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# Determination of midazolam and its metabolite as a probe for cytochrome P450 3A4 phenotype by liquid chromatography–mass spectrometry

Hideko Kanazawa [a,*], Akiko Okada [a], Eri Igarashi [a], Megumu Higaki [b], Takako Miyabe [c], Tadashi Sano [c], Ryouhei Nishimura [c]

[a] *Department of Physical Chemistry, Kyoritsu College of Pharmacy, 1-5-30 Shibakoen, Minato-ku, Tokyo 105-8512, Japan*
[b] *Institute of Medical Science, St. Marianna University School of Medicine, 2-16-1 Sugao, Miyamae-ku, Kanagawa 216-8512, Japan*
[c] *Faculty of Agriculture, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, Japan*

**Abstract**

This study demonstrated the analysis of midazolam and its metabolites by liquid chromatography–mass spectrometry (LC–MS) with a sonic spray ionization (SSI) interface. The analytical column was a YMC-Pak Pro $C_{18}$ (50 mm × 2.0 mm i.d.) using 10 mM ammonium acetate (pH 4.8)–methanol (1:1) at a flow rate of 0.2 ml $min^{-1}$. The drift voltage was 100 V. The sampling aperture was heated at 110 °C and the shield temperature was 230 °C. The lower limits for the detection of midazolam and 1′-hydroxymidazolam were 26.3 and 112.76 pg injected, respectively. The calibration curves for midazolam and 1′-hydroxymidazolam were linear in the range of 0.1–5 μg $ml^{-1}$. Within-day relative standard deviations was less than 7%. The method was applied to the determination of midazolam in monkey plasma, and the analysis of midazolam and its metabolites in an in vitro study with recombinant cytochrome P450 (CYP) 3A4. This method is sufficiently sensitive and useful to elucidate the kinetics of midazolam metabolite formation. We also investigated the effect of propofol on the metabolism of midazolam using recombinant CYP3A4. Propofol competitively inhibited the metabolism of midazolam to 1′-hydroxymidazolam by CYP3A4.


*Keywords:* Sonic spray ionization; Interfaces, LC–MS; Midazolam; Cytochromes; Propofol

## 1. Introduction

Midazolam, 8-chloro-6-(2-fluorophenyl)-1-methyl-4*H*-imidazo[1,5-*a*][1,4]benzodiazepine, is used for intravenous sedation and the induction of anesthesia. In humans, it has been widely used for cardiac surgery in combination with opioids. Oral midazolam is commonly used for procedural sedation in children, despite the lack of a product license for the oral route of administration and a low bioavailability [1–4]. The oxidative metabolism of drugs in the liver is catalyzed by substrate-specific or selective cytochrome P450 (CYP), a superfamily of hemoproteins that catalyze the metabolism of a large number of clinically important drugs [5–7]. Hepatic drug oxidation is a major source of interindividual variations in drug pharmacokinetics and therapeutic response. Midazolam is widely used as a probe to measure the activity of CYP3A4, an important member of the cytochrome P450 superfamily of drug-metabolizing enzymes [8–11]. This benzodiazepine undergoes oxidative metabolism to one major metabolite, 1′-hydroxymidazolam, in humans, a pathway which seems to be mediated almost exclusively by CYP3A isoforms [12,13].

In vitro cytochrome P450 assays are used in metabolism studies in support of early phases of drug discovery to investigate, e.g., metabolic stability, enzyme inhibition and induction by new chemical entities. LC–UV and GC–MS are traditional analytical tools that support of such studies [8–15]. However, these tools typically comprise different methods of relatively low throughput for the various metabolites of the probe reactions. In recent years, liquid chromatography–mass spectrometry (LC–MS) methods have been developed to increase the throughput [16–22].

This study demonstrates determination of midazolam in monkey plasma, and the analysis of midazolam and its metabolites in an in vitro study with recombinant CYP3A4 by LC–MS with a sonic spray ionization (SSI) interface. Additionally, in an in vitro study, the effect of propofol (2,6-diisopropylphenol) on the metabolism of midazolam was studied with recombinant CYP3A4. Propofol is a short-acting anesthetic commonly used in clinical practice.

* Corresponding author. Tel.: +81-3-5400-2657; fax: +81-3-5400-1378.
*E-mail address:* kanazawa-hd@kyoritsu-ph.ac.jp (H. Kanazawa).

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Propofol is frequently used in combination with midazolam and other anesthetics, and has been shown to inhibit their metabolism and that of those compounds [23–29].

## 2. Experimental

### 2.1. Chemicals

Midazolam (Dormicum), propofol (Rapinovet) and intralipid (fat emulsion preparation) were purchased from Yamanouchi Pharmaceuticals (Tokyo, Japan), Shering-Plough (Osaka, Japan) and Otsuka Pharmaceuticals (Tokyo, Japan), respectively. 1′-Hydroxymidazolam was from Daiichi (Tokyo, Japan).

β-Diphosphopyridine nucleotide disodium salt (NADP), glucose-6-phosphate (G6P), glucose-6-phosphate dehydrogenase (G6PDH) and magnesium chloride were purchased from Wako (Osaka, Japan). Human liver microsomes and recombinant human CYP3A4 were purchased from Gentest (Worburn, MA, USA). All other reagents and solvents were of analytical grade.

### 2.2. LC–MS conditions

The assay was developed using a model M-8000 ion trap LC–MS system (LC/3DQMS, Hitachi, Tokyo, Japan) with sonic spray ionization interface. The analytical column was a YMC-Pack Pro $C_{18}$ (50 mm × 2.0 mm i.d., YMC, Japan) operated at 25 °C. The drift voltage was 100 V. The sampling aperture was heated at 110 °C and the shield temperature was 230 °C. Nitrogen gas was used as a carrier gas with output pressure of 0.50 MPa and ion source inlet pressure at 0.39 MPa. The mobile phase for analysis of midazolam and its metabolite was methanol–10 mM ammonium acetate (1:1) at a flow rate of 0.2 ml $min^{-1}$.

For analysis of propofol and its metabolite, methanol–water (65:35) as a mobile phase at a flow rate of 0.2 ml $min^{-1}$ with YMC-Pack Pro $C_{18}$ (50 mm × 2.0 mm i.d.). The other LC–MS conditions were the same as that for midazolam.

### 2.3. Sample preparation

Blood samples were collected into heparinized tubes and separated by centrifugation for 10 min at 2000 × *g*. The samples were stored frozen at −20 °C until analysis. To remove proteins prior to injection, the plasma sample was pretreated with a solid-phase extraction. A 490 μl volume of the plasma sample added 10 μl of internal standard (detomidine, 5 mg $ml^{-1}$) was loaded to an Empore disk cartridge, $C_{18}$ (3 M, St. Paul, MN, USA), after conditioning the cartridge with methanol, water and 10 mM ammonium acetate. A 5 ml volume of 10 mM ammonium acetate as a washing solvent was passed through the cartridge. The sample fraction was obtained by elution with 3 ml of methanol. After evaporated to dryness using a CVE-2000 centrifugal evaporator (EYELA, Tokyo, Japan) at 42 °C, the residue was dissolved in 500 μl of the eluent and a 5 μl volume of the sample was injected into an LC–MS system.

Plasma samples were collected from three healthy monkeys (Japanese macaques) before and 20, 40, 60, 150 min after a single oral dose of 1 mg $kg^{-1}$ midazolam (Dormicum). A 200 μl volume of the plasma was loaded into an Empore disk cartridge. After the same treatments as described earlier, the residue was dissolved in 20 μl of the eluent and a 1 μl volume of the sample was injected into an LC–MS system. Animal study was conducted under the guidelines provided by the Primate Research Institute, Kyoto University.

### 2.4. Calibration curves

Known amounts of midazolam and 1′-hydroxymidazolam in the concentration range 0.1–5.0 μg $ml^{-1}$ were added to blank plasma samples. Calibration curves were constructed by plotting the peak-area ratios of drug or metabolite peaks to that of the internal standard against known concentrations. The data were subjected to linear regression analysis.

### 2.5. Method validation of determination of midazolam and its metabolite

The known amounts of midazolam and 1′-hydroxymidazolam were added to the monkey plasma, and each of the resulting solutions was assayed with proposed method. Within-day precisions were determined by preparing and analyzing on the same day five replicates at two concentrations. Between-day precisions were assessed by analyzing each day for 5 days. Relative standard deviation (R.S.D.) values were used as the index of precision.

### 2.6. In vitro experimental

The basic incubation medium contained 100 mM potassium phosphate buffer (pH 7.4), a NADPH-regenerating system (1.3 mM NADP, 3.3 mM G6P, 0.4 units $ml^{-1}$ G6PDH, and 3.3 mM $MgCl_2$) and midazolam (15.3 μM). The final incubation volume was 0.5 ml. After preincubation at 37 °C for 5 min, 30 pmol of recombinant CYP3A4 was added to the mixture and incubation was carried out at 37 °C for 10 min. Adding 0.5 ml of cold acetonitrile stopped the reaction. After the addition of an internal standard (10 μl of 5 mg $ml^{-1}$ detomidine), the mixture was centrifuged at 13,000 × *g* for 5 min, and 0.5 ml of the supernatant was evaporated to dryness using a CVE-2000 centrifugal evaporator and dissolved in 1 ml of 10 mM ammonium acetate. To remove phosphate buffer prior to injection, the sample was pretreated with a solid-phase extraction. A 1 ml of the sample was loaded to an Empore disk cartridge, $C_{18}$. After the same treatments as plasma sample, the residue was dissolved in 100 μl of the eluent and a 5 μl volume of the sample was injected into an LC–MS system.

*2.6.1. Inhibition studies of midazolam 1′-hydroxylation by propofol and metabolism of propofol by human liver microsomes*

The metabolism of midazolam was measured with 30 pmol CYP3A4 in the absence or presence of a range of propofol concentrations. The concentrations of propofol were 0, 25, 50, 100, 200 and 400 μM. CYP3A4 was incubated with 15.3 μM midazolam. For a kinetic analysis, the midazolam concentration ranged from 1 to 10 μM. The effects of propofol (100 μM) on the formation of 1′-hydroxymidazolam were studied. The concentrations of propofol employed in the present study were comparable to the liver concentration obtained from a previously reported in vivo study [23].

Metabolism of propofol by human liver microsomes was measured using almost the same method used for midazolam metabolism with CYP3A4. Instead of CYP3A4, human liver microsomes containing 100 μg of protein were used. Propofol was initially prepared in methanol solution, and the final concentration in the incubation mixture was adjusted to 20 μM. The final concentration of methanol was less than 0.4%.

## 3. Results and discussion

### 3.1. Calibration curves and precision

LC–MS with an SSI interface was used for the analysis of midazolam ($M_r$ 325.77) and 1′-hydroxymidazolam ($M_r$ 342.77) with methanol–10 mM ammonium acetate (pH 4.8) (1:1) as the eluent at a flow rate of 0.2 ml $min^{-1}$. The peak areas were calculated based on the selected-ion chromatograms of midazolam and 1′-hydroxymidazolam at *m/z* 326 and 342, respectively, under positive conditions. The linear relationship calculated between the peak-area ratio and the concentration of midazolam (or 1′-hydroxymidazolam) from 0.1 to 5 μg $ml^{-1}$ and the correlation coefficients (*r*)

Table 1
Within- and between-day precision and accuracy for the determination of midazolam and 1′-hydroxymidazolam

| | Concentration (μg $ml^{-1}$) | Within-day R.S.D. (%) | Between-day | |
|---|---|---|---|---|
| | | | R.S.D. (%) | Accuracy (%) |
| Midazolam | 0.1 | 4.6 | 4.7 | 95.6 |
| | 0.5 | 2.7 | 1.8 | 97.8 |
| 1′-Hydroxymidazolam | 0.1 | 6.6 | 8.1 | 90.8 |
| | 0.5 | 5.5 | 2.4 | 95.7 |

Precision is expressed as relative standard deviation (R.S.D.) and accuracy as the assayed concentration relative to the actual concentration (%).

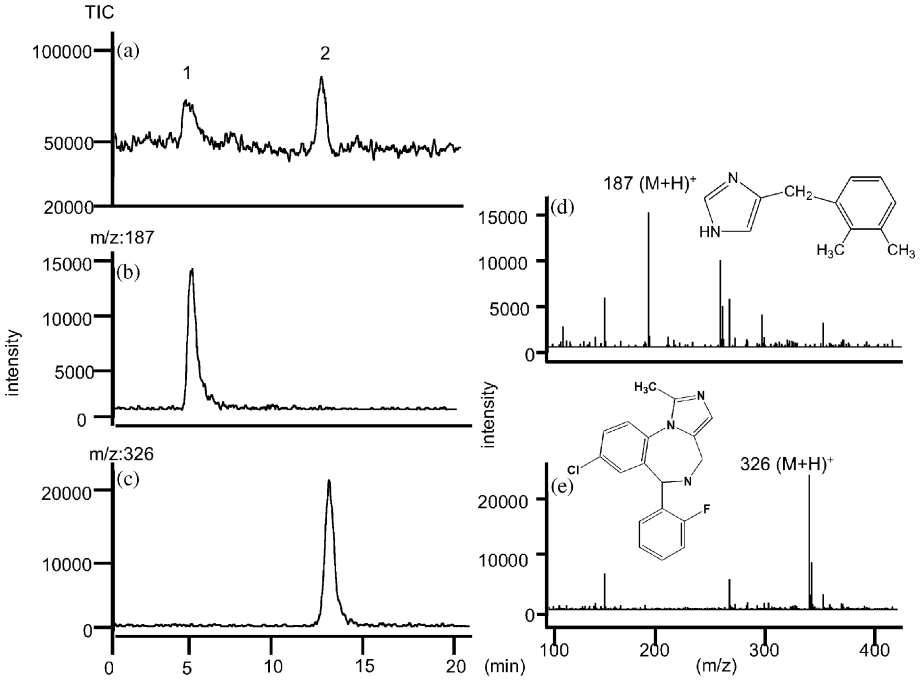


Fig. 1. Total ion chromatogram, mass chromatograms and mass spectra of an extract of plasma sample obtained from monkey administered midazolam (I.S.: detomidine). Peak numbers: (1) detomidine; (2) midazolam. Injection volume is 1 μl.

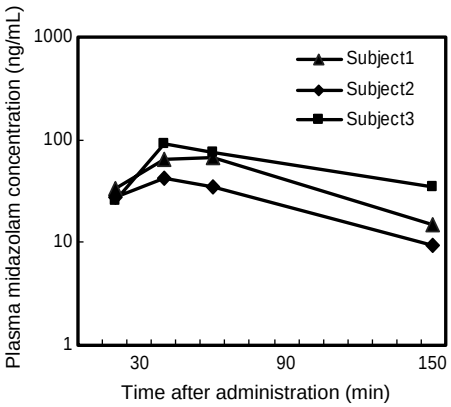


Fig. 2. Plasma concentration–time curves for midazolam.

were as follows:

midazolam : $y = 5.56x + 0.28$ $(r^2 = 0.964)$,

1′-hydroxymidazolam : $y = 1.06x - 0.003$ $(r^2 = 0.983)$.

The lower limits for the detection of midazolam and 1′-hydroxymidazolam were 26.3 and 112.76 pg injected at a signal-to-noise ratio of 3, respectively. The recoveries of midazolam and 1′-hydroxymidazolam with solid-phase extraction were 102.3 and 100.8%. The precision of the method was established from 5 assays. Within- and between-day precision and accuracy data are shown in Table 1. The present method is sufficiently sensitive and accurate to measure kinetic parameters.

### 3.2. Application to the quantitation of midazolam in monkey plasma

The present method was used for the determination of midazolam ($M_r$ 325.77) and its metabolites in monkey plasma. Fig. 1 shows the total ion chromatogram (TIC), mass chromatograms and the mass spectra under positive-ion conditions of an extract of plasma sample obtained from the subject following oral administration of the mixture. Protonated molecular ions, $[M + H]^+$, of the internal standard and midazolam were clearly observed at *m/z* 187 and 326, respectively, as base peaks. There was no interference from the extracted components in monkey plasma. The mass spectrum of midazolam was almost the same as that obtained by a direct analysis. However, the metabolites of midazolam were not observed in this condition. Plasma concentration–time curves for midazolam is shown in Fig. 2. The maximal concentration of midazolam in plasma ($C_{max}$) and time to reach $C_{max}$ ($t_{max}$) were 40–90 ng ml$^{-1}$ and 40–60 min, respectively. The present method may be applicable to the pharmacokinetic studies of midazolam in biological samples.

### 3.3. In vitro metabolism

#### 3.3.1. Oxidation of midazolam by recombinant CYP3A4

Midazolam was incubated in vitro with recombinant 3A4 isoform. The incubation mixture and other conditions are described in Section 2. Following recombinant CYP3A4 incubations, the chromatographic peaks were

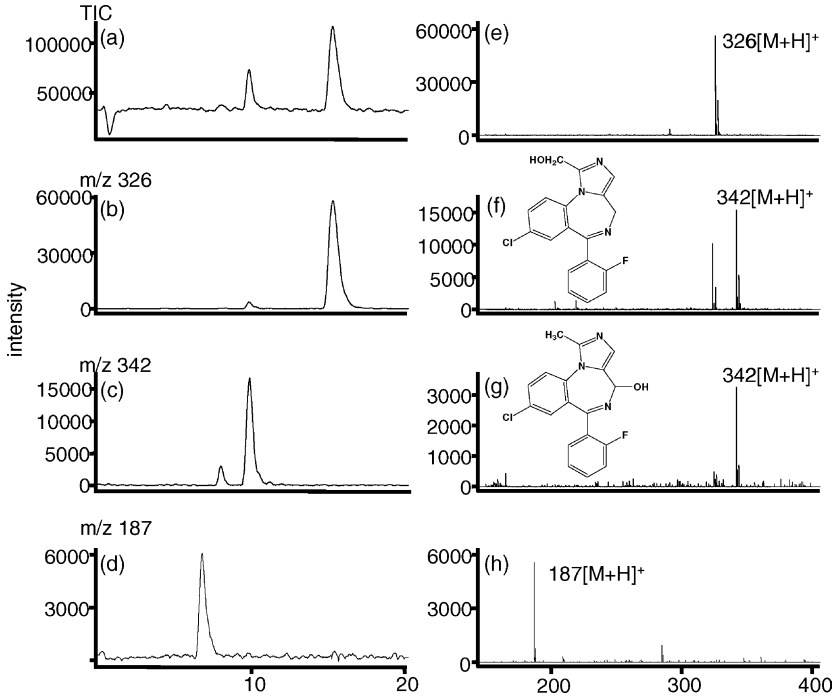


Fig. 3. Total ion chromatogram, mass chromatograms and the mass spectra of midazolam and its metabolites under positive-ion conditions at a drift voltage of 100 V. TIC (a), mass chromatograms at *m/z* 326 (b), *m/z* 342 (c), *m/z* 187 (d), and mass spectra of midazolam (e), 1′-hydroxymidazolam (f), 4-hydroxymidazolam (g), detomidine (I.S.) (h). Mobile phase is methanol–10 mM ammonium acetate (1:1) at a flow rate of 0.2 ml min$^{-1}$. Other conditions are described in Section 2.

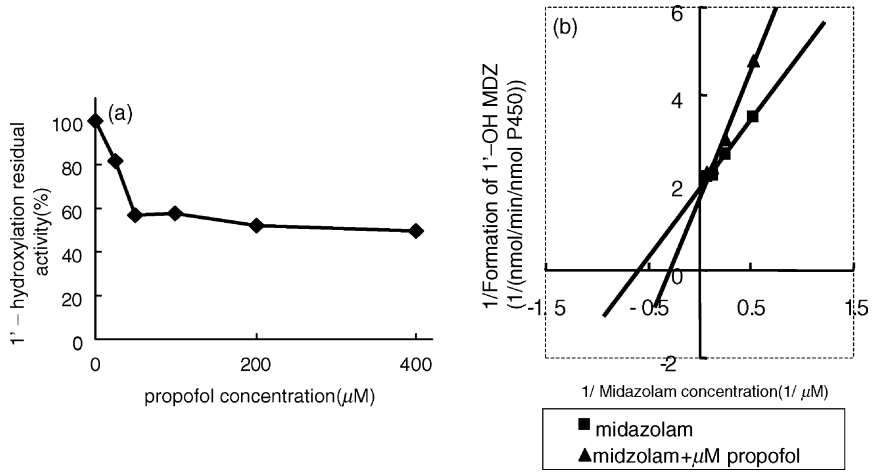


Fig. 4. (a) Effect of propofol on midazolam 1′-hydroxylation. (b) Lineweaver–Burk plots for the 1′-hydroxylation of midazolam by CYP3A4.

detected with retention times corresponding to those of 1′-hydroxymidazolam (9.88 min), 4-hydroxymidazolam (8.03 min) and midazolam (12.29 min), as shown in Fig. 3. There was no interference from the extracted components of the incubation system. Protonated molecular ions, $[M + H]^+$, of the internal standard, 1′-hydroxymidazolam, 4-hydroxymidazolam and midazolam were clearly observed at *m*/*z* 187, 342, 342 and 326, respectively, as base peaks. The fragment ion $[M - H_2O]^+$ of 1′-hydroxymidazolam was observed at *m*/*z* 324.

### 3.3.2. Inhibition of midazolam metabolism by propofol

When midazolam was incubated with recombinant 3A4, 1′-hydroxymidazolam was the metabolite detected by LC–MS. The effect of inhibitory propofol against CYP3A4 on midazolam hydroxylation was examined (Fig. 4a). With concentrations ranging from 25 to 100 μM, propofol showed inhibitory effect on CYP3A4-catalyzed midazolam hydroxylation. Single-phase straight curves were obtained from Lineweaver–Burk plots (Fig. 4b) for the 1′-hydroxylation of midazolam by CYP3A4 in the absence or presence of propofol. The maximum rate of metabolism ($V_{max}$) and the Michaelis–Menten constant ($K_m$) for the formation of 1′-hydroxymidazolam were estimated to be 3.455 μM and 0.596 nmol $min^{-1}$ $nmol^{-1}$ CYP with propofol by linear regression from Lineweaver–Burk double-reciprocal plots, respectively. And those of without propofol were 1.683 μM and 0.540 nmol $min^{-1}$ $nmol^{-1}$ CYP, respectively. These results were indicated that formation of 1′-hydroxymidazolam by CYP3A4 may be competitively inhibited by propofol.

### 3.4. Metabolism of propofol by human liver microsomes

The inhibition mechanism of midazolam metabolism by propofol was not clarified. Therefore, we studied in vitro propofol metabolism by CYP3A4 and human liver microsomes. Fig. 5a shows the mass chromatogram at *m*/*z* 177 and the mass spectrum of propofol ($M_r$ 178.27) under negative-ion conditions, respectively, at drift voltage of 40 V. The mass chromatogram was obtained with methanol–water (65:35) as a eluent at a flow rate of 0.2 ml $min^{-1}$. The mass spectrum of propofol was almost the same as that obtained

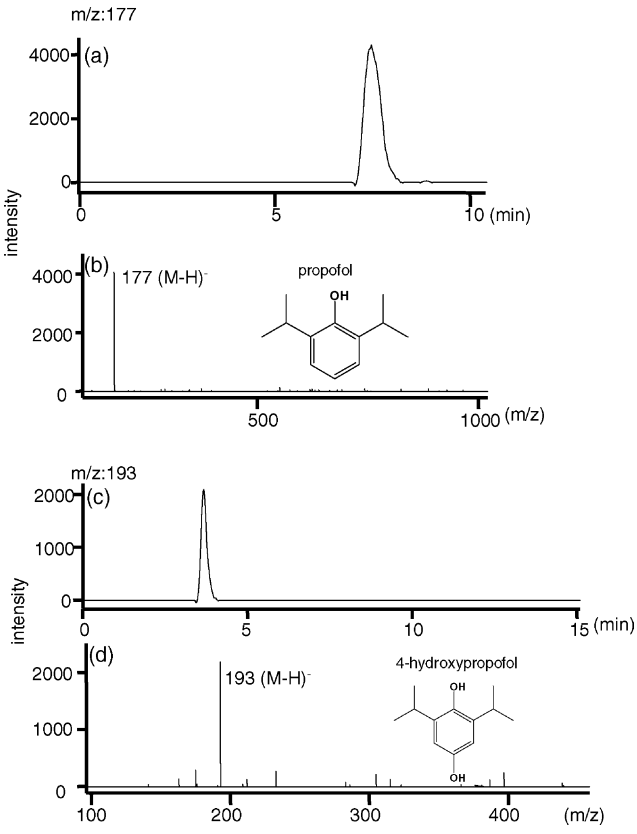


Fig. 5. (a) Mass chromatogram and the mass spectrum of propofol under negative ion conditions at a drift voltage of 40 V. (b) Mass chromatogram at *m*/*z* 177 and mass spectrum of propofol. (c) Mass chromatogram and the mass spectrum of propofol metabolites by human liver microsomes. (d) Mass chromatogram at *m*/*z* 193 and mass spectrum of the peak of propofol metabolite. Mobile phase is methanol–water (65:35) at a flow rate of 0.2 ml $min^{-1}$. Other conditions are described in Section 2.

by a direct analysis. Deprotonated molecular ion, $[M-H]^-$, of propofol was observed at *m*/*z* 177 as the base peak. Following the human liver microsomes incubations, the chromatographic peak was detected with retention time 3.5 min corresponding to propofol metabolite, 4-hydroxypropofol, as shown in Fig. 5b. There was no interference from extracted components of the incubation system. Deprotonated molecular ion, $[M-H]^-$, of 4-hydroxypropofol was clearly observed at *m*/*z* 193 as base peak. However, the peak of 4-hydroxypropofol was not observed by CYP3A4 incubation under the same LC–MS conditions. The present studies were indicated that CYP3A4 is not involved in the metabolism of propofol.

## 4. Conclusion

The present method is more sensitive than our previous reported LC–MS technique [20]. A recently published LC–MS technique published by Shiran et al. [22] when the system used a 100 mm × 4.6 mm i.d. column packed with 3 μm Luna $C_{18}$ (Phenomenex, Cheshire, UK) stationary phase is more rapid than the present method. However, their method was not detected 4-hydroxymidazolam. Furthermore, the present method has the advantage of a small sample volume and reduction in organic solvent consumption using semi-microcolumn instead of conventionally-sized column.

In this study, we achieved a analysis of midazolam and its metabolites by LC–MS. The method was applied to the quantitation of midazolam in monkey plasma and an in vitro study with recombinant CYP3A4. LC–MS in the SSI mode is very useful for polar analytes, such as midazolam metabolite. Using the present method, we have shown that propofol competitively inhibits the metabolism of midazolam 1′-hydroxylation. We also achieved analysis of propofol and its metabolite by LC–MS. The present method is sufficiently sensitive and accurate to study the kinetics of the formation of midazolam metabolites and drug–drug interactions.

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