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Assay of Midazolam in Human Plasma by Gas-Liquid Chromatography with Nitrogen-Phosphorus Detection

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A sensitive and specific method is described for the determination of midazolam in human plasma. The drug was extracted from 1 mL of carbonate buffered plasma (pH 9.6) with 8 mL of diethyl ether. Famprofazone was used as internal standard. The organic phase was evaporated to dryness. The residue was dissolved in methanol for the direct analysis by gas chromatograph-nitrogen phosphorus detector system. In the concentration range of 1-5000 ng/mL, the calibration curve was linear. The coefficients of variation from the precision test were < 6% at the range of the concentration of 0.10-2.00 μg/mL and the detection limit for midazolam in 1 mL of plasma was 0.5 ng. This assay is more sensitive, selective, simple and rapid than earlier methods. Plasma midazolam concentrations were determined by this method after administration of midazolam.

Introduction

Midazolam [8-chloro-6-(2-fluorophenyl)-1-methyl-4H-imidazo(1,5-a)(1,4)-benzodiazepine] (Figure 1) is a benzodiazepine used as a premedicant and sedative in surgical and other procedures and for the induction of anaesthesia.

It is also used in the management of severe insomnia. Excessive drowsiness, sedation and ataxia are the most frequent adverse effects. Respiratory depression and hypotension have occurred after intravenous use for conscious sedation.[1] Although blood level studies have been described, currently available methods for determination of the drug in plasma are not suitable for pharmacokinetic studies in which single doses may be compared. Several methods based on different principles have been proposed for the determination of midazolam. For the determination of nanogram amounts of midazolam in biological fluids, the most commonly used methods employ high performance liquid chromatography (HPLC)[2-13] and gas chromatography (GC).[14-21] HPLC methods are not sensitive enough to determine the trace levels (down to 1 ng/mL) of midazolam present in human plasma. Several GC methods[14,18] measured plasma concentrations of midazolam by a single-step extraction procedure with a mixture of chloroform and ethylacetate (80 : 20),[14] or n-hexane-dichloromethane (70 : 30).[18] These solvents such as chloroform and dichloromethane are very toxic for human.

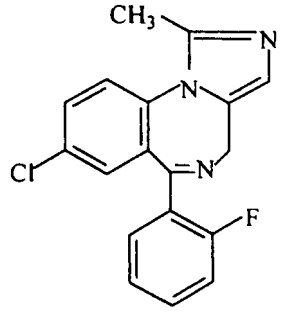

Figure 1. The structure of midazolam.

We propose a GC separation with NPD after a single extraction with diethyl ether for the selective and highly sen-

sitive determination of midazolam in human plasma.

Experimental

Chemicals. Midazolam was donated by Dong-Aa Medical School (Pusan, Korea). Famprofazone, which was chosen as internal standard, was supplied by Ed. Geistlich Sohne (Wolhysen, Switzerland). The following reagents were used: diethyl ether, methanol, potassium carbonate and sodium bicarbonate (Merck, Darmstadt, Germany).

Stock solution. Standard solutions of midazolam and famprofazone (1 mg/mL) were prepared by dissolving 10 mg of each drug in 10 mL of methanol and kept at 4 °C. Under these conditions the solutions were stable for several weeks. Working solutions (10 and 1 μg/mL) were prepared by sequential dilutions.

Drug administration and sample collection. Three male patients aged 64, 43 and 51) were studied. After the induction of anaesthesia, midazolam was administered by i.v. loading of 0.25 mg/kg for 10 sec. 5 mL of venous blood was collected in heparinized syringe at intervals of 1, 3, 5, 10, 20, 30, 40 min, 1, 1.5, 2, 2.5, 3, 4, 5, 6, 8, 10, 12 h. The blood samples were centrifuged (2000 rpm for 5 min) and the plasma was separated and stored at −20 °C until analysis.

Extraction procedure. For each analysis 1.0 mL of human plasma was used. 10 μL of famprofazone solution (10 μg/mL) were added as internal standard to each sample of plasma in a glass centrifuge tube. 8 mL of diethyl ether and 50 mg of $NaHCO_3$+K_2CO_3 were added, and tubes were then stoppered and shaked mechanically for 20 min and centrifuged for 5 min at 750 g. The organic layer was transferred to a 15 mL glass centrifuge tube, and evaporated to dryness at reduced pressure, dissolved in 100 μL of methanol. 2 μL of the methanolic solution was injected into GC.

Calibration curve and quantitation. The standard curve of midazolam was constructed by adding 1, 5, 25, 50, 100, 250, 500, 1000, 2500 and 5000 ng of the authentic and 100 ng famprofazone to 1.0 mL of drug-free plasma. Peak area ratios for drug/internal standard were used to calculate the calibration curves, the slope of which was used in the quantitation of the drugs in plasma. For the calculation of extraction recovery, samples were prepared by adding 100 and 500 ng of the authentic in 1.0 mL of drug-free plasma. 8 mL of diethyl ether and 50 mg of $NaHCO_3$+ K_2CO_3 were added to each sample, and tubes were then stoppered and shaked mechanically for 20 min and centrifuged for 5 min at 750 g. The organic layer was transferred to a 15 mL glass centrifuge tube and internal standard was added organic phases. Organic solvent was evaporated to dryness at reduced pressure and dissolved in 100 μL of methanol. Recovery was evaluated by comparing peak area ratios for drug/internal standard after extraction from plasma and standards at 100 and 500 ng/mL concentration without extraction.

Gas chromatography (GC). All GC experiments were performed with a Hewlett-Packard (Avondale, P.A., USA) 5890 gas chromatograph with a nitrogen phosphorus detector (NPD). All injection were made by a HP 7673A Auto sampler, GC conditions for the plasma analyses are listed in Table 1.

Table 1. Gas chromatographic conditions used for the determination of midazolam

Parameter	Condition
Column	HP fused silica capillary, cross linked 5% phenyl methyl silicon (HP-5, 25 m × 0.2 mm I.D. × 0.33 μm F.T.)
Detector temp.	300 °C
Initial temp.	100 °C
Program rate	20 °C
Final temp. (time)	300 °C (2 min)
Carrier gas	Helium
Flow rate	14.1 psi (97 kPa)
Auxillary gas flow	Helium at 25 mL/min
Hydrogen flow	4.0 mL/min
Air flow	100 mL/min
Split ratio	1 : 10

Gas chromatography-mass spectrometry. All mass spectra were obtained with a Hewlett-Packard 5890/5971 instrument. The ion source was operated in the electron ionization mode (EI; 70 eV, 150 °C). Full-scan mass spectra (m/z 40-450) were recorded for analyte identification. An HP cross-linked 5% phenylmethylsilicone capillary column (HP-5MS, 30 m × 0.25 mm I.D.; film thickness 0.25 μm) was used. Samples were injected in the split mode with a split ratio of 1 : 10. The flow rate of helium as carrier gas was 1.23 mL/min (97 kPa). The GC operating temperatures were as follows: injector temperature, 280 °C; transfer line temperature, 280 °C; oven temperature, programmed from 100 °C at 20 °C/min to 300 °C.

Results and Discussion

Figure 2 shows typical chromatograms of plasma samples before and after dosing midazolam at 0.25 mg/kg. The drug

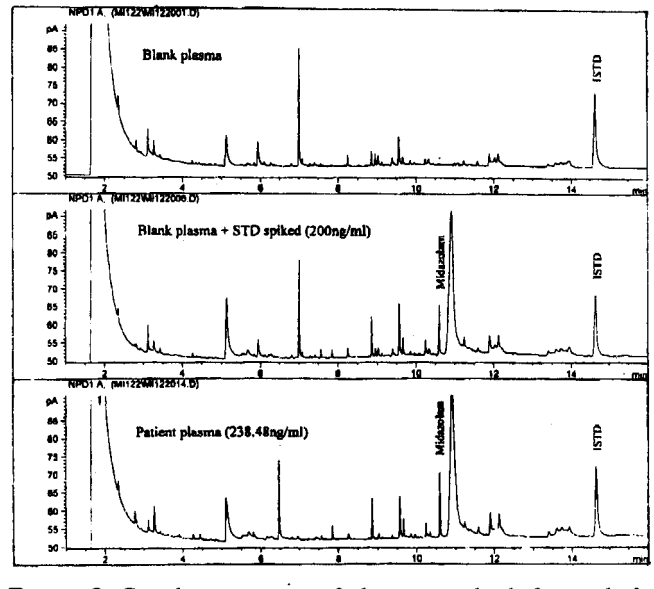

Figure 2. Gas chromatograms of plasma samples before and after dose of midazolam at 0.25 mg/kg.

Table 2. Precision and accuracy of the determination of midazolam concentration in a 1.0 mL sample of human plasma

Concentration added (μg/mL)	Concentration found (N=3)	
	Mean (μg/mL)	C.V. (%)
0.100	0.109	5.25
0.500	0.489	4.32
2.000	1.924	3.37

and internal standard were eluted at 10.61 and 14.67 min, respectively, under the present chromatographic conditions. Extraction recovery was evaluated by analyzing spiked plasma samples and standards. In these experiments, internal standard was added after transfer of organic phases. The extraction yield from plasma was nearly complete and reasonably consistent (92%) at the two concentrations (100 and 500 ng/mL). The detection limit with a 1.0 mL plasma sample was 0.5 ng/mL at S/N of 3. Linear calibration curves were observed over the 1 ng/mL-5 μg/mL range. The correlation coefficient was 0.9993 and the line of best fit was y=0.8454 x-0.0012, where x is the analyte concentration (ng/mL) and y is the peak area ratio of the analyte to internal standard. The intra-assay precision was determined by estimating the variation on normalized peak area ratios (*i.e.*, peak area ratios of midazolam divided by peak area of the corresponding internal standard). The coefficient of variation varied from 3.37 to 5.25% (Table 2). The intra-day coefficient of variation was 6.37% when it was determined by analyzing samples spiked with 100 ng/mL on three different days.

No interfering peak from endogeneous substances was observed. The major feature of NPD is to overcome the interfering problems of the quantitative assay. The high selectivity for nitrogen-containing compounds seems to provide a means for the further simplication of existing methods, by reducing the need for purification of the plasma extract prior to the chromatography, thus reducing the time required for analysis. In this way, we reduced the time necessary to prepare twenty samples for GLC injection to less than 1 h.

The present method was used for the determination of midazolam in plasma samples of patients receiving a dose of 0.25 mg/kg midazolam. In one person, the maximum plasma concentration of midazolam was 1412 ng/mL. The variation of the midazolam plasma concentration was described by a two-compartment pharmacokinetic model, and its first elimination half-life was about 9.5 min (Figure 3). The results from other two persons were similar to that previously reported. Generally the pharmacokinetics of midazolam can be influenced by age, physiology, and hepatic and renal function of patients.[21-23] Thus, monitoring the midazolam concentration in blood can be helpful to prevent intoxication.

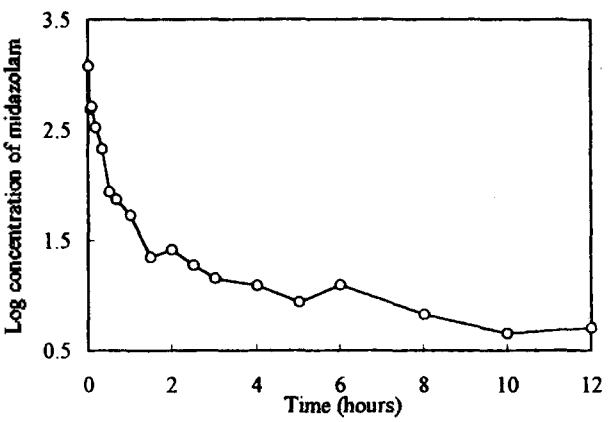

Figure 3. Variation of the midazolam plasma concentration from a patient after its administration at a dose of 0.25 mg/kg.

In summary, the present method was suitable for rapid analysis of midazolam. Sample preparation was minimal, and there was no interference from endogeneous substances.

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