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Note

High-performance liquid chromatographic assay to determine midazolam and flumazenil simultaneously in human plasma

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Midazolam is a potent weakly basic (pK_a = 6.0) imidazobenzodiazepine. The basic nitrogen in position 2 of the imidazo ring system makes it possible to form water-soluble salts, thereby producing stable aqueous solutions. The formation of a closed (and thus non-ionized) ring structure at a physiological pH of 7.4 [1] contributes to a painless intravenous injection in contrast to, for example, diazepam. For this reason, and because of its relatively short elimination half-life of 2–5 h, midazolam is the most suitable benzodiazepine for use in anaesthesia. Elimination of midazolam occurs predominantly by hydroxylation and subsequent glucuronization in the liver. Estimated total plasma clearance values range from 268 to 630 ml/min [2].

Midazolam is used for premedication, induction of anaesthesia, hypno/sedation during employment of regional techniques, and as a hypnotic component during maintenance of anaesthesia [3,4]. In these applications, advantage is taken of its strong sedative and hypnotic properties. Besides, midazolam has anticonvulsant, muscle relaxant and anterograde amnesic properties. Although midazolam is rapidly eliminated from the body, prolonged postoperative sedation or hypnosis may occur, in particular when high doses of midazolam have been administered as a hypnotic component during maintenance of anaesthesia [4].

Flumazenil, which is, like midazolam, an imidazobenzodiazepine, binds to the benzodiazepine receptor [5] but antagonizes in a competitive way the effects produced by benzodiazepine agonists [6]. Clinical applications of flumazenil include reversal of undesirable postoperative sedation or hypnosis and unwanted side-effects of benzodiazepines. In addition, it may be used as a diagnostic tool in intoxicated patiens. Clinically, flumazenil has a relatively rapid onset, but a short duration of action after administration of high doses of midazolam [4]. Therefore, permanent antagonism of benzodiazepine effects after administration of high doses of an agonist, requires repeated injections of flumazenil. The short

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duration of action of flumazenil may be related to its pharmacokinetics. Its mean elimination half-life and total plasma clearance are 70 min and 897 ml/min, respectively [7].

Gas chromatographic and high-performance liquid chromatographic (HPLC) methods have been used to measure midazolam [8–16] and flumazenil [17–20]. These procedures are intended to measure one drug only. However, since flumazenil is usually administered after previous administration of a benzodiazepine agonist, it would be advantageous to have an assay available to measure both drugs simultaneously. This paper describes an HPLC method for the simultaneous assay of midazolam and flumazenil. It has been applied for a study on the pharmacokinetic and pharmacodynamic interactions between midazolam and flumazenil in volunteers.

EXPERIMENTAL

Chemicals

The following chemicals were analytical grade: diethyl ether, ethanol, disodium hydrogenphosphate 12-hydrate, sodium dihydrogenphosphate 1-hydrate, sodium hydroxide and triethylamine (TEA). Dichloromethane was spectroscopic grade. These chemicals were all obtained from E. Merck (Darmstadt, F.R.G.). Water and methanol were HPLC grade and were purchased from J. T. Baker (Deventer, The Netherlands). Flumazenil, midazolam, their metabolites, and their internal standards were donated by F. Hoffmann-La Roche (Basle, Switzerland). The molecular structures of these compounds are shown in Fig. 1.

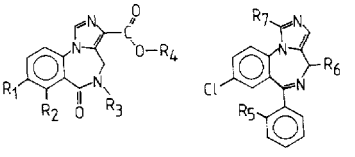
Calibration standards

A stock solution was made of 50 mg of flumazenil in 250 ml of ethanol. Working solutions were prepared by diluting the stock solution with ethanol to obtain 200 ng, 100 ng, 75 ng, 50 ng, 25 ng, 5 ng and 2 ng per 25 μl. The corresponding internal standard solution (Ro 15-3505, **I**) was made by dissolving 50 mg of **I** in 250 ml of ethanol, and then diluting with ethanol to obtain a working solution of 75 ng/25 μl.

A stock solution was made of 50 mg of midazolam maleate in 250 ml of ethanol. Working solutions were prepared by diluting the stock solution with ethanol to obtain 800 ng, 600 ng, 400 ng, 200 ng, 50 ng, 30 ng and 15 ng of midazolam per 25 μl. The internal standard solution was made by dissolving 50 mg of climazolam in 250 ml of ethanol, and then diluting with ethanol to obtain a working solution of 190 ng/25 μl. All solutions were stored at 4°C.

Extraction procedure

A 25-μl volume of a standard solution of flumazenil and midazolam, or ethanol, and 25 μl of the internal standard solution of **I** and climazolam were added to 1 ml of human plasma and mixed with 1 ml of buffer solution (0.1 *M*



Compound	R_1	R_2	R_3	R_4	R_5	R_6	R_7
flumazenil	F	H	CH_3	C_2H_5			
N-desmethyl ester metabolite	F	H	H	C_2H_5			
acid metabolite	F	H	CH_3	H			
N-desmethyl acid metabolite	F	H	H	H			
Ethyl 7-chloro,5,6-dihydro-5-methyl-6-oxo-4H-imidazo [1,5-a] [1,4] benzodiazepine-3-carboxylate, internal standard (I)*	H	Cl	CH_3	C_2H_5			
midazolam					F	H	CH_3
1-hydroxy-midazolam					F	H	CH_2OH
4-hydroxy-midazolam					F	OH	CH_3
1,4-dihydroxy-midazolam					F	OH	CH_2OH
desmethyl-midazolam					F	H	H
climazolam, internal standard					Cl	H	CH_3

* Ro 15-3505

Fig. 1. General structures of flumazenil (left), midazolam (right), their metabolites and their internal standards.

Na_2HPO_4, adjusted to pH 10.5 with NaOH) in a centrifuge tube. Samples were extracted with 5 ml of diethyl ether–dichloromethane (60:40, v/v) on a Vortex whirl-mixer for 30 s and then centrifuged for 10 min at 2000 *g* and 4°C. The organic phase was transferred to another centrifuge tube and washed on a Vortex whirl-mixer for 30 s with 1 ml of the buffer solution and then centrifuged for 5

TABLE I

CONTROLLER SETTINGS

Time (min:s)	% B	Time (min:s)	% B
00:00	6.20	17:30	97.50
05:30	6.20	21:00	97.50
05:40	40.00	21:30	6.20
17:00	40.00		

min at 2000 *g* and 4°C. The organic phase was transferred to a tapered evaporation tube and evaporated to dryness in a water-bath at 40°C under a gentle stream of pure nitrogen. The residue was dissolved in 50 μl of mobile phase, and 1–15 μl were injected into the HPLC apparatus.

Instrumentation

A modular HPLC system was used, consisting of two SF 400 pumps, an SF 450 programmer, an SF 757 absorbance detector equipped with a 12-μl flow cell, a static mixer (all Applied Biosystems, Ramsey, NJ, U.S.A.), an SpH99 a column thermostat, a Promis II auto-injector (Spark Holland, Emmen, The Netherlands) and a stainless-steel column (100 × 4.6 mm I.D.) prepacked with CP-Microspher C_{18}, 3 μm (Chrompack, Middelburg, The Netherlands). For data acquisition an SP 4290 integrator and an Epson PC-E/HD, with chromatographic software (Spectra-Physics, San Jose, CA, U.S.A.) were used.

Operating conditions

Chromatography was performed in reversed-phase mode using two solvents: A, methanol–buffer (6 g/l $NaH_2PO_4 \cdot H_2O$ and 1 ml/l TEA, adjusted to pH 7.00 with NaOH) (1:2); and B, methanol–water (4:1). The gradient profile is shown in Table I. The flow-rate of the mobile phase was 1.5 ml/min, and the oven temperature was 40°C. The absorption wavelength of the detector was 220 nm with a filter rise-time of 0.5 s. The total run time was 33 min.

RESULTS

Typical chromatograms of blank and spiked human plasma are shown in Fig. 2. The retention times were 5.0 min for flumazenil, 6.4 min for **I**, 15.5 min for midazolam and 17.0 min for climazolam. No endogenous components and none of the known metabolites of flumazenil and midazolam interfered with the assay (Table II). The results of eight calibrations carried out on eight different days with plasma from eight male human volunteers showed coefficients of variation (C.V.) of the peak-height ratios of flumazenil (2–200 ng/ml) and midazolam (30–800

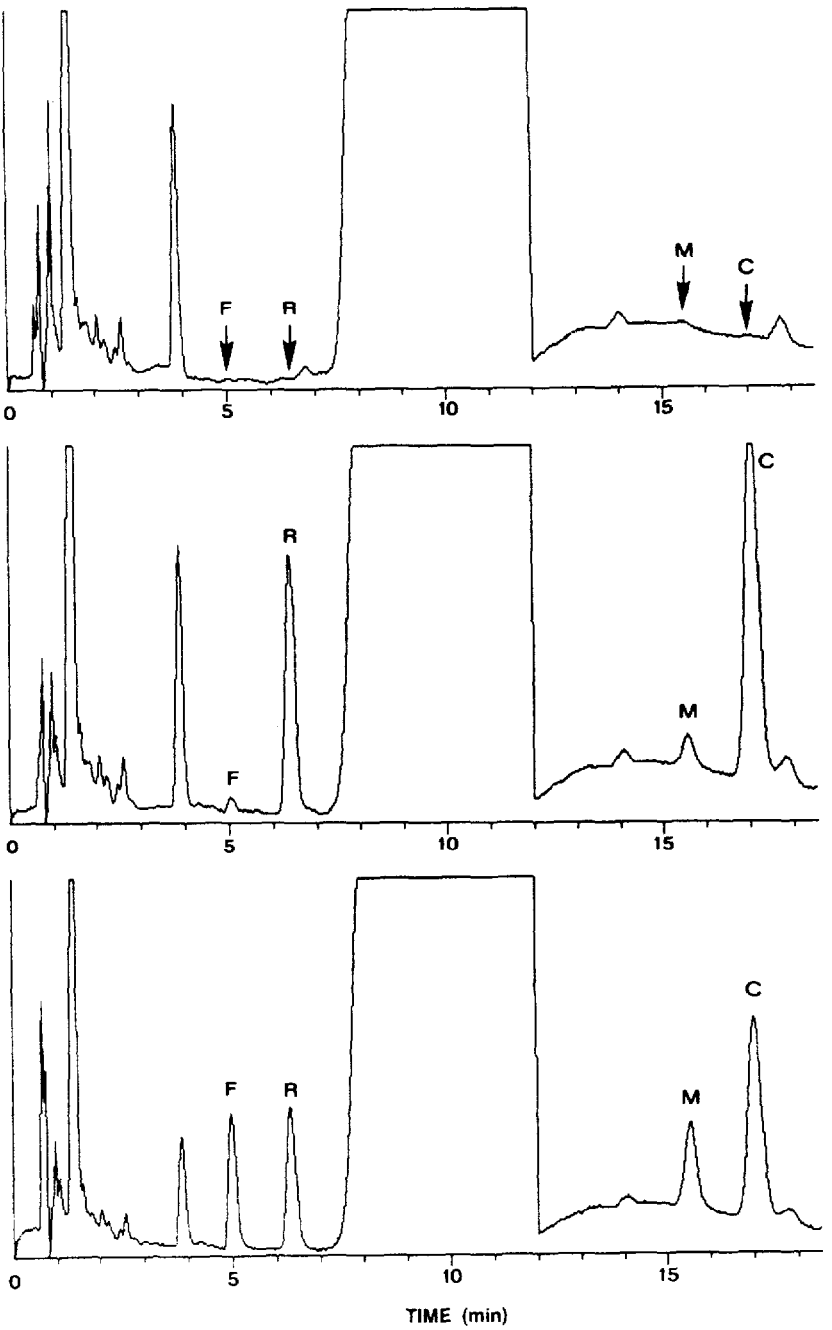


Fig. 2. Chromatograms of blank plasma (upper), plasma spiked with 5 ng/ml flumazenil and 15 ng/ml midazolam (middle), and 100 ng/ml flumazenil and 75 ng/ml midazolam (lower). Peaks: F = flumazenil; R = I (75 ng/ml); M = midazolam; C = climazolam (190 ng/ml).

TABLE II

RELATIVE RETENTION TIMES OF FLUMAZENIL, MIDAZOLAM, THEIR METABOLITES AND THEIR INTERNAL STANDARDS

Compound	Relative retention time	Compound	Relative retention time
Acid metabolite of flumazenil	0.07	4-Hydroxymidazolam	2.48
N-Desmethyl ester metabolite of flumazenil	0.61	1-Hydroxymidazolam	2.68
Flumazenil	1.00	Desmethylmidazolam	2.89
Internal standard of flumazenil (**I**)	1.32	Midazolam	3.49
1,4-Dihydroxymidazolam	2.03	Climazolam	3.86

ng/ml) to their respective internal standards of 3.2–12% (mean 5.6%) and 3.0–5.6% (mean 4.3%), respectively. Calibration curves were obtained by weighted least-squares linear regression analysis (weight factor $1/y^2$) of the peak-height ratio of flumazenil/**I** *versus* the concentration of flumazenil, and the peak-height ratio of midazolam/climazolam *versus* the concentration of midazolam. The calibration line for flumazenil was linear in the investigated range (2–200 ng/ml) with a correlation coefficient varying from 0.9996 to 0.9999. The recovery was 90% for flumazenil and 94% for **I** over the investigated range. The calibration line for midazolam was linear in the investigated range (30–800 ng/ml) with a correlation coefficient varying from 0.9959 to 0.9999. The recovery was 77% for midazolam and 73% for climazolam over the investigated range. Deviations between the spiked concentration and the concentration determined with the aid of the calibration lines, varied on average from −2.1% to +3.8% for flumazenil (2–200 ng/ml), and from −2.8 to +2.9% for midazolam (30–800 ng/ml). The detection limit for flumazenil was 0.3 ng/ml plasma, and 2 ng of flumazenil could be quantified with acceptable precision. The detection limit for midazolam was 1 ng/ml plasma, and 30 ng of midazolam per ml plasma could be quantified with acceptable precision.

DISCUSSION

The development of the combined assay of midazolam and flumazenil is related to the fact that flumazenil is a competitive benzodiazepine antagonist. Therefore measured flumazenil concentrations should always be interpreted against the background of the concentrations of the benzodiazepine agonist. This obviates the need for an assay that enables simultaneous determination of both the agonist and antagonist. Determination of each drug with separate assays is definitely more elaborate and more time-consuming.

In previous studies we have used isocratic HPLC methods to measure plasma concentrations of midazolam and flumazenil after administration of each drug

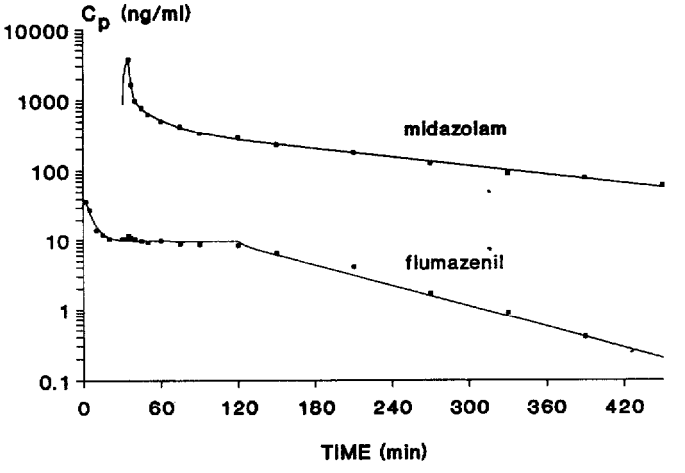


Fig. 3. Plasma concentration (C_p)–time profiles of midazolam and flumazenil in a representative volunteer. Flumazenil was infused at a rate of 0.45 mg/min for 2 min, then at 0.0087 mg/min for 118 min. This infusion rate was aimed at attaining and maintaining a steady-state concentration of 10 ng/ml from 30 to 120 min. Midazolam was infused at a rate of 6 mg/ml for 5 min, starting 30 min after the flumazenil infusion.

alone [7,21]. The performance of these methods was satisfactory, but modification was considered desirable, because in the midazolam assay and unidentified plasma constituent with a retention time of 2 h was sometimes present in the chromatograms. With the present procedures late peaks were never observed. This was accomplished by the inclusion of a column clean-up step in the procedure.

The assay described here enables simultaneous quantification of flumazenil and midazolam in the clinically relevant concentration ranges. Occasionally, plasma concentrations of midazolam may well exceed the documented 800 ng/ml. This will, for example, be the case when a high dose of midazolam is rapidly infused (Fig. 3). In this situation samples containing more than 800 ng/ml are first diluted with blank plasma before the extraction. This is done, because, in our experience, the calibration curve is not always linear at the higher concentrations, depending on the condition of the column.

Minor modifications of the procedure, described here enable quantification of each drug alone. The performance of the modified procedures are at least as good as the performance of the combined assay. In this respect it is noteworthy that the detection limit of flumazenil is slightly lower when the drug is analysed alone. This is attained by changing the absorption wavelength. In the present assay the wavelength is set at 220 nm, which is the absorption maximum for midazolam. In the assay of flumazenil alone, the wavelength can be set to 245 nm, the absorption maximum for flumazenil.

None of the known metabolites of midazolam and flumazenil interferes with the assay of these drugs. One metabolite of flumazenil, the N-desmethyl acid metabolite, was not available when the assays were developed. However, given its hydrophilic properties, it is very unlikely that it will interfere, because the extraction yield is likely to be poor and, in addition, its retention time should be much shorter than that of flumazenil.

The method described here has been used to measure plasma concentrations of midazolam and flumazenil in more than 1000 samples, collected from a total of 20 volunteers in two different studies on the pharmacokinetics and pharmacodynamics of midazolam and the interaction between midazolam and flumazenil. In the latter study flumazenil was infused according to a Wagner infusion scheme, based on previously determined pharmacokinetic data [7] and aimed at attaining and maintaining a constant flumazenil concentration of 10 or 20 ng/ml from 30 to 120 min after the start of the infusion. In addition, 30 or 60 mg of midazolam were infused in 5 min, starting 30 min after the start of the flumazenil infusion. The plasma concentration profiles, obtained in one of the volunteers, receiving a flumazenil infusion, aimed at a 'steady state' of 10 ng/ml, and 30 mg of midazolam, are shown in Fig. 3. Averaged over all eight volunteers, the mean (± S.D.) concentrations of flumazenil during the steady-state period were 8.9 ± 1.1 ng/ml and 18.3 ± 1.3 ng/ml. Mean (± S.D.) total plasma clearance, steady-state volume of distribution and elimination half-life values were 960 ± 78 ml/min, 87 ± 14 l and 72 ± 8 min for flumazenil, and 344 ± 31 ml/min, 55 ± 5 l and 133 ± 22 min for midazolam.

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