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Short communication

Determination of midazolam and its unconjugated 1-hydroxy metabolite in human plasma by high-performance liquid chromatography

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Abstract

A selective and sensitive high-performance liquid chromatographic method for the analysis of midazolam and its unconjugated 1-hydroxy metabolite in plasma samples was developed. The compounds were extracted from plasma by a liquid–liquid extraction procedure with diethyl ether. Mean analytical recoveries were 87% and 86% at a concentration of 300 ng/ml for midazolam and 1-hydroxymidazolam, respectively, and the quantification limit was 2 ng/ml for a plasma volume of 1 ml. The separation of midazolam, 1-hydroxymidazolam and flurazepam (internal standard) was achieved on a Spherisorb 5 CN column using methanol–2-propanol (75:25, v/v) containing 0.015% perchloric acid at a flow-rate of 1.5 ml/min. The method is sensitive enough for monitoring midazolam and also the unconjugated form of the active metabolite in plasma during pharmacokinetic studies.

1. Introduction

Midazolam is a weakly basic benzodiazepine widely used in anaesthesia and for sedation of mechanically ventilated patients in intensive care units [1]. In man, midazolam is predominantly metabolised by hepatic oxidation to 1-hydroxymidazolam and 4-hydroxymidazolam. The major metabolite, 1-hydroxymidazolam, has a shorter elimination half life than midazolam; however, it may contribute to the pharmacodynamic effect of the drug [2]. The effect of the metabolite is further reduced by glucuronidation [3]. Limited data are available on the circulating concentration of the unconjugated form of 1-hydroxymidazolam in plasma essentially due to the lack of sensitivity of the methods published previously.

Several chromatographic methods [4–13] have been reported for the analysis of midazolam and metabolites in plasma. Gas chromatographic methods [4–7] appear more sensitive than HPLC methods; however, they require sample derivatization for the simultaneous analysis of

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midazolam and the hydroxylated metabolite. The limits of quantification of the HPLC methods [8–13] range from 5 to 50 ng/ml for a minimum sample volume of 1 ml. More sensitive methods are required for accurate drug monitoring and the investigation of midazolam and 1-hydroxymidazolam in human plasma.

This paper describes a sensitive, isocratic HPLC method for the determination of midazolam and the unconjugated form of 1-hydroxymidazolam. The method was applied to drug monitoring in intensive care patients.

2. Experimental

2.1. Chemicals

Midazolam, 1-hydroxymidazolam and internal standard, flurazepam, were generously supplied by Hoffman-La Roche (Basle, Switzerland). Biorad drug-free serum was used for the calibration curve (Biorad, Ivry-sur-Seine, France).

Methanol, 2-propanol, ethanol (both of Uvasol grade), diethyl ether (pro-analysi), 70% perchloric acid (Suprapur) were purchased from Merck (Nogent-sur-Marne, France). Borate buffer (Normapur) was from Sigma (Isle d'Abeau, France).

2.2. Apparatus and chromatographic conditions

The chromatographic system consisted of Hewlett-Packard 1050 Series with a computer HP Vectra 486/33 M using HP Chem software and HP Disk Jet 510 (Hewlett-Packard, Evry, France). The column (150 × 4.6 mm I.D.) was packed with Spherisorb CN, 5 μm (Touzart et Matignon, Vitry-sur-Seine, France). The mobile phase consisted of methanol–2-propanol (75:25, v/v) containing 0.015% perchloric acid. The flow-rate was 1.5 ml/min and the detection wavelength was 215 nm. The analyses were performed at ambient temperature.

2.3. Standards

Stock solutions of midazolam, 1-hydroxymidazolam and flurazepam were prepared at a concentration of 100 mg/l in absolute ethanol. Those solutions were diluted in absolute ethanol in order to obtain appropriate working standard solutions, stable at 4°C for 2 weeks.

2.4. Sample collection and storage

Blood samples (5 ml) were collected in heparinized tubes and centrifuged without delay at low temperature. Plasma was decanted and stored at −20°C until analysis. Plasma samples can be stored for up to 6 months at −20°C before analysis without any modification in the concentration of the compounds.

2.5. Extraction procedure

Plasma (1 ml) containing 300 μl of 0.1 *M* borate buffer, pH 9 and 25 μl of internal stan-

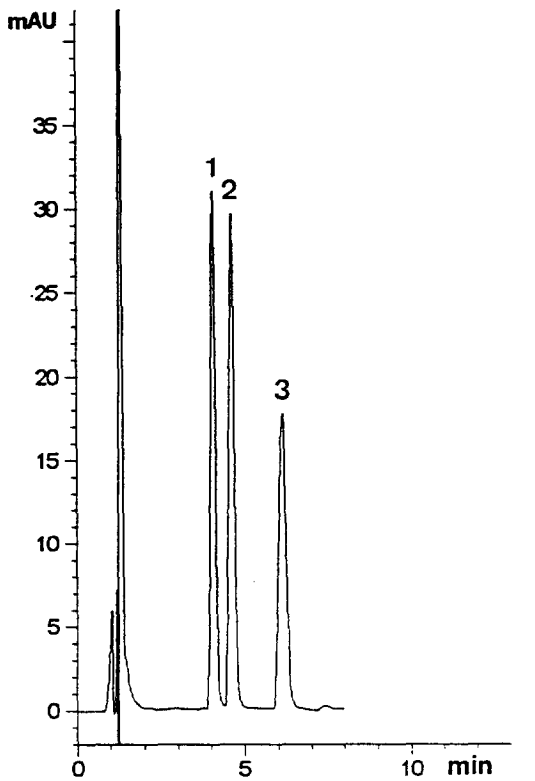

Fig. 1. Chromatographic separation of midazolam, 1-hydroxymidazolam and flurazepam (I.S.). Chromatographic conditions are described in Section 2. Peaks: 1 = 1-hydroxymidazolam (300 ng/ml); 2 = midazolam (300 ng/ml); 3 = flurazepam (300 ng/ml).

dard were extracted with 5 ml of diethyl ether by mixing for 10 min at 60 rpm. After centrifugation at 1500 *g* at 15°C for 5 min, the supernatant was transferred into a conical glass tube and evaporate at 45°C under a gentle nitrogen stream. The dry residue was reconstituted in 100 μl of mobile phase.

3. Results and discussion

Midazolam is a benzodiazepine with an imidazole ring which confers the basicity of the molecule.

The retention of midazolam, 1-hydroxymidazolam and flurazepam (I.S.) was strongly influenced by the concentration of the acid. An increase of perchloric acid content decreased significantly the retention of the compounds and this effect was used to reduce the mobile phase flow-rate to 1.5 ml/min compared to 3 ml/min flow-rate used by Blackett et al. [11]. The retention behaviour of the compounds was less affected by the concentration of methanol and 2-propanol. From these data, the concentration of the organic modifiers and the acid content was defined to achieve the optimal separation of the compounds with an analysis time less than 7 min (Fig. 1).

Analytical recovery of midazolam, 1-hydroxymidazolam and internal standard was determined by addition of known concentration of compounds to plasma and comparison of peak area with those obtained by direct injection of standards. Mean recoveries at a concentration of 100 and 300 ng/ml are given in Table 1. The relationship between the peak area and the concentration was linear up to 1200 ng/ml with a correlation coefficient greater than 0.999 for both compounds. The minimum detectable amount defined as signal-to-noise ratio of 4 was found to be 1 ng for midazolam and 1-hydroxymidazolam. The quantification limit was 2 ng/ml with a coefficient of variation less than 15% for a 1-ml sample volume.

The intra-day and inter-day precision and accuracy are given in Table 2. The Spherisorb CN column has demonstrated a long lifetime: ca. 500 samples were injected without any deterioration of its performance. In contrast to published

Table 1
Analytical recoveries of midazolam, 1-hydroxymidazolam and internal standard from spiked plasma

Compound	Concentration (ng/ml)	Recovery (%)	C.V. ($n = 6$) (%)
Midazolam	100	87	3
	300	87	6
1-Hydroxymidazolam	100	84	4
	300	86	6
Internal standard	150	80	3

Table 2
Precision and accuracy

Compound	Concentration added (ng/ml)	Concentration found (ng/ml)	C.V. (%)	R.E. (%)
Intra-assay ($n = 6$)				
Midazolam	10	10.1	1.9	1.0
	100	99.2	4.4	−0.8
1-Hydroxymidazolam	10	10.5	6.6	5.0
	100	98.5	2.9	−1.5
Inter-assay ($n = 6$)				
Midazolam	10	10.4	4.3	4.0
	100	101.0	5.6	1.0
1-Hydroxymidazolam	10	10.4	6.5	4.0
	100	101.8	2.7	1.8

C.V. is coefficient of variation. R.E. is relative error: (concentration found–concentration added) × 100/(concentration added).

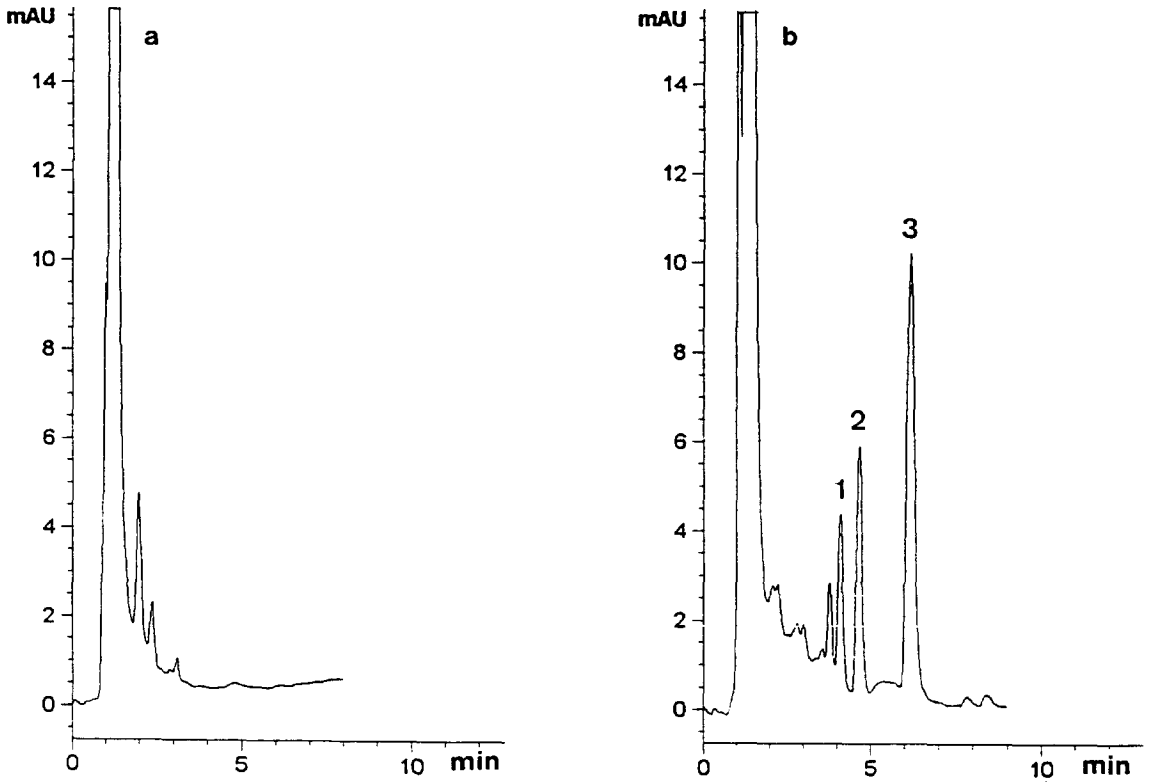

Fig. 2. Chromatograms of (a) blank plasma and (b) plasma sample collected 4 h after 0.2 mg/kg i.v. bolus followed by 0.1 mg/kg/h i.v. infusion of midazolam over 2 h from an intensive care patient. Peaks: 1 = 1-hydroxymidazolam, 29 ng/ml; 2 = midazolam, 49 ng/ml; 3 = flurazepam, 150 ng/ml.

methods using cyanopropyl bonded phases [11,12], there was no interference with other benzodiazepines or drugs commonly used in intensive care units. The perchloric acid content of the mobile phase contributes to the selectivity of the method.

The present method was used for monitoring midazolam and unconjugated 1-hydroxy-midazolam to intensive care patients. Typical chromatograms of a blank plasma and a plasma sample from a patient receiving 0.2 mg/kg i.v. bolus followed by 0.1 mg/kg/h i.v. infusion over 2 h are shown in Fig. 2. Significant concentrations of midazolam and of its unconjugated metabolite were recovered 6 h post-dose.

The HPLC method described is simple, selective and sensitive enough to monitor midazolam and also the unconjugated form of the active metabolite in plasma during pharmacokinetic studies. Pharmacokinetic investigation of midazolam and its metabolite involves very sensitive methods with a quantification limit reaching 2 ng/ml and low plasma volume (up to 1 ml) with respect to the numerous blood samples required.

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