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Note

Rapid and sensitive high-performance liquid chromatographic assay for midazolam and 1-hydroxymidazolam, the major metabolite, in human serum

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Midazolam is a member of a new range of imidazobenzodiazepines currently under investigation. It is water soluble at pH < 4 but is highly lipophilic at physiological pH [1]. The drug is a powerful sedative with a rapid onset and short duration of action following a single intravenous dose. It is rapidly absorbed after oral administration but with a poor bioavailability ranging from 36 to 50% [2–4]. Like diazepam, midazolam also has anticonvulsant, muscle relaxant and anterograde amnesic properties [5]. The pharmacoloical properties and therapeutic uses of midazolam have been reviewed by several authors [1,6,7]. Following intravenous administration midazolam undergoes rapid distribution ($t_{1/2}$=0.15–0.30 h) [8] and elimination ($t_{1/2}\beta$=1.5–3.5 h) [2–4] leading to very low serum levels within a few hours. The major product of hepatic oxidation of midazolam is 1-hydroxymidazolam which is less pharmacologically active than the parent compound. A second metabolite, though minor, is 4-hydroxymidazolam which is also less pharmacologically active than midazolam. The overall pharmacological effect of the metabolites is further reduced by rapid glucuronidation [7,9,10]. The 4-hydroxymidazolam glucuronidate makes up only 3% of the metabolites found in urine. It is therefore unlikely to be present in the unconjugated form in serum

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in significant amounts [1]. The 1-hydroxymidazolam glucuronidate makes up 50–70% of the total dose eliminated in urine and so is more likely to be found in the unconjugated form in serum.

In order to characterise the pharmacokinetics of midazolam in humans, therefore, a sensitive assay is required to measure both parent drug and 1-hydroxmidazolam at levels below 10 ng/ml. A rapid, simple and sensitive high-performance liquid chromatographic (HPLC) method to measure both compounds is described.

EXPERIMENTAL

Material and reagents

Midazolam, 1-hydroxymidazolam and the internal standard flurazepam were supplied by Roche Products (Welwyn Garden City, U.K.). The internal standard was used as a 4 μg/ml aqueous solution. The methanol and 2-propanol were HPLC-grade reagents from Rathburns (Walkerburn, U.K.). Diethyl ether, sodium hydroxide and 70% perchloric acid (all analytical-reagent grade) were obtained from BDH (Poole, U.K.).

Chromatography

The solvent was delivered by a Model 300/2 constant-flow reciprocating pump (Applied Chromatography Systems, Macclesfield, U.K.) and the sample injection was carried out through a Model 7125 Rheodyne syringe loading valve fitted with a 20-μl loop. The column was a 150 mm × 5 mm stainless-steel tube packed with Spherisorb 5 CN (Phase Separations, Queensferry, U.K.). The mobile phase was monitored at 215 nm using a Model 750/11 UV monitor (Applied Chromatography Systems). The peak heights were recorded on a Model 3401-M flat bed chart recorder (Laboratory Data Control, Stone, U.K.). The mobile phase consisted of methanol–2-propanol (75:25) containing 0.02% perchloric acid and the flow-rate was 3 ml/min. The chromatography of an extract from a serum standard containing midazolam, 1-hydroxymidazolam and internal standard is illustrated in Fig. 1. The retention times of these and some concurrently administered drugs used in intensive care units are given in Table I.

Sample preparation

Serum (1 or 2 ml) was pipetted into a 10-ml glass screw-cap tube to which were added 50 μl of internal standard and 50 μl of 1 mol/l sodium hydroxide, using a Hamilton repeating mechanism fitted with a 2.5-ml syringe (Phase Separations), followed by 8 ml of diethyl ether. The contents were mixed using a rotary mixer at 32 rpm (Baird and Tatlock, Dagenham, U.K.) for 10 min and then centrifuged using an MSE Minor S (Fisons, Crawley, U.K.) at 1300 *g* for 5 min. Approximately 7.5 ml of the supernatant were transferred to a conical glass tube containing an antibump granule. These tubes were placed in a sample concentrator (Tehne, Cambridge, U.K.) set at 60°C and the ether was then evaporated off under a stream of air. The sample was reconstituted in 50 μl methanol and 40 μl were injected into the loop.

Fig. 1. Chromatograms of (a) blank serum, (b) spiked serum containing 100 ng/ml midazolam (2), 1-hydroxymidazolam (1) and internal standard, flurazepam (3) and (c) patient sample.

TABLE I

RETENTION TIMES RELATIVE TO FLURAZEPAM OF MIDAZOLAM AND OTHER COMPOUNDS

Compound	Relative retention time	Compound	Relative retention time
Propofol	0.09	Hyosine	0.61
Lorazepam	0.13	Diazepam	0.66
Oxazepam	0.22	Etomidate	0.66
Desalkylflurazepam	0.33	1-Hydroxymidazolam	0.66
Triazolam	0.40	Atropine	0.69
Nordiazepam	0.42	Pethidine	0.70
4-Hydroxymidazolam	0.44	Codeine	0.81
Nitrazepam	0.44	Midazolam	0.81
Bromazepam	0.47	Flurazepam	1.00 (=3.4 min)
Alprazolam	0.56	Ranitidine	4.36
Lignocaine	0.59		

RESULTS AND DISCUSSION

The use of a 2-ml sample volume enabled the quantification of midazolam and 1-hydroxymidazolam down to 2 ng/ml with a coefficient of variation (C.V.) of between 2.1 and 7.6% for midazolam and its metabolite for the concentrations 5, 15, 120 and 350 ng/ml (Table II). A comparison with a 1-ml sample volume gave

TABLE II

INTRA-ASSAY COEFFICIENTS OF VARIATION FOR MIDAZOLAM AND 1-HYDROXYMIDAZOLAM USING A 1-ml AND 2-ml SAMPLE VOLUME

Concentration (ng/ml)	Coefficient of variation (%)				*n*	
	Midazolam		1-Hydroxymidazolam		2 ml	1 ml
	2 ml	1 ml	2 ml	1 ml		
5	2.8	–	7.6	–	10	–
15	6.3	2.6	6.7	4.7	10	9
120	3.6	1.7	5.4	2.7	10	8
350	2.1	6.2	2.1	6.7	10	7

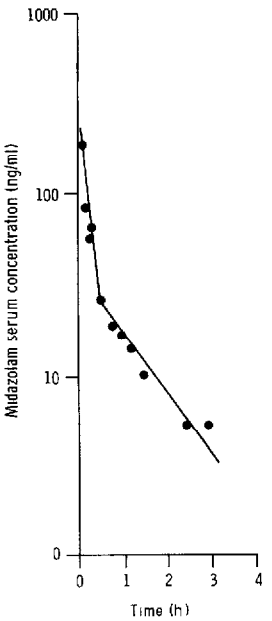

Fig. 2. Concentration–time profile for a single intravenous 5-mg dose of midazolam in a representative patient. Concentration of midazolam at 5 min = 181 ng/ml and at 3 h = 5.3 ng/ml.

a lower limit of detection of 10 ng/ml with a C.V. between 1.7 and 7% for 15, 120 and 350 ng/ml (Table II).

The extraction procedure gave a recovery from bovine serum of 100.5 and 93.2% for midazolam and 99.9 and 98.5% for the metabolite at concentrations of 100 and 300 ng/ml, respectively. A typical chromatogram is shown in Fig. 1 where it can be seen that the analytes elute within 4 min and there are no endogenous interfering peaks.

The relationship between peak height and concentration was linear up to 1.5 μg/ml with $r=0.988$ and 0.992 and intercepts of -0.13 and -0.28 ng/ml for midazolam and the metabolite, respectively. The calibration line of 2–25 ng/ml

was also linear with $r = 0.996$ and 0.995 and intercepts of -0.018 and -0.002 ng/ml for midazolam and the metabolite, respectively.

From Table I it can be seen that a number of drugs coelute with either midazolam or the 1-hydroxymidazolam metabolite. For all these except the anticholinergic drugs their are clinically acceptable alternatives. Such alternatives are morphine for codeine (morphine is poorly soluble in diethyl ether), propofol for etomidate and lorazepam for diazepam.

Following a single intravenous dose of 5 mg midazolam in six subjects the mean ($\pm$S.D.) serum concentration of midazolam at 5 min was 435 ± 315.18 ng/ml decreasing rapidly to 8.23 ± 5.72 ng/ml at 3 h. (Fig. 2). Blood samples were taken at intervals up to 8 h post-dose but at 4 h and beyond there were no detectable levels of midazolam. The metabolite 1-hydroxymidazolam was not detectable throughout the study time course. The lack of detectable levels of metabolite are perhaps not surprising considering the route of administration and the rapid glucuronidation of 1-hydroxymidazolam.

CONCLUSION

The method presented here is a simple, quick and sensitive assay for the simultaneous quantification of midazolam and the major metabolite 1-hydroxymidazolam. It enables the determination of patient serum levels of midazolam both for routine monitoring purposes and for pharmacokinetic studies.

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