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Short communication

Simultaneous determination of midazolam and flumazenil concentrations in human plasma by gas chromatography

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Abstract

A gas chromatographic method for the simultaneous determination of midazolam and flumazenil concentrations in human plasma has been developed, using clinazolam as the internal standard. A mixture of chloroform and ethyl acetate (80:20) was used to extract the two compounds. A 2-μl volume of the reconstituted sample was injected using a 1:20 split injection mode. Intra-day coefficients of variation ranged from 2 to 6.9%. The assay was linear over the range 3–1000 ng/ml. This assay was subsequently used to analyze samples from a human pharmacokinetic study.

1. Introduction

Midazolam, a 1,4-benzodiazepine, is commonly used as a short-acting hypnotic and for induction of anesthesia. Recently flumazenil, a benzodiazepine receptor antagonist, was marketed for the complete or partial reversal of benzodiazepine sedation following anesthesia induction, and for the management of benzodiazepine overdose. As flumazenil is often administered to reverse midazolam sedation, it is desirable to have at one's disposal an assay which will quickly and accurately measure both compounds.

Several methods have been reported for assaying midazolam or flumazenil alone. These include a radio-receptor assay, high-performance liquid chromatographic (HPLC) assays, and gas–liquid chromatographic methods [1–10]. Additionally, a reversed-phase liquid chromatographic method which simultaneously quantitates midazolam and flumazenil concentrations has been described [2]. As this method is both labor- and time-intensive, we developed a gas-chromatographic method for the simultaneous determination of midazolam and flumazenil concentrations. This assay was subsequently utilized for determining midazolam and flumazenil concentrations in plasma samples obtained during a

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human pharmacokinetic–pharmacodynamic study.

2. Experimental

2.1. Materials

Midazolam free base, flumazenil, and clinazolam were provided by Hoffman-LaRoche (Nutley, NJ, USA). The chemical structures are shown in Fig. 1. All other chemicals were of analytical-reagent grade.

2.2. Preparation of stock and working solutions

Stock solutions of midazolam, flumazenil, and clinazolam (internal standard) were prepared by separately dissolving 10 mg of each compound in 10 ml of methanol.

A working solution of midazolam/flumazenil was prepared by diluting 0.1 ml of stock midazolam and flumazenil with drug-free plasma

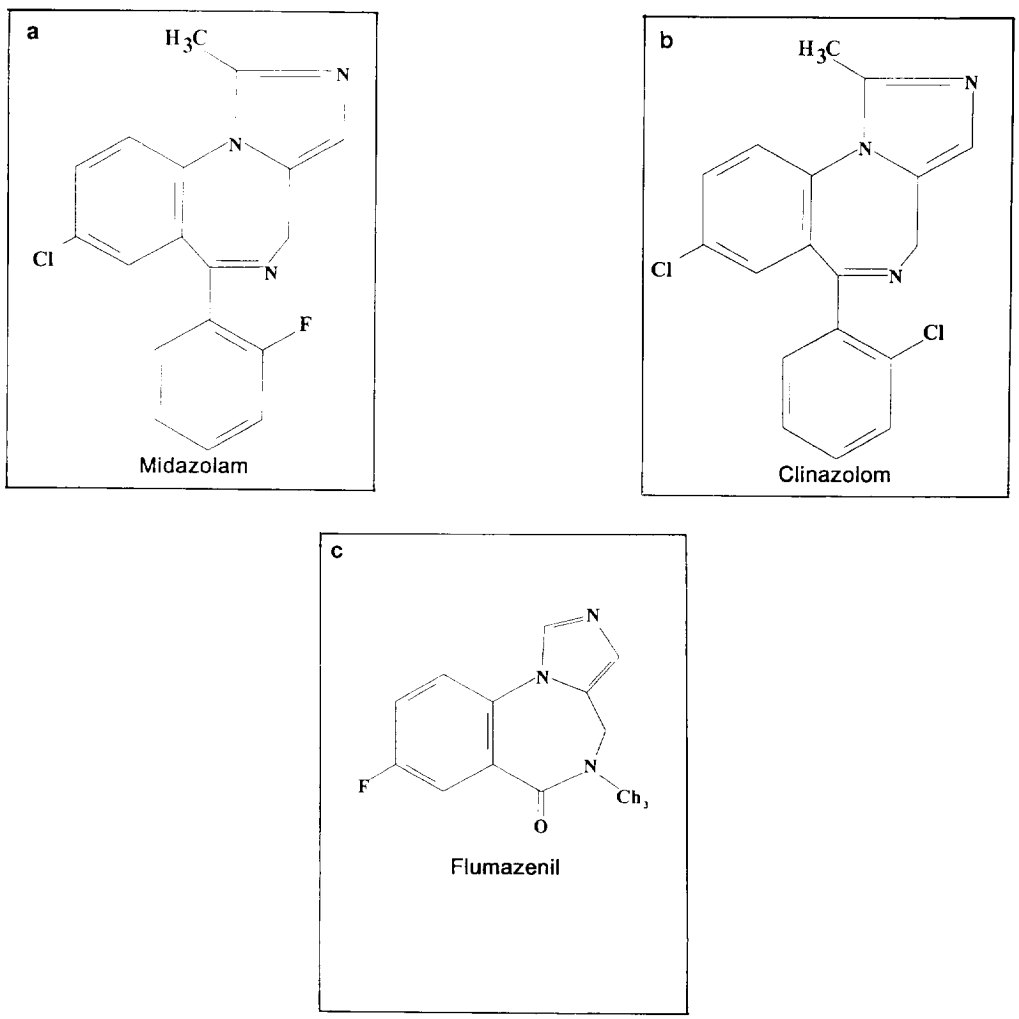

Fig. 1. (a) midazolam, (b) clinazolam, (c) flumazenil

to a final concentration of 100 ng/ml. Clinazolam working solution (2.5 μg/ml) was prepared by diluting 25 μl of stock solution in 10 ml of distilled water.

2.3. Extraction

The frozen plasma samples were thawed at room temperature. Samples were prepared by adding 0.2 ml of internal standard solution, 1 ml of the plasma sample, and 0.5 ml of a saturated sodium borate solution to a 10-ml screw-top tube and then vortex-mixing for 5 s. Midazolam, flumazenil and clinazolam were extracted with 5 ml of a chloroform–ethyl acetate solution (80:20, v/v). The tubes were rotated for 10 min and then centrifuged for 5 min at 1800 *g*. All of the organic phase was then transferred into 7-ml conical centrifuge tubes and evaporated in a 70°C sand bath aided by the flow of air. The residue was reconstituted with 50 μl of chloroform and 2 μl was injected onto the GC system using a 1:20 split-ratio injection mode.

2.4. Gas chromatography

A Hewlett-Packard 5890A GC (Palo Alto, CA, USA) equipped with a nitrogen–phosphorous detector and a Hewlett-Packard 3390A integrator was used with a J and W Scientific (Folsom, CA, USA) RTX-5 capillary column (30 m × 0.32 mm I.D., 0.25 μm film thickness). Injector and oven operating temperatures were 260°C, while the detector temperature was 300°C. The carrier gas was helium at an inlet pressure of 103 kPa.

2.5. Calibration

The standards used in the calibration curves had concentrations of 3, 6, 12.5, 31.25, 50, 62.5, 125, 250, 500, and 1000 ng/ml. These were prepared by dissolving the appropriate volumes of working solution in 1 ml of drug-free plasma. The calibration curve was obtained from the least-squares linear regression of the peak-height ratio of midazolam or flumazenil to the internal standard versus the theoretical concentration of midazolam or flumazenil.

3. Results

Fig. 2 shows a chromatogram of a blank human plasma sample, a human plasma sample spiked with 3 ng/ml of midazolam and flumazenil, and a sample from a representative human subject. The flumazenil peak was detected at approximately 7 min, the midazolam peak at approximately 8 min and the clinazolam peak at about 12 min. A complete run required about 15 min.

The calibration curves for both midazolam and flumazenil were linear ($r^2 = 0.999$) over the range of 3–1000 ng/ml. In order to more accurately characterize the lower concentration range, the curve was divided into two parts: 3–250 ng/ml and 31.25–1000 ng/ml. For the lower concentration range the least-squares linear regression equations were $y = 1.01x - 1.71$ and $y = 1.01x - 0.25$ for midazolam and flumazenil. At the higher concentration range, the equations were $y = 1.0x + 0.43$ and $y = 1.0x - 0.34$.

The intra-assay precision was determined using 25, 100 and 500 ng/ml of midazolam and flumazenil. Ten samples of each concentration were analyzed within a run. Table 1 lists the intra-day assay variation for the two compounds. The intra-day coefficients of variation were all less than 7%. The inter-day coefficients of variation were determined by extracting and analyzing plasma samples spiked with 100 ng/ml of midazolam/flumazenil on eleven different days over a 2-week period. The inter-day coefficients of variation were 5.8% for flumazenil and 2.6% for midazolam. The lower limit of detection for midazolam and flumazenil was 3 ng/ml. Percent recovery of flumazenil and midazolam ranged from 89 to 106%. For concentrations < 50 ng/ml the recovery was around 90% and for those > 50 ng/ml the recovery was close to 100%.

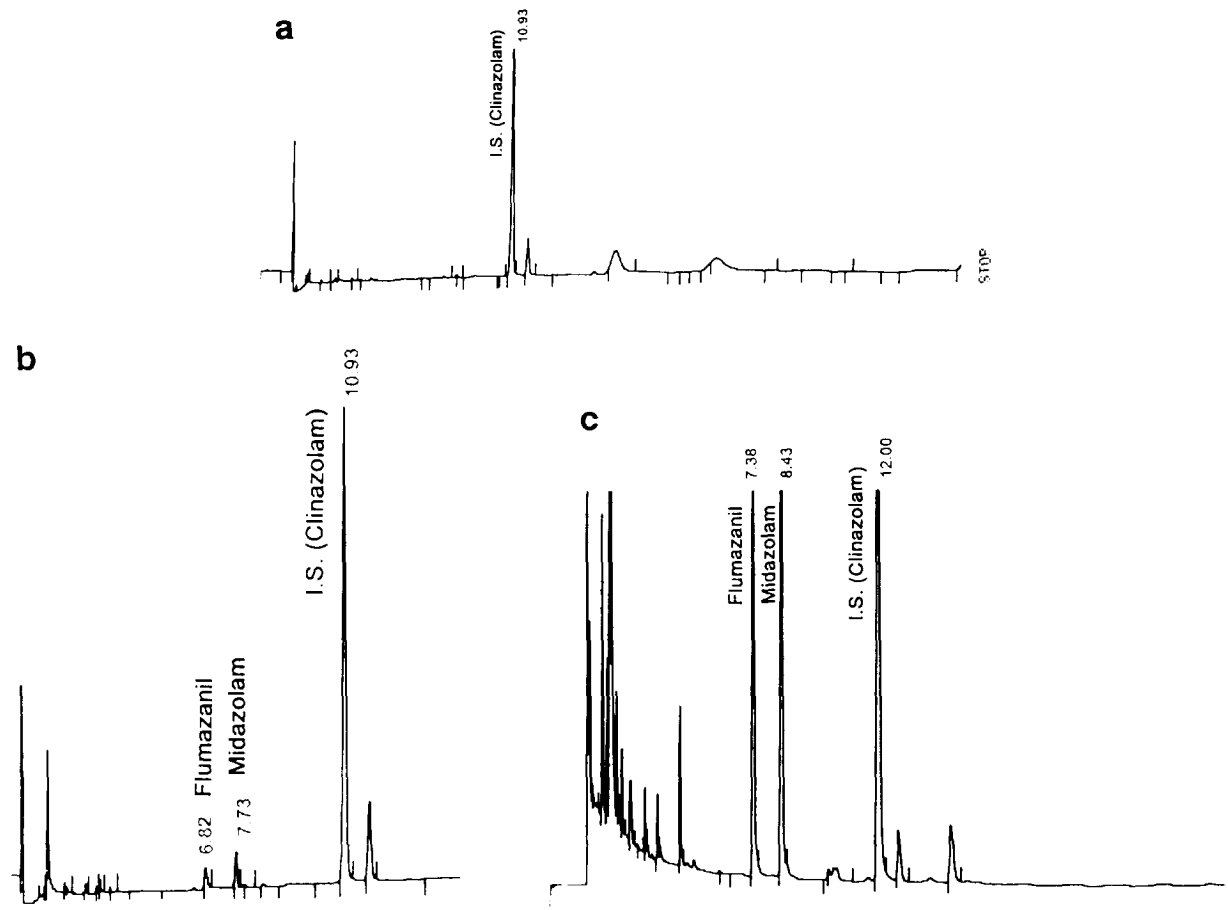

Fig. 2. (a) Chromatogram of a blank human plasma sample: (b) chromatogram of a human plasma sample spiked with 3 ng/ml of midazolam and flumazenil: (c) chromatogram of a human plasma sample following an intravenous dose of midazolam and flumazenil.

4. Discussion

The assay described here is linear over the range 3–1000 ng/ml, which encompasses the human therapeutic range of each drug. Expected plasma concentrations following a standard 1-mg dose of flumazenil range from 2 to 20 ng/ml, and therapeutic concentrations of midazolam range from 200 to 400 ng/ml. Additionally, this assay is a simple and precise method for determining midazolam and flumazenil concentrations simultaneously.

The method described here was used to measure plasma concentrations of midazolam and flumazenil in a human pharmacokinetic–pharmacodynamic study. The plasma concentrations obtained for a representative subject are shown in Figs. 3 and 4. The pharmacokinetic values obtained for both drugs are consistent with those

Table 1
Intra-day assay coefficients of variation (n = 10)

Added (ng/ml)	Coefficient of variaton (%)	
	Midazolam	Flumazenil
25	4.4	4.4
100	2.0	2.5
500	6.9	2.9

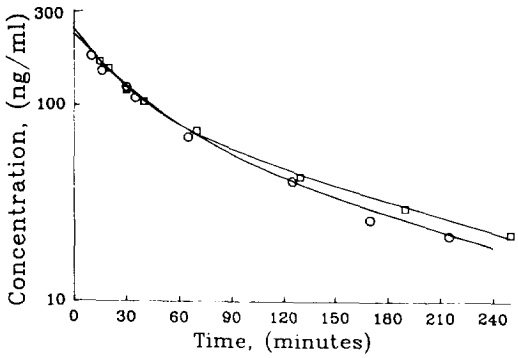

Fig. 3. Midazolam plasma concentrations in a representative subject after an intravenous bolus of midazolam alone (○) and with flumazenil reversal (□).

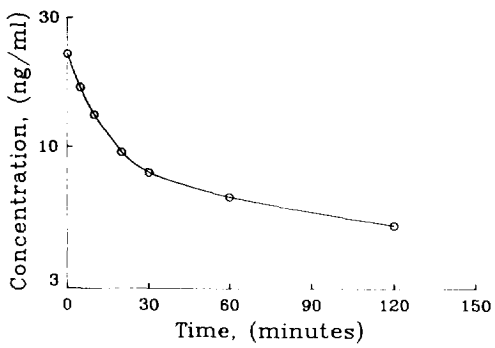

Fig. 4. Flumazenil plasma concentrations after an intravenous bolus of flumazenil (1 mg) in a representative subject.

previously reported. Therefore this assay is useful for pharmacokinetic studies and therapeutic monitoring in humans.

Acknowledgement

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References

[1] L. Aaltonen, J.J. Himberg, J. Kanto and A. Vuori, *Int. J. Clin. Pharmacol.*, 23 (1985) 247.
[2] A.A. Vletter, A.G.L. Burm, L.T.M. Breimer and J. Spierdijk, *J. Chromatogr.*, 530 (1990) 177.
[3] C.V. Puglisi, J. Pao, F.J. Ferrara and J.A.F. deSilva, *J. Chromatogr.*, 344 (1985) 199.
[4] J. Vasiliades, *J. Chromatogr.*, 228 (1982) 195.
[5] J. Vasiliades, *J. Chromatogr.*, 225 (1981) 266.
[6] I.F.I. de Kroon, P.N.J. Langendijik and P.N.F.C. de Goede, *J. Chromatogr.*, 491 (1989) 107.
[8] C.D. Syracuse, B.R. Kuhnert, C.J. Kaine, A.C. Santos and M. Finster, *J. Chromatogr.*, 380 (1986) 145.
[8] A. Blackett, S. Dhillon and J.A. Cromarty, *J. Chromatogr.*, 433 (1988) 326.
[9] E.K. Fukada, N. Choma and P.P. Davis, *J. Chromatogr.*, 491 (1989) 97.
[10] M. Zell and U. Timm, *J. Chromatogr.*, 491 (1989) 97.