ELSEVIER

Journal of Chromatography B, 654 (1994) 69–75

JOURNAL OF CHROMATOGRAPHY B: BIOMEDICAL APPLICATIONS

Automated determination of midazolam in human plasma by high-performance liquid chromatography using column switching

Rolf Lauber*, Marcel Mosimann, Michael Bührer, Alex Martin Zbinden

Institute for Anaesthesiology and Intensive Care, Section of Research, University Hospital, 3010 Bern, Switzerland

(First received August 16th, 1993; revised manuscript received January 11th, 1994)

Abstract

An automated gradient high-performance liquid chromatographic method using a column-switching technique was developed in order to determine and quantify midazolam (separated from the metabolite α-hydroximidazolam) in human plasma. After dilution with an internal standard (flurazepam) solution, containing 20% acetonitrile, 400 μl of the plasma samples were injected onto a precolumn (17 × 4.6 mm I.D., C_{18} Corasil 37–53 μm) and retained. Proteins and polar plasma components were washed out using a 0.1 *M* sodium hydroxide solution, followed by an equilibration with a phosphate buffer of pH 8.0. After column-switching midazolam and flurazepam were eluted and transferred to the analytical column (RP-select B) in the backflush mode, separated by gradient elution and detected at 230 nm by ultraviolet detection. Precision of replicate analyses on the same day was 1.5% for midazolam and 0.7% for flurazepam. Recovery of midazolam was in the range 80–89% and the detection limit was 2 ng/ml plasma.

1. Introduction

Midazolam (Fig. 1) is a basic benzodiazepine with a relatively short half-life of 2–5 h. It can be used as induction agent for general anesthesia [1–3]. With a p*K*a of 6.15, the imidazole nitrogen is basic enough to form salts that will not dissociate when dissolved in water and at a pH lower than 6 the imine bond of the azepine ring is broken which results in a pH-dependent equilibrium between the ring-closed and the ring-open form [4,5]. In the method described we took advantage of the fact that the azepine ring is fully closed above pH 7.4 and the molecule therefore becomes non-soluble in water but is stable in hydrolytic solutions because of the imidazole ring.

Numerous methods for the detection of midazolam have been described such as radioreceptor-assay [6], gas chromatography [7–11], gas chromatography–mass spectrometry [12–14] and high-performance liquid chromatography (HPLC) [15–21] where the plasma samples are prepared by liquid–liquid extraction. Recently solid-phase extraction has also been described [22] in combination with a HPLC–MS assay [23]. Our method differs from the standard procedures in that the plasma samples diluted

* Corresponding author.

0378-4347/94/$07.00
SSDI 0378-4347(94)00017-Y

with internal standard solution were directly injected onto a RP-18 precolumn (PC) where the automated solid-phase extraction took place. The main goal was a time reduction of the whole analysis.

2. Experimental

2.1. Materials and reagents

Acetonitrile (gradient grade), sodium hydroxide, potassium dihydrogen phosphate and orthophosphoric acid 85% (pro analysi) were purchased from Merck (Zürich, Switzerland), water was distilled twice in an all-glass apparatus. Reference substances I–III (Fig. 1) were provided by Hoffmann-La Roche (Basle, Switzerland) and sodium citrated human blood plasma for standard curves was obtained from the blood donation centre of the Swiss Red Cross (SRK, Berne, Switzerland).

2.2. Solutions and standards

A standard solution of 1 mg midazolam in 10 ml acetonitrile (in a volumetric flask) was further diluted with acetonitrile to 100 μg/ml (standard solution 1), 10 μg/ml (standard solution 2) and 1 μg/ml (standard solution 3). These standard solutions were added to the blank plasma samples, giving calibration standard solutions in a concentration range of 10 to 2000 ng/ml plasma.

A stock solution of the internal standard (I.S.) with 1 mg flurazepam in 5 ml acetonitrile was prepared in a volumetric flask. To a mixture of 3.6 ml of 2 *M* sodium hydroxide solution and 2.5 ml of the I.S. stock solution 200 ml acetonitrile was added and diluted with bidistilled water to a volume of 1000 ml, producing an I.S. solution with a concentration of 500 ng flurazepam per ml.

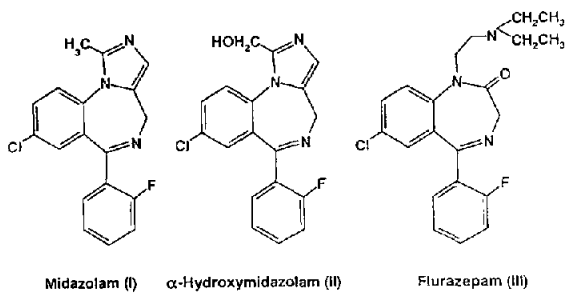

Fig. 1. Chemical structures.

2.3. Columns and mobile phases

The precolumn (PC; 17 × 4.6 mm I.D.; Methrohm, Herisau, Switzerland) was dry-packed with Bondapak C_{18} Corasil, 37–50 μm (Millipore, Volketswil, Switzerland) and used with 5-μm highgrade steel sieves (Metrohm) to avoid column blocking. The analytical column (AC) was a LiChroCART filled with LiChrospher 60 RP-select B 5 μm (250 × 4 mm I.D.; Merck). To protect the AC, a guard column (GC) LiChroCART (4 × 4 mm I.D.; Merck) RP-select B was used.

Mobile phase M1 was a 0.1 *M* sodium hydroxide solution. Mobile phase M2 consisted of a 0.02 *M* phosphate buffer (2.7 g potassium dihydrogen phosphate in 1 l bidistilled water) adjusted with 2 *M* sodium hydroxide solution to pH 8.0 with a pH-meter 691 (Metrohm). Mobile phase M3 consists of two components: A = 0.02 *M* phosphate buffer pH 2.4 (2.7 g sodium hydroxide in 1 l bidistilled water, adjusted with phosphoric acid 85%), B = acetonitrile.

2.4. Chromatographic system

Fig. 2 shows a diagram of the column-switching system. For purging the PC we used pump P1 (L-6000 pump; Merck) equipped with an electronically triggered low pressure valve (Merck) for the change of the mobile phases M1 and M2 at a flow-rate of 1 ml/min.

The automatic sampler (AS; AS-2000, Merck) injected 400 μl of diluted plasma onto the PC. The low pressure gradient pump P2 (L-6200 Intelligent Pump; Merck) with a dynamic mixing chamber (MIX; Merck) delivered the gradient mobile phase M3 (different flow-rates, see Table 1) in order to wash the retained substances from the PC onto the AC. Separated compounds were

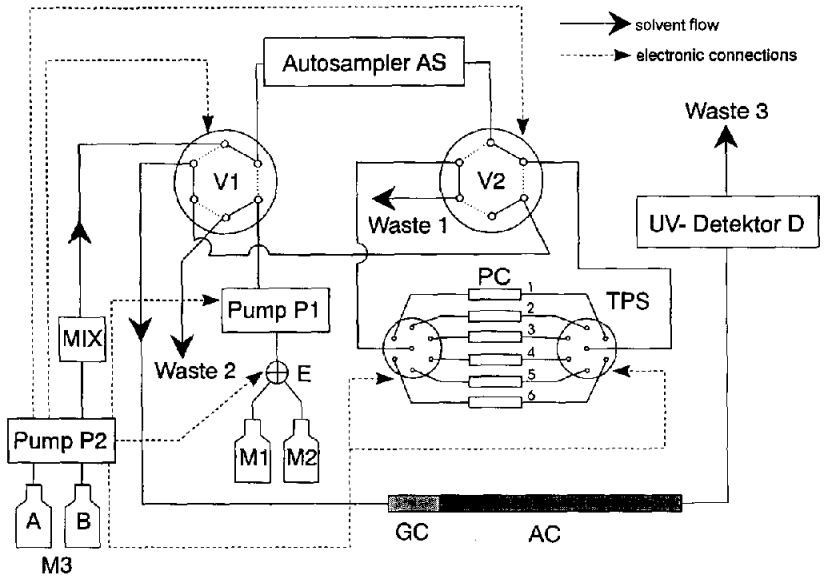

Fig. 2. Diagram of the column-switching chromatographic system. The valves V1 and V2 are shown in position O (for further details see text and Table 1).

detected with a UV-Vis detector (Spectroflow 783, Applied Biosystems, Foster City, CA, USA) at 230 nm. For data acquisition and control of the AS an integrator D-2500 Chromato-Integrator (Merck) was used. The electronic valves V1 and V2 (H.S. Valve 7000 E, Prolab, Reinach/Switzerland) of the column-switching system, the tandem precolumn selector (TPS) with two 6-port valves (Prolab) for the changing of the PCs, pump P1, valve E and the gradient program were controlled by the gradient pump P2. Integrator, gradient pump P2 and the AS were connected via a Private Area Network (PAN), the AS giving the starting signal for the analyses.

2.5. Analytical procedure

A 600-μl volume of plasma and 600 μl of I.S. solution were mixed in a 1.5 ml Eppendorf vial and shaken. After centrifugation (3 min at 1500 *g*), the supernatant was transferred by pipette into an AS vial (crimp vials 8002H with Teflon septum 8003, Infochroma, Zug, Switzerland) and 400 μl were injected. The samples were kept at room temperature in the AS before injection. The whole chromatographic system was purged after each analysis. The total sequence of automated sample analysis required 28.1 min, including conditioning of GC/AC. The different steps of the assay are shown in Table 1.

2.6. Calculation with calibration curve

Six standard plasma samples were prepared within the concentration range of 10–2000 ng/ml midazolam and were measured at the beginning and end of each set of unknown plasma samples. The standard curve was calculated from the ratio of the midazolam peak area divided by the peak area of the I.S. Based on the two standard curves a linear least-squares regression curve was computed, which gave us the calibration curve for the interpolation of unknown concentrations in the human plasma samples from the measured peak area. All data calculations were carried out with MathCAD software (MathSoft, Cambridge, MA, USA) on a personal computer (MS-DOS).

Table 1
Gradient- and valvecontrol

Time (min)	Gradient M3		Flow M3 (mℓ/min)	Electronically set event at pump P2	Comment (Abbreviations see text and Fig. 2)
	%A	%B			
0.0	76	24	1.00		V1 = 1; V2 = 0; injection of diluted plasma sample onto PC with M1; conditioning of AC with M3
4.0				20	V1 = 1; V2 = 1; polar component washed out of PC to waste 2 in backflush mode
5.5				30	Valve E switches from M1 to M2
9.0			1.000		Start of increasing the flow through GC/AC
10.0	76	24	1.500	11	V1 = 0; V2 = 1; elution of retained substances in backflush mode to GC/AC with P2; start of gradient
10.5				62	Pump P1 off
13.0				10	V1 = 1; V2 = 1; P2 switched directly onto AC
21.0	66	34			End of gradient
21.9	66	34			End of flush at same gradient
22.0	50	50		11 42	V1 = 0; V2 = 1; start of purging the system with P2 and M3; TPS switches to next PC if planned
25.0				52 31	P1 on; valve E switches from M2 to M1
26.0				10	V1 = 1; V2 = 1; start of conditioning PC with M1 for the next injection; purging GC/AC goes on
28.0	50	50	1.500		End of purging GC/AC
28.1	76	24	1.000	21	V1 = 1; V2 = 0; ready for next injection

3. Results and discussion

3.1. Sample preparation

Early publications described the method of column-switching for plasma samples without dilution or addition of an I.S. [24–26]. When developing the method we found that the PC had a tendency to get blocked. A recent publication pointed out the possibility of dilution [27] which had the effect that 30 plasma samples could be repeatedly retained onto the PC without blocking, furthermore the reproducibility of replicate plasma samples improved, as the protein binding of midazolam is loosened by the addition of acetonitrile. As a solution with a pH $\geq$ 7.4 causes a closure of the azepine ring in midazolam, sodium hydroxide solution was added. This high pH also results in the denaturation of plasma proteins which could be rinsed away in a next chromatographic step, thus not affecting the resulting chromatogram.

3.2. Chromatography and column-switching

The mobile phase M1 (0.1 *M* sodium hydroxide) was required in order to dissolve the plasma components not used in the analysis and

wash them out from the PC to waste. Before switching to the AC, however, the PC had to be purged and equilibrated with phosphate buffer M2, to prevent the AC from damage. Mobile phase M2 was adjusted to pH 8.0 where the ring system of midazolam still remains closed and thus apolar and where the hydroxide ion activity does not deteriorate the AC. Using a flow-rate of 1 ml/min the clean-up process of the samples required a total of 10 min, switching 4 min after injection to backflush modus and changing the mobile phase after 5.5 min.

Fig. 3 shows typical chromatograms of a plasma sample and a plasma blank. In order to separate the plasma peaks from midazolam a gradient was required improving the quality of the peak at the same time. Better results were obtained with the RP-select B column than with the normal RP-18 column. After approximately 700 injections the AC was replaced. Deviation of retention time during a day's analysis (24 h) was: ± 0.3 min. After each set of patients plasma samples and standards (20–25 injections) the TPS switched automatically to the next PC. In the case of a too high pressure in the chromatographic system automated change of the PC was possible as well.

In a 24-h period more than 40 patients plasma samples and standards could be analyzed quantitatively with a preparation time of only one hour.

3.3. Limit of quantification

The limit of quantification for midazolam in plasma samples was 10 ng/ml. Fig. 3 shows a chromatogram of a spiked plasma sample at this concentration. The limit of detection at a signal-to-noise ratio of 3:1 was 2 ng/ml plasma.

3.4. Linearity

The correlation of peak-area ratios and concentrations of I and III was linear in the range 10–2000 ng/ml. The coefficients of determination (r^2) were better than 0.999.

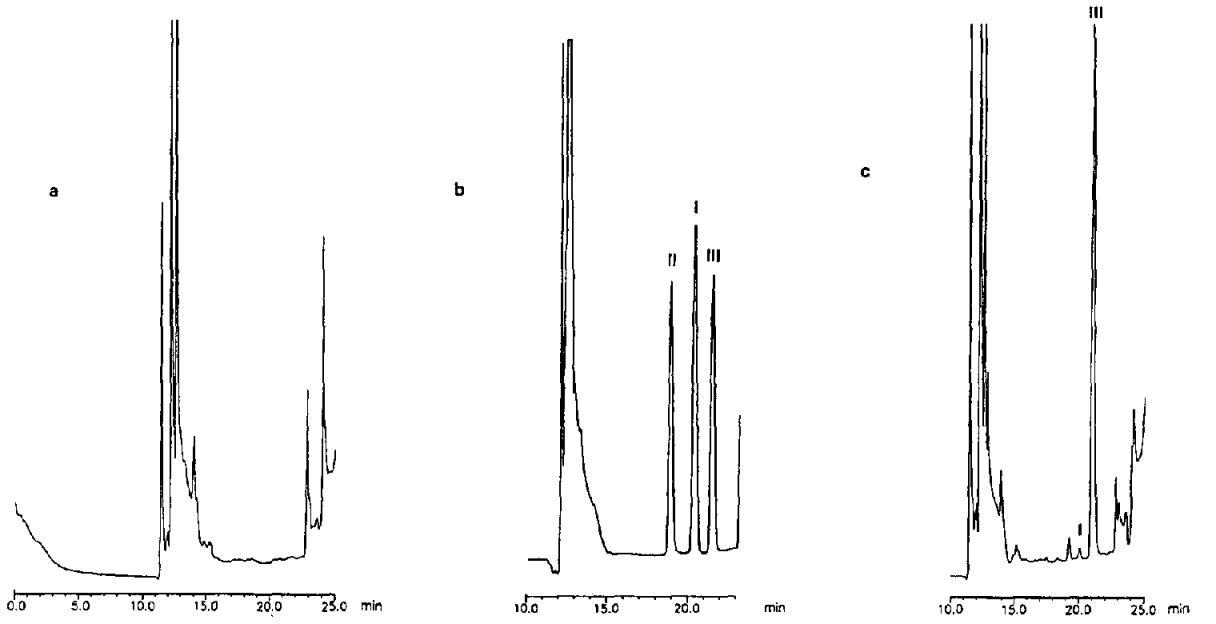

Fig. 3. (a) Chromatogram from blank sample. (b) Chromatogram from plasma sample containing 800 ng/ml midazolam (I), spiked with 700 ng/ml α-hydroxymidazolam (II) and 400 ng/ml flurazepam (III). (c) Chromatogram from plasma sample at quantification limit of 10 ng/ml I.

3.5. Recovery

The first few undiluted plasma injections resulted in an inadequate recovery and produced blocked PCs, which confirms that the dilution of the plasma samples is essential. The fraction of protein-bound midazolam is >90% [28] and is not influenced by age or by sex [29]. Table 2 shows recovery values with the relative standard deviations (R.S.D.) of three samples of midazolam and one of flurazepam. The recovery from plasma was determined by replicate analyses ($n = 5$) of spiked plasma samples, followed by replicate injections ($n = 5$) of a standard solution directly onto the AC (100% value). For the direct injections onto the AC the sixth position of the TBS was replaced by a capillary instead of a column. Valve V1 was switched to position 0 where the flow coming from the gradient pump P2 went directly onto the AS, then from valve V2 (position 1) through the TBS unit back to V2, further to V1 and finally to the AC.

3.6. Reproducibility

The precision of the inter-assay reproducibility (defined as R.S.D. of day-to-day analyses) was evaluated over the concentration range 105–1680 ng/ml of plasma. All samples were freshly prepared, spiked with midazolam and measured with duplicate injections on 5 days during a period of 3 weeks. Table 3 shows the results. The intra-assay reproducibility was obtained by replicate analyses ($n = 20$) of one specimen of spiked plasma samples (300 ng/ml) on the same day and gave the following precision (R.S.D.): midazolam 1.47%, flurazepam 0.73% and as ratio 1.27%. Furthermore we cross-checked our results of 10 samples in the range 10 to 1500 ng/ml with a gas chromatographic method established in forensic medicine [30] and found deviations of less than ± 14%. The intra-assay reproducibility of this forensic assay is less than ± 15% (R.S.D.) at a plasma concentration of 200 pg/ml.

Reproducibility measurements showed that the reproducibility could be enhanced when three spiked plasma samples were injected before starting a new batch as conditioning runs for the PC. This was then applied in all our analyses.

Table 2
Recovery values of midazolam and flurazepam

Concentration (ng/ml)	Recovery (%)	R.S.D. (%)
Midazolam		
10	83.1	1.86
60	87.5	0.46
400	89.3	1.09
Flurazepam		
100	92.3	0.62

Table 3
Inter-assay reproducibility for midazolam

Concentration (ng/ml)	R.S.D.
105	2.25
420	1.47
840	0.84
1680	0.83

injections; $n = 5$.

3.7. Stability

The plasma samples can be stored at a temperature of − 20°C for at least 6 months without changing the analytical results [31]. We had, however, an initial problem as the analyses of plasma samples stored more than 12 h in the AS at room temperature showed a lot of interfering peaks in the chromatogram. We can only guess that this was because plasma components, like proteins, had decomposed. The problem was solved by adjusting the dilution volume of the acetonitrile–sodium hydroxide solution. In the AS the samples were then stable during 24 h without cooling.

4. Conclusion

Using the column-switching technique, a fully automated gradient HPLC method with direct

injection of diluted plasma samples was developed and successfully applied to more than 1200 plasma samples from patients. Time-consuming extraction steps were therefore avoided and in a 24-h period more than 40 plasma samples containing midazolam could be analyzed quantitatively with a preparation time of only one hour.

5. Acknowledgement

This work was supported in part by the University Research Foundation of Berne. The authors thank Dr. Ch. Crevoisier, Hoffmann-La Roche, for the donation of substances I–III, Dr. L. Ulrich (Institut für Rechtsmedizin, Bern) for the cross-check of our analyses and Mrs. M. Leggoe for the translation and typing of the manuscript.

6. References

[1] H. Allonen, G. Ziegler and U. Klotz, *Clin. Pharmacol. Ther.*, 30 (1981) 653.
[2] M.T. Smith, M.J. Eadie and T.O. Brophy, *Eur. J. Clin. Pharmacol.*, 19 (1981) 271.
[3] D.J. Greenblatt and R.I. Shader, *Anesthesiology Review*, XIII, 3S (1986) 7.
[4] M. Gerecke, *Br. J. Clin. Pharmac.*, 16 (1983) 11S.
[5] R.J. Fryer, *Anesthesiology Review*, XII, 3S (1985) 11.
[6] L. Aaltonen, J.J. Himberg, J. Kanto and A. Vuori, *Int. J. Clin. Pharmacol. Ther. Toxicol.*, 23 (1985) 247.
[7] R.M. Arendt, D.J. Greenblatt and W.A. Garland, *Pharmacology*, 29 (1984) 158.
[8] P.J. Howard, E. McClean and J.W. Dundee, *Anaesthesia*, 40 (1985) 664.
[9] C.D. Syracuse, B.R. Kuhnert, C.J. Kaine, A.C. Santos and M. Finster, *J. Chromatogr.*, 380 (1986) 145.
[10] H.R. Ha, B. Funk, P.O. Maitre, A.M. Zbinden, F. Follath and D.A. Thomson, *Clin. Chem.*, 34 (1988) 676.
[11] I.F.I. de Kroon, P.N.J. Langendijk and P.N.F.C. de Grede, *J. Chromatogr.*, 491 (1989) 107.
[12] J. Vasiliades and T. Sahawneh, *J. Chromatogr.*, 228 (1982) 195.
[13] F. Rubio, S. Chen, T. Crews, F. DeGrazia, W.A. Garland and M. Barbalas, *J. Chromatogr.*, 421 (1987) 281.
[14] C. Drouet-Coassolo, C. Aubert, P. Coassolo and J.P. Cano, *J. Chromatogr.*, 487 (1989) 295.
[15] T.B. Vree, A.M. Baars, L.H.D. Booij and J.J. Driessen, *Arzneim.-Forsch.*, 31 (1981) 2215.
[16] J. Vasiliades and T.H. Sahawneh, *J. Chromatogr.*, 225 (1981) 266.
[17] C.V. Puglisi, J. Pao, F.J. Ferara and J.A. de Silva, *J. Chromatogr.*, 344 (1985) 199.
[18] 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.
[19] A. Blackett, S. Dhillon, J.A. Chromarty, R. Horne and G. Richards, *J. Chromatogr.*, 433 (1988) 326.
[20] A.A. Vletter, A.G.L. Burm, L.T.M. Breimer and J. Spierdijk, *J. Chromatogr.*, 530 (1990) 177.
[21] P.J. Hayball, D.G. Cosh and J. Wrobel, *J. Chromatogr.*, 528 (1990) 526.
[22] V. Sautou, J. Chopineau, M.P. Terrisse and P. Bastide, *J. Chromatogr.*, 571 (1991) 298.
[23] H. Kanazawa, Y. Nagata, Y. Matsushima, N. Takai, H. Uchiyama, R. Nishimura and A. Takeuchi, *J. Chromatogr.*, 631 (1993) 215.
[24] D.J. Popovitch, E.T. Batts and C.J. Lancaster, *J. Liq. Chromatogr.*, 1 (1978) 469.
[25] W. Roth, K. Beschke, R. Jauch, A. Zimmer and F.W. Koss, *J. Chromatogr.*, 222 (1981) 13.
[26] W. Voelter, T. Kronbach, K. Zech and R. Huber, *J. Chromatogr.*, 239 (1982) 475.
[27] R. Wyss and F. Bucheli, *J. Chromatogr.*, 424 (1988) 303.
[28] J.X. de Vries, J. Rudi, I. Walter-Sack and R. Conradi, *Biomed. Chromatogr.*, 4 (1990) 28.
[29] J.M. Servin, *Eur. J. Anaesthesiol.*, 4 (1987) 1.
[30] L. Ulrich and F. Sager in W. Holczabek (Editor), *Beiträge zur gerichtlichen Medizin*, Verlag Franz Deuticke, Wien, Vol. 46, 1988, p.135.
[31] R. Lauber and M. Mosimann (1992), personal communication.