond-Elut cartridges (Analytichem International).

The HPLC system consisted of successive Merck-Hitachi (Darmstadt, Germany components: gradient (L5000 LC controller), pump (655 A-11 liquid chromatograph), detector (LCM system variable-wavelength monitor), integrator (DS 2000 chromato-integrator). A LiChrocart 4 mm × 4 mm I.D. LiChrosorb 100 RP 18 5-μm precolumn (Merck) and a LiChrocart 125 mm × 4 mm I.D. LiChrospher 100 RP 18 endcapped 5-μm analytical column (Merck) were used.

Chemicals

All reagent were of analytical-reagent grade (>99% purity). They included acetonitrile (Prolabo, Paris, France), methanol (Carlo Erba, Milan, Italy) and tetrahydrofuran (Fluka, Buchs, Switzerland).

Potassium phosphate buffer (0.01 *M*, pH 5.6) was obtained by diluting 10 ml of 1 *M* potassium phosphate buffer (pH 5.6) to 1 l with distilled water. The 1 *M* buffer was prepared by mixing 94.8 ml of 1 *M* potassium dihydrogenphosphate solution (KH_2PO_4, Merck Darmstadt, Germany) with 5.2 ml of 1 *M* dipotassium hydrogenphosphate solution (K_2HPO_4, Merck). Reagents used for solid-phase extraction were methanol, distilled water, acetonitrile–distilled water (30:70, v/v) and acetonitrile–distilled water (15:85, v/v).

Chromatographic parameters

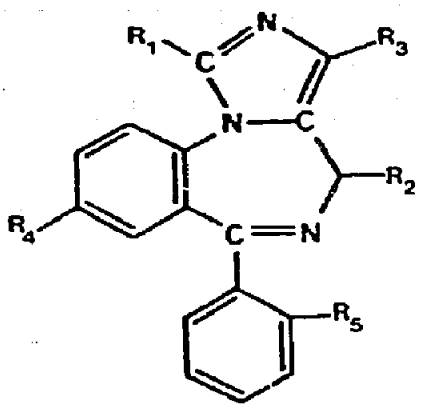
The mobile phase was methanol–acetonitrile–tetrahydrofuran–potassium phosphate buffer (0.01 *M*, pH 5.6) (25:28:2:50, v/v). The analysis was performed in isocratic mode. The flow-rate was 1.3 ml/min. The UV detector wavelength was 254 nm. A constant injection volume was obtained with a 20-μl injection loop.

Standards

The analytical standards used were midazolam (I), 1-hydroxymethylmidazolam (II), 4-hydroxymidazolam (III) and climazolam (IV, internal standard). They were obtained from Hoffman-La Roche (Basel, Switzerland). These compounds were of pharmaceutical-grade purity (>99%), and their structures are given in Fig. 1. Standards of compounds I, II, III and IV were prepared at 1 mg/ml in methanol. Dilutions of these stock solutions were used to prepare the appropriate working standard solutions (mixed standard solutions).

Solid-phase extraction

The extraction procedure consisted of the following four steps.



Compound	R_1	R_2	R_3	R_4	R_5
Midazolam (I)	CH_3	H	H	Cl	F
1-Hydroxymethylmidazolam (II)	CH_2OH	H	H	Cl	F
4-Hydroxymethylmidazolam (III)	CH_3	OH	H	Cl	F
Internal standard, climazolam (IV)	CH_3	H	H	Cl	Cl

Fig. 1. Structures of compounds I, II, III and IV.

Sample preparation. Samples were prepared in haemolysis tubes (for one use), according to their type: (i) preparation of samples used for calibration: 1 ml of control plasma (human plasma from Blood Transfusion Centre of Clermont-Ferrand, France) to which 100 μl of standard solution were added; (ii) preparation of control blank: 1 ml of control plasma to which 100 μl of methanol were added; (iii) preparation of the sample itself: 1 ml of patient's plasma to which 100 μl of internal standard solution were added.

These plasma samples were deproteinated by the addition of 1 ml of acetonitrile–water (30:70). After homogenization (vortex-mixing for 10 s), the samples were centrifuged for 5 min at 4000 *g*.

Conditioning of the Bond-Elut cartridges. Methanol (2 ml) and then distilled water (2 ml) were run through the cartridges. This was followed by passage of the plasma sample (first prepared) through the conditioned cartridges.

Washing. Acetonitrile–water (15:85) (2 ml) was run through the cartridges, and these were then left to dry for 3 or 4 min.

Elution. The cartridges were eluted with four 200-μl volumes of methanol, and the eluate was evaporated under nitrogen. The residue was taken up in 100 μl of methanol.

TABLE I

VALIDATION OF THE METHOD: INTER-DAY ASSAY VARIABILITY

Concentration added (μg/ml)	Compound I		Compound II		Compound III	
	Concentration found[a] (μg/ml)	Coefficient of variation (%)	Concentration found[a] (μg/ml)	Coefficient of variation (%)	Concentration found[a] (μg/ml)	Coefficient of variation (%)
0.050	0.051 ± 0.005	9.8	0.057 ± 0.007	12.3	0.059 ± 0.006	10.2
0.100	0.101 ± 0.008	7.9	0.102 ± 0.012	11.8	0.103 ± 0.008	7.8
0.200	0.202 ± 0.008	4.0	0.199 ± 0.017	8.5	0.205 ± 0.013	6.3
0.300	–	–	0.284 ± 0.023	8.1	0.313 ± 0.010	3.2
0.400	0.383 ± 0.017	4.4	–	–	–	–
1.000	0.992 ± 0.041	4.1	0.985 ± 0.023	2.3	1.050 ± 0.038	3.6
Average		6.0		8.6		6.2

[a] Mean ± standard deviation; $n = 10$.

TABLE II

VALIDATION OF THE METHOD: INTER-DAY ASSAY VARIABILITY

Concentration added (μg/ml)	Compound I		Compound II		Compound III	
	Concentration found[a] (μg/ml)	Coefficient of variation (%)	Concentration found[a] (μg/ml)	Coefficient of variation (%)	Concentration found[a] (μg/ml)	Coefficient of variation (%)
0.050	0.049 ± 0.003	6.1	0.058 ± 0.004	6.9	0.053 ± 0.002	3.8
0.100	0.105 ± 0.004	3.8	0.104 ± 0.009	8.6	0.108 ± 0.008	7.4
0.200	0.214 ± 0.011	5.1	0.206 ± 0.015	7.3	0.216 ± 0.016	7.4
0.300	–	–	0.316 ± 0.017	5.4	0.320 ± 0.014	4.2
0.400	0.384 ± 0.021	5.4	–	–	–	–
1.000	1.043 ± 0.027	2.6	0.979 ± 0.028	2.9	1.052 ± 0.033	3.1
Average		4.6		7.0		5.2

[a] Mean ± standard deviation; $n = 10$.

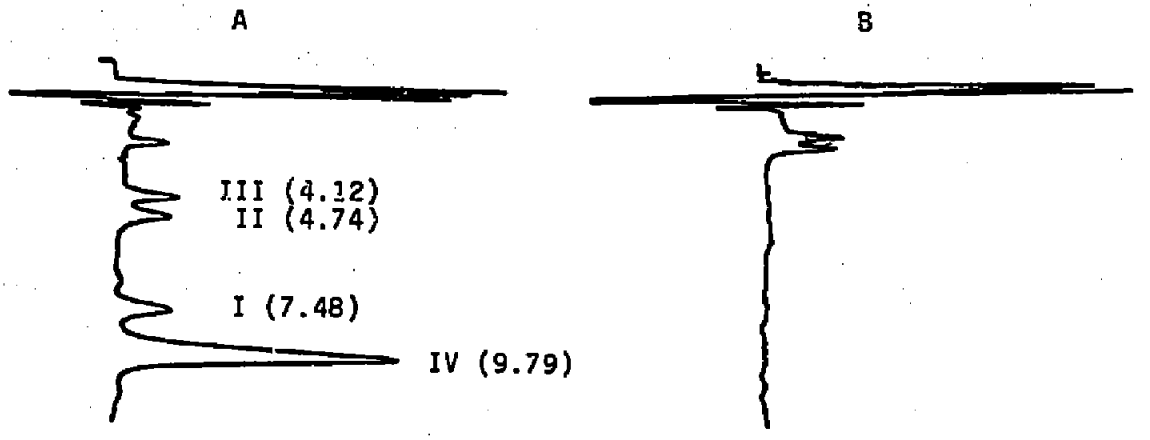


Fig. 2. (A) Chromatogram of a plasma extract containing 0.1 μg/ml I, II and III and 1 μg/ml internal standard (IV). (B) Chromatogram of a blank plasma extract.

Calibration

Calibration curves were obtained by plotting the peak-area ratios (the peak area of compounds I, II or III to the peak area of the internal standard, IV) as a function of the concentration of I, II or III per ml of plasma. The concentration of compound I, II or III in a patient sample was calculated by interpolation from the standard curve (power equation: $y = ax + b$).

RESULTS

Fig. 2 shows a chromatogram of a plasma sample containing 0.1 μg/ml of each compound (I, II and III), and a chromatogram of a blank plasma extract. The relationships between the concentrations of compounds I, II and III and the peak-area ratios demonstrated a linear response to the UV detector for concentrations in the range 0.05–1 μg/ml (the correlation coefficient was 0.999 for all three compounds), with slopes of 0.0017, 0.0013 and 0.0011 for I, II and III respectively, and corresponding intercepts of −0.020, −0.018 and −0.016. Table I and II show the precision of the method for compounds I, II and III, which was evaluated with the intra- and inter-assay variabilities.

The limit of quantitation was 0.05 μg/ml of plasma for each compound. The overall recoveries of I, II and III from plasma were 94.5 ± 6.1, 93.2 ± 5.9 and 93.7 ± 4.2%, respectively.

DISCUSSION

Several papers have described HPLC methods for the analysis of midazolam and its metabolites, but they used liquid–liquid extraction. In this study, the solid-phase extraction was preferred because of its rapidity, its convenience, the low volume of solvents required, the small amount of plasma required and the

good recoveries. However, care should be taken at the evaporation step, which is performed under nitrogen flow. It is important to monitor the nitrogen flow to ensure that it is slow and constant, to ensure minimal loss of product.

For the analysis, the pH of the potassium phosphate buffer in the mobile phase plays an important role. The assay was first carried out at pH 7.4. Then the pH was reduced, to favour the separation of the peak of metabolite III from the peak of metabolite II. A pH value of 5.6 was finaliy chosen.

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