Journal of Chromatography, 528 (1990) 526–530
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 5260

Note

Rapid, sensitive determination of human serum midazolam by high-performance liquid chromatography

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(First received September 19th, 1989; revised manuscript received February 5th, 1990)

We describe here a rapid, sensitive high-performance liquid chromatographic (HPLC) analysis of midazolam in human serum. The method was developed to enable us to conduct a pharmacokinetic study of this sedative in mechanically ventilated patients in our intensive-care unit. Plasma or serum concentrations of midazolam (and its major hydroxylated metabolite in many cases) have been determined by numerous chromatographic methods. These include gas chromatographic (GC) assays (with either electron-capture or nitrogen-sensitive detection [1–4]) and gas chromatographic–mass spectrometric (GC–MS) methods (electron-impact and negative-ion chemical ionization [5,6]). A HPLC method was preferred because sample derivatization could be avoided and reduced financial expense incurred compared with GC and GC–MS methods.

Previously published HPLC methods were rejected in favour of the method described below since either an internal standard was not included [7], the method lacked sufficient sensitivity [8] or exhaustive multiple extractions

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during sample work-up were required [9]. The method described here involves a single extraction step and the inclusion of a structurally related benzodiazepine as an internal standard (clonazepam). The limit of detection for the determination of serum midazolam is 25 ng/ml at a signal-to-noise ratio of 5.

EXPERIMENTAL

Materials

Mobile phase acetonitrile (HPLC-grade) was purchased from Waters Assoc. (Sydney, Australia). Dichloromethane, sodium acetate and sodium hydroxide were of analytical grade (Ajax Chemicals, Sydney, Australia). Fresh distilled water was further purified by passage through a charcoal column (Norganic® cartridge, Waters Assoc., Milford, MA, U.S.A.) immediately prior to use. Midazolam hydrochloride and its chief metabolite 1-hydroxymidazolam were a generous gift of Hoffman-La Roche (Sydney, Australia) and the internal standard, clonazepam, was purchased as an injection solution (1 mg/ml) from Roche.

Chromatographic equipment and conditions

The mobile phase, 20% (v/v) acetonitrile in 0.020 mol/l sodium acetate, was adjusted to a final pH of 3.0 and filtered through a 0.22-μm pore filter prior to use. It was pumped at 2 ml/min with a Model 510 pump (Waters Assoc.). Chromatographic resolution was achieved using Nova-Pak CN cartridge (10 cm$\times$8 mm I.D.; Waters Assoc.) radially compressed with a Model RCM-100 compression module (Waters Assoc.).

The eluent was monitored at 232 nm with a variable-wavelength ultraviolet absorbance detector (Model 490, Waters Assoc.) and the system was maintained at room temperature (24°C). Samples (0.20 ml) were injected via a Wisp® autoinjector (Waters Assoc.) and data handling and peak integration were carried out with a Model 840 chromatography data station (Waters Assoc.).

Sample preparation

To 1 ml of serum in a borosilicate glass culture tube (10 ml capacity with PTFE-lined screw cap) were added 0.10 ml of internal standard (clonazepam, 10 mg/l in distilled water), 0.20 ml of 4.0 mol/l aqueous sodium hydroxide, 1.0 ml of distilled water and 5 ml of dichloromethane. Each tube was mixed on a vortex for 30 s and centrifuged for 5 min at 3000 *g*. The upper (aqueous) layer was removed by vacuum aspiration and a further 3 ml of dichloromethane were added. Tubes were mixed on a vortex, centrifuged and any residual upper layer was removed as described above. The organic layer (approximately 8 ml) was transferred to a glass centrifuge tube (15 ml capacity) and evaporated to dry-

ness under a stream of nitrogen at 50°C. The dried residue was taken up in 0.25 ml of mobile phase and 0.20 ml injected into the HPLC column.

Calibration

To 1.0 ml of drug-free serum was added 0.10 ml of aqueous midazolam as the hydrochloride salt to give six standard concentrations in the range 25–1000 ng/ml (expressed as midazolam base). Each standard was treated in identical fashion to those samples of unknown midazolam concentration as described above under *Sample preparation.*

Calibration curves in the range of midazolam concentrations from 25 to 1000 ng/ml were constructed as the peak-area ratios of midazolam to internal standard. Calibration curves were linear in this range and passed through the origin. The mean normalised peak-area ratio was calculated for each set of standard (run concurrently with each set of unknowns) and used to calculate serum midazolam concentrations in our samples. The mean ($\pm$S.D.) coefficient of variation for fifteen sets of calibration standards was 6.3$\pm$1.9%. The inter-day variability of the assay over a period of a week ($n=5$) was determined by analysing samples spiked with 100 ng of midazolam. The mean ($\pm$S.D.) coefficient of variation was 7.9 $\pm$2.3%. Least-squares linear regression analysis was used to determine the slope, intercept and correlation coefficient for the 25–1000 ng/ml midazolam concentration range; the mean values ($n=5$) were 0.00308, −0.03 and 0.999, respectively.

The analytical recovery of midazolam, 1-hydroxymidazolam and internal standard was measured by comparing the chromatographic peak areas of biological samples spiked with 100 ng of each compound to those obtained from direct injection of aqueous solutions of the compounds. The recovery, using the sample preparation technique described above, was 95 and 85% for midazolam and 1-hydroxymidazolam, respectively. The recovery of internal standard was approximately 80%.

RESULTS AND DISCUSSION

Retention times for midazolam and the internal standard were 7.5 and 6.2 min, respectively (Fig. 1). Peaks of interest, including the chief metabolite (1-hydroxymidazolam) were suitably resolved and free of any interfering peaks. The midazolam metabolite (1-hydroxymidazolam) peak (6.9 min) was only occasionally seen in patient sera implying its rapid clearance from plasma and its reported [10] appearance as the glucuronide in urine.

A serum midazolam concentration–time profile is depicted in Fig. 2 for one of our study subjects immediately after ceasing administration of 2.5 mg/h midazolam as a continuous intravenous infusion (infused over 24 h). Institutional ethics committee approval was granted to conduct the study.

Although study subjects were administered morphine concurrently with

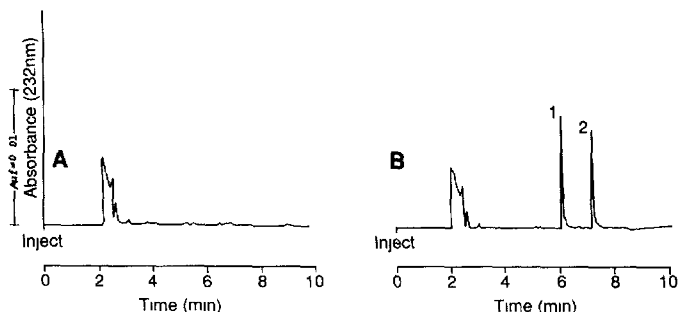

Fig. 1. Chromatograms of (A) drug-free serum and (B) serum spiked with (1) internal standard (clonazepam) and (2) midazolam (250 ng/ml).

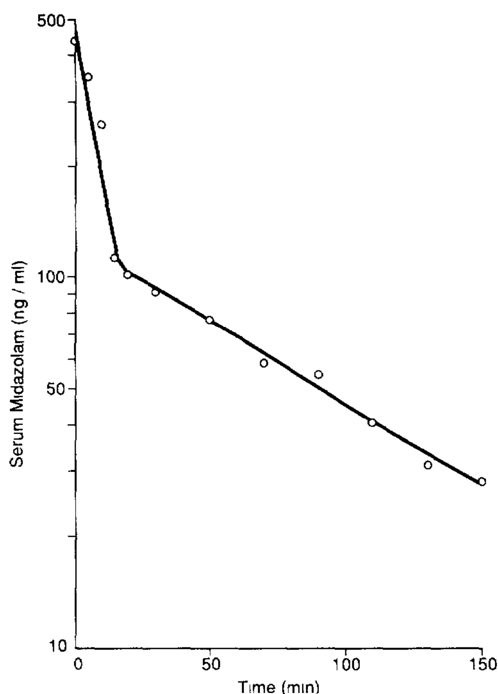

Fig. 2. Log-linear serum (venous) midazolam concentration-time profile for a human subject after ceasing a 24-h midazolam intravenous infusion of 2.5 mg/h. Time zero refers to the corresponding serum midazolam concentration immediately upon ceasing the infusion.

midazolam, control experiments demonstrated that this compound eluted close to the void volume (approximately 2 min). While coadministration of other benzodiazepines with midazolam would be extremely unlikely, a range of these compounds were tested to see if they were resolved from peaks of interest using this HPLC system. Retention times of flurazepam, diazepam, oxazepam and

nitrazepam were 8.9, 8.0, 4.9 and 5.5 min, respectively. This is not an exhaustive range of benzodiazepines but represents commonly used compounds each of which was resolved from midazolam, 1-hydroxymidazolam and the internal standard. While developing the sample preparation method outlined above, a white sera-derived flocculent material appeared in the organic layer after the first mixing/centrifuge step. This was presumed to be due to minor emulsification of the two liquid phases. The addition of a further 3 ml of dichloromethane and a second mixing/centrifuge step forced this material to the surface of the organic layer where it was easily removed.

In summary, we have described a rapid sensitive HPLC assay for the determination of serum midazolam with a limit of detection of 25 ng/ml. Utilizing a moderately polar stationary phase (cyanopropylsilane) permits a reduction in the amount of organic solvent used in the mobile phase with a subsequent reduction in analytical costs.

REFERENCES

1 R.M. Arendt, D.J. Greenblatt and W.A. Garland, Pharmacology, 29 (1984) 158.
2 P.J. Howard, E. McClean and J.W. Dundee, Anaesthesia, 40 (1985) 664.
3 C.D. Syracuse, B.R. Kuhnert, C.J. Kaine, A.C. Santos and M. Finster, J. Chromatogr., 380 (1986) 145.
4 H.R. Ha, B. Funk, P.O. Maitre, A.M. Zbinden, F. Follath and D.A. Thomson, Clin. Chem., 34 (1988) 676.
5 F. Rubio, S. Chen, T. Crews, F. DeGrazia, W.A. Garland and M. Barbalas, J. Chromatogr., 421 (1987) 281.
6 C. Drouet-Coassolo, C. Aubert, P. Coassolo and J.P. Cano, J. Chromatogr., 487 (1989) 295.
7 T.B. Vree, A.M. Baars, L.H.D. Booij and J.J. Driessen, Arzneim.-Forsch., 31 (1981) 2215.
8 C.V. Puglisi, J. Pao, F.J. Ferrara and J.A. de Silva, J. Chromatogr., 344 (1985) 199.
9 J. Vasiliades and T.H. Sahawneh, J. Chromatogr., 225 (1981) 266.
10 T.G. Clausen, J. Wolff, P.B. Hansen, F. Larsen, S.N. Rasmussen, J.S. Dixon and C. Crevoisier, Br. J. Clin. Pharmacol., 25 (1988) 457.