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Sensitive and specific determination of midazolam and 1-hydroxymidazolam in human serum by liquid chromatography–electrospray mass spectrometry

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Abstract

A liquid chromatographic–mass spectrometric technique was designed for the determination of the anaesthetic benzodiazepine midazolam (MID) and its active metabolite 1-hydroxymidazolam (1-OHM), with the aim of conducting pharmacokinetic/pharmacodynamic studies. MID and 1-OHM were extracted from alkalinised (pH 9.5) spiked and clinical serum samples using a single step, liquid–liquid extraction procedure with diethyl ether–2-propanol (98:2, v/v). The chromatographic separation was performed on a Nucleosil C_{18}, 5 μm (150×1 mm I.D.) column, using a gradient of acetonitrile in 5 m*M* ammonium formate, pH 3.0 as the mobile phase, delivered at a flow-rate of 50 μl/min. The compounds were ionised in the ionspray source of an atmospheric pressure mass spectrometer, fragmented by in-source collisions and the pseudomolecular and fragment ions detected in the selected ion monitoring mode. The recovery was between 79 and 87% for MID, between 83 and 87% for 1-OHM and 81.5% for methylclonazepam. The limit of detection was 0.2 μg/l for MID and 0.5 μg/l for 1-OHM, the limit of quantitation (LOQ) was 0.5 μg/l for both. Linearity was verified from these LOQs up to 2000 μg/l and the method was found accurate and precise over this range. It was successfully applied to a preliminary study to establish the concentration versus time curve of MID and 1-OHM in a patient administered midazolam by continuous infusion.

Keywords: Midazolam; 1-Hydroxymidazolam

1. Introduction

Midazolam (MID), 8-chloro-6-(2-fluoro-phenyl)-1-methyl-4H-imidazo-[1,5-a][1,4]-benzodiazepine is a short acting benzodiazepine, widely used in clinical practice for induction of anaesthesia and for sedation of mechanically ventilated patients, in intensive care units. MID is rapidly and intensively metabolised, via CYP 450 3A isoforms to 1-hydroxymidazolam (1-OHM) and to a lesser extent to 4-hydroxy-midazolam. These hydroxy metabolites are further conjugated as glucuronides. If both 1-OHM and its conjugated metabolite bind to the cerebral benzo-diazepine receptor in vitro, only the former is known

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to be pharmacologically active, whereas the latter has only been suspected as having a sedative effect when it accumulates in patients with renal failure [1].

A major problem encountered in patients administered midazolam by continuous infusion is delayed awakening. Though midazolam yields a clearly defined, E-max type concentration–effect relationship [2,3], it is not clear yet whether this inter-patient variability is due only to pharmacogenetic or pathological differences in CYP 3A activity [4], to age and liver function [5], to renal function [6] or if accumulation of midazolam active metabolites is concerned [1]. Therefore, further studies are needed to investigate the processes potentially involved.

The analytical techniques published so far for MID, and eventually some of its metabolites, have mainly involved high-performance liquid chromatography (HPLC) with UV detection [7–12], gas chromatography (GC) [13–17], or even GC–mass spectrometry (MS) [18]. HPLC techniques have generally shown higher limits of detection (LODs) than GC techniques, except for a recently published capillary-HPLC procedure which yielded limits of quantitation (LOQs) of 1 and 0.5 ng/ml for MID and 1-OHM, respectively. Martens and Banditt [18] reported a very sensitive and specific GC–MS technique for the determination of MID, 1-OHM and 4-OHM, the only drawback of which was a long extraction and derivatisation procedure. Lausecker et al. [19] even used MID and three of its metabolites as examples for a comparison of liquid chromatography–tandem mass spectrometry (LC–MS–MS) and capillary zone electrophoresis–tandem mass spectrometry (CZE–MS–MS); liquid–liquid extraction from human plasma was found more suitable than solid-phase extraction and CZE–MS–MS more sensitive than LC–MS–MS. However, such techniques still require expensive instruments that are not available in most laboratories, whereas benchtop single-quadrupole LC–electrospray (ES) MS devices are now affordable and widely available in analytical laboratories.

In view of a pharmacokinetic/pharmacodynamic study of midazolam and 1-OHM in mechanically ventilated patients administered midazolam by continuous infusion, the aim of the present study was to design a sensitive, specific and rapid method for the determination of these two compounds in a large number of serum samples.

2. Experimental

2.1. Reagents

MID, 1-OHM and methylclonazepam (internal standard, I.S.) were gifts of Produits Roche (Neuilly-sur-Seine, France). Acetonitrile and methanol of HPLC quality were purchased from Carlo-Erba (Milan, Italy). Diethyl ether and 2-propanol of HPLC quality, as well as sodium carbonate and sodium hydrogencarbonate, both of R.P. Normapur purity, were from Prolabo (Fontenay-sous-bois, France). Ammonium formate and formic acid (99% pure) were from Sigma (St. Louis, MO, USA). Purified water was obtained in the laboratory using a Milli-Q water system (Millipore, Bedford, MA, USA).

Stock solutions of MID, 1-OHM and I.S. were prepared at 1 g/l in methanol and were kept at −20°C in the dark for a maximum of three months. Each day, working solutions containing both midazolam and its metabolite at 100, 10, 1, 0.1 and 0.01 mg/l and a working solution of I.S. at 5 mg/l were prepared by appropriate dilution with purified water.

A 0.5 *M* carbonate–hydrogencarbonate, pH 9.50 buffer was prepared by mixing a solution of 0.5 *M* sodium carbonate, pH 9.50 with an equal amount of a solution of 0.5 *M* sodium hydrogencarbonate.

A mixture of diethyl ether–2-propanol (98:2, v/v) was used for the extraction of the analytes from serum.

2.2. Sample preparation

To 2 ml of serum were added 50 μl of a 5 mg/l internal standard solution, 2 ml of the carbonate-hydrogencarbonate, pH 9.50 buffer and 8 ml of the extraction solvent mixture, in a 15 ml borosilicate tube. The tubes were reciprocally agitated for 15 min, then centrifuged at 3000 rpm (1600 *g*) for 5 min. The organic phase was transferred to a 10-ml borosilicate tube and evaporated to dryness at 30°C under a gentle stream of nitrogen. The dry extract was redissolved in 50 μl of a mixture of acetonitrile–5 m*M* ammonium formate, pH 3.0 (40:60, v/v), of which 2 μl was injected into the chromatographic system.

A set of calibrating standards at 0, 0.5, 1, 5, 10, 50, 100, 1000 and 2000 μg/l of MID and 1-OHM was prepared with each series, by spiking blank serum samples with the appropriate working solution.

2.3. Liquid chromatography–mass spectrometry

The HPLC system consisted of a series 200 autosampler, a series 200LC high-pressure gradient, micro-flow-rate chromatographic pump (Perkin-Elmer, Foster City, CA, USA) and a Nucleosil C_{18}, 5 μm (150×1 mm I.D.) column (LC-Packings, San Francisco, CA, USA). The mobile phase, delivered at a flow-rate of 50 μl/min at +20°C, was a gradient of acetonitrile in 5 m*M* ammonium formate, pH 3.0 (40% acetonitrile during 1 min, increased to 70% in 9 min and decreased to 40%, i.e., original conditions, between 10th and 11th min).

The detection was performed using an API-100 mass spectrometer (Sciex, Concord, Canada), equipped with an ionspray source. High-purity nitrogen was used as nebulisation and curtain gas. In-source fragmentation induced dissociation was adjusted by varying the acceleration voltage (''orifice voltage'') in the intermediate pressure zone, between atmospheric and high vacuum, in order to obtain the highest possible intensity for one quantitation ion and one confirmation ion per compound, including the internal standard. Acquisition was made in the selected ion monitoring mode of positive ions, with a dwell time of 100 ms. The ions selected and corresponding orifice voltage are reported in Table 1. The other main parameter settings were: nebulisation gas flow 0.95 l/min; curtain gas flow 1.16 l/min; ionspray voltage 5000 V.

2.4. Validation

Recoveries were determined in triplicates at concentrations of 5, 50 and 500 ng/ml. For each concentration, three serum samples were fortified with MID, 1-OHM and I.S. and three others with I.S. only. They were extracted as previously described. The dry extracts of the fortified samples were redissolved in 50 μl of reconstitution solvent, while the extracts of the blank samples were redissolved with 50 μl of reconstitution solvent containing the respective nominal amounts of MID and 1-OHM. The latter were used as neat standards.

Within-day precision and accuracy were determined at four concentrations, by preparing and analysing on the same day six replicates for each level. Between-day precision and accuracy, as well as linearity and LOQ were assessed by analysing each day for six days a set of samples spiked at 0.2 0.5, 1, 2, 5, 10, 20, 50, 100, 200, 500, 1000 and 2000 ng/ml, prepared in advance in volumetric flasks, aliquoted and stored at −20°C until analysed. Precision, expressed as the coefficient of variation (CV) of the measured values, was expected to be less than 15% at all concentrations, except for the limit of quantitation, for which 20% was acceptable. In the same way, accuracy was evaluated using the mean relative error (MRE), which had to be less than 15% of theoretical values at each concentration level except for the limit of quantitation, for which 20% was acceptable. Therefore, LOQ was defined as the

Table 1
Chromatographic retention times and selected ions of midazolam and 1-hydroxymidazolam and methylclonazepam (I.S.)

Compound	Retention time (min)	Selected ions (u)	Orifice voltage (V)
1-Hydroxymidazolam	6.9	**342.4**[a]	40
		324.4	55
Midazolam	7.3	**326.4**	60
		291.4	90
Methylclonazepam (I.S.)	10.7	**331.0**	60
		284.0	70

[a] Quantitation ions are in bold characters.

lowest concentration yielding between-day precision CV and MRE of less than 20% each. The specificity of the method was evaluated by analysing 10 serum samples, from four healthy volunteers and six patients not treated with MID.

3. Results and discussion

Though in the chromatographic system used, the retention times of MID and 1-OHM were very similar (Table 1) and the chromatographic peaks not totally resolved on the total ion chromatogram, their specific identification and quantitation were possible using their characteristic ions, and selected reconstructed chromatograms, owing to MS (Fig. 1). A gradient of acetonitrile was used to get methylclonazepam (I.S.), a not therapeutically used benzodiazepine, closer to the compounds of interest. The full-scan mass spectra of MID and 1-OHM are presented in Fig. 2. The ions selected and their respective optimised fragmentation (''orifice'') voltage are reported in Table 1. For each compound, the ratio of the confirmation ion to that of the quantitation ion was automatically compared to the theoretical one by the quantitation software used (Turboquan, Sciex).

Owing to the selectivity of MS, a simple, single-step liquid–liquid extraction could be used, which was revealed to be efficient in terms of purification. In particular, the analysis of 10 blank serum samples of different origins (healthy volunteers and patients) showed no interfering peak on the reconstructed chromatograms. Extraction recovery for MID and 1-OHM was, respectively, 79.3 and 82.8 at 5 μg/l; 87.6% and 83.6% at 50 μg/l; and 83.4% and 87.4% at 500 μg/l. The extraction recovery of I.S. was 81.5% at 250 μg/l.

The LOD, defined as the lowest tested concentration yielding a signal-to-noise ratio higher than 3, was 0.2 μg/l for midazolam and 0.5 μg/l for 1-hydroxymidazolam, using a 2-ml sample volume.

The within-day precision, as well as the between-day precision and accuracy were satisfactory under all the concentrations tested (Table 2). The LOQ deduced from between-day precision and accuracy was 0.5 μg/l for both midazolam and its metabolite. The linearity of the compound-to-I.S. peak area ratio versus the theoretical concentration was verified from this LOQ up to 2000 μg/l for both analytes: using a $1/x$ weighted linear regression, the correlation coefficients were typically better than 0.99 and the curvature, tested by analysis of variance on a set of six calibration curves, was not significant. Such a large linearity range is useful for analysing, in a same run, samples with a large concentration range, as is the case in a pharmacokinetic study. Moreover, the effect of dilution of concentrated samples with water was tested in the range 1/2 to 1/10 using triplicates of spiked blank samples and showed a mean relative error of less than 10% (NS).

A previously published GC–MS procedure yielded lower LODs than the present technique (MID: 0.2 ng/ml; 1-OHM and 4-OHM: 0.1 ng/ml), but similar LOQs. However, our method is more sensitive than most of the HPLC or GC techniques published so far, the detection limits of which were generally higher than 1 μg/l [7–10,12–17], and even more sensitive than the LC–ES-MS–MS technique previously described, which obtained LODs of 0.5 and 2 μg/l for MID and 1-OHM, respectively [19]. The lower LODs obtained with a single quadrupole instrument herein can probably be explained by the use of a larger sample volume and of a chromatographic column of smaller internal diameter. This latter assumption is further illustrated by the method of Eeckhoudt et al. [11], that used a capillary HPLC technique (0.8 mm I.D. column) and yielded the same LOQ as ours for 1-OHM, despite a smaller sample volume (1 ml), while the LOQ of MID was twice as high as ours. In a recent review, we insisted on the gain in sensitivity brought about by microchromatography, especially as atmospheric pressure ionisation sources provide a signal proportional to the concentration, and not to the amount of the compounds in the chromatographic effluent [20].

This method was actually used in a clinical experiment, preliminary to a pharmacokinetic/pharmacodynamic study that was approved by the local ethics committee. In a mechanically ventilated patient administered 10 mg/h midazolam by continuous intravenous infusion for 56 h, blood samples were drawn from an antecubital vein at 12 h and 36 h during the infusion and at 0, 5, 20, 40 min, 1, 2, 4, 6, 8, 24, 48 and 72 h after the end of it. The concentration-versus-time curves of MID and 1-

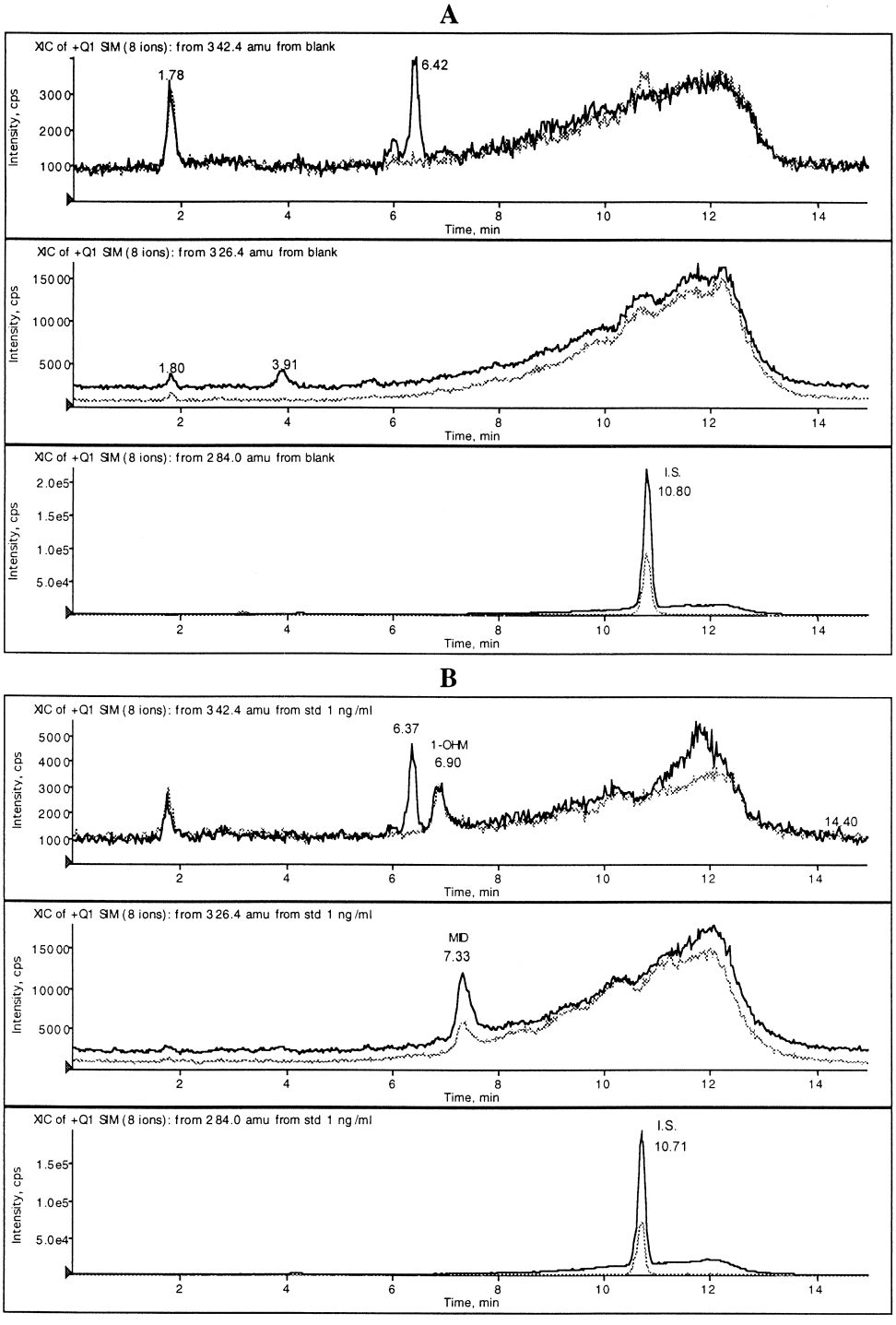

Fig. 1. Reconstructed mass chromatograms obtained from (A) a blank serum and (B) a serum sample spiked at 1 ng/ml with midazolam and 1-hydroxymidazolam (bold lines: quantitation ions; dash lines: confirmation ions).

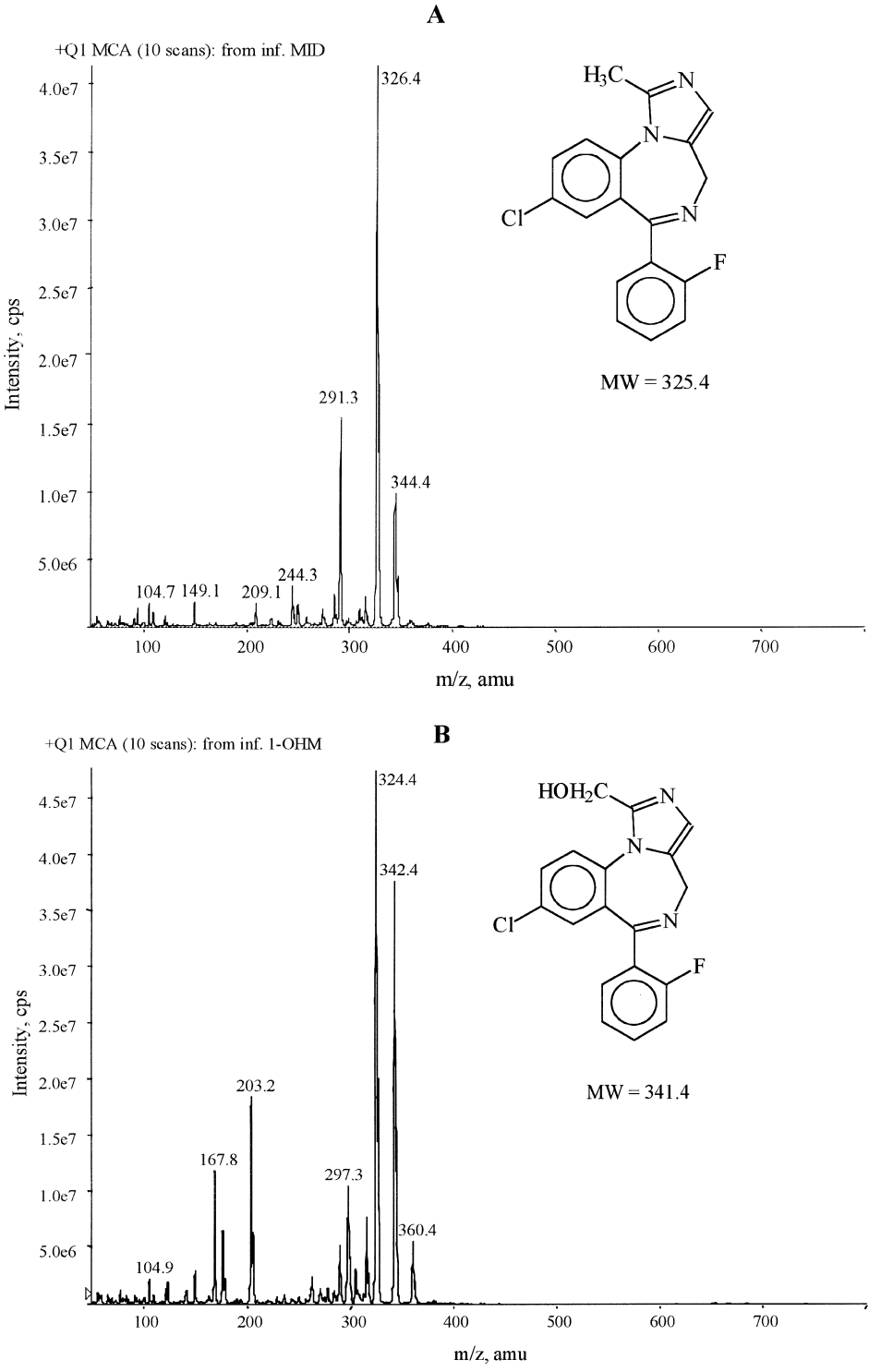

Fig. 2. Chemical structures and mass spectra of (A) midazolam and (B) 1-hydroxymidazolam.

Table 2
Within- and between-day precision, accuracy and linearity of the LC–ES-MS determination of midazolam and 1-hydroxymidazolam in human serum

Concentration (μg/l)	Within-day precision (n=6) CV (%)		Between-day (n=6)			
			Precision CV (%)		Mean relative error (%)	
	Midazolam	1-OH-midazolam	Midazolam	1-OH-midazolam	Midazolam	1-OH-midazolam
0.2	–	–	24.7	ND[a]	19.5	ND
0.5	–	–	18.1	7.6	3.0	3.8
1	–	–	11.7	9.6	0.0	1.7
2	7.4	6.4	3.4	5.1	7.9	3.9
5	–	–	5.9	5.5	5.2	4.0
10	12.3	14.3	8.5	7.3	2.8	2.6
20	2.7	2.5	6.3	7.7	1.9	1.2
50	7.1	12.1	2.6	2.3	0.3	1.4
100	–	–	7.3	7.6	1.2	0.0
200	9.7	7.3	7.4	14.7	0.6	3.2
500	–	–	11.4	8.4	7.3	8.3
1000	8.7	7.9	5.6	8.2	0.9	0.3
2000	–	–	3.0	5.3	2.1	1.6
Mean correlation coefficient					r=0.9978 (n=6)	r=0.9978 (n=6)

[a] ND=Not detected.

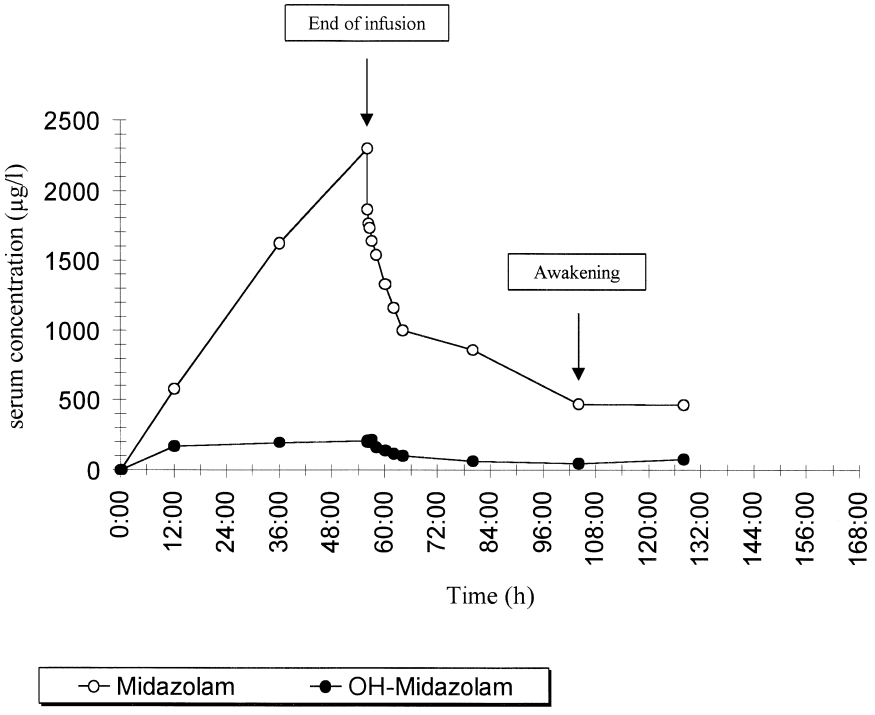

Fig. 3. Concentration versus time curves of midazolam and 1-hydroxymidazolam in a patient administered midazolam intravenously during 56 h, who awakened 48 h after the end of infusion.

OHM are shown in Fig. 3. This particular patient had a delayed awakening (48 h after the end of infusion), corresponding to residual blood concentrations of 474 and 47 μg/l for MID and 1-OHM, respectively.

In conclusion, the validated LC–ES-MS method described herein is very sensitive, and one of the most specific techniques published so far, due to mass spectrometry. Moreover, its rapidity and simplicity (except maybe for the instruments used) makes it a convenient technique for large clinical studies. Finally, it is based on the same instruments and chromatographic conditions as the other LC–MS methods that we have published so far, dedicated to various therapeutic drugs, abused drugs or other xenobiotics [21–24]. This allows different methods to be run on the same day, which is convenient for a laboratory performing a number of analytical procedures in different fields.

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