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Simple method for determination of triazolam in human plasma by high-performance liquid chromatography/tandem mass spectrometry

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Abstract

Triazolam was analyzed from human plasma samples by high-performance liquid chromatography (HPLC)-tandem mass spectrometry (MS/MS) with an MSpak GF polymer column (50 mm × 4.6 mm i.d., particle size 6 μm), which enabled direct injection of crude biological samples. Separation of triazolam, and lorazepam as the internal standard (IS) was carried out using 10 mM ammonium acetate (pH 3.56)–0.1% formic acid and an acetonitrile gradient elution. Both compounds formed base peaks due to $[M+H]^+$ ions by HPLC/ESI-MS, and product ions were produced from each $[M+H]^+$ ion as seen by HPLC–MS/MS. Quantification of triazolam and the IS in plasma samples was made by selective reaction monitoring using each base peak of product ions of HPLC–MS/MS. The recovery range of triazolam spiked into plasma was 86.4–92.7%. The regression equation for triazolam showed excellent linearity in the range of 0.25–20 ng/mL, and the detection limit was 0.1 ng/mL. Intra- and inter-day precisions for triazolam in plasma samples were not greater than 12.4%. Accuracy for the drug was in the range of 88.0–101.4%. Data obtained after oral administration of triazolam in male and female subjects are also presented.
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1. Introduction

Triazolam is used for short-term treatment of insomnia, and belongs to a family of drugs known as benzodiazepines [1,2]. Many patients develop a dependence on this drug, which is often involved in intoxications.

Several methods have been reported for the determination of triazolam in various matrices using gas chromatography (GC) [3–5], GC–mass spectrometry (GC–MS) [6–9], high-performance liquid chromatography (HPLC) [10–13], and HPLC–MS [14–19]. In general, triazolam has been determined by chromatographic methods following liquid–liquid extraction or solid-phase extraction to remove impurities contained in human body fluids. Although these methods may be successful in extracting drugs from biological fluids, the large amounts of organic solvent used in the extraction procedure causes problems with regard to health and the environment. Moreover, these methods are time-consuming and tedious and often require preconcentration of the extract prior to instrumental analysis. To circumvent such sample pretreatments, HPLC columns such as internal-surface reversed-phase silica supports which enable direct injection of biological samples into HPLC have been developed [19–22]. These columns are usually used in a column-switching arrangement.

Recently, a new HPLC polymer stationary phase, an MSpak GF column consisting of a highly cross-linked hard gel of polyvinyl alcohol, was developed for use in HPLC–MS in Japan. In the present study, we established simple HPLC–MS/MS methods for analyzing triazolam by direct injection of human plasma samples using the MSpak GF column without the need

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of an extraction procedure and without the column-switching technique.

2. Experimental

2.1. Materials

Triazolam and lorazepam were provided by Sumitomo Pharmaceutical (Osaka, Japan) and Sawai Pharmaceutical (Osaka, Japan), respectively. Syringe filters with polypropylene membranes (pore size 0.2 μm) were purchased from Whatman (Clifton, NJ, USA). Other common chemicals were of the highest purity, and commercially available. Whole blood samples were obtained from healthy volunteers. Samples were centrifuged at 1700 × *g* for 10 min at 4 °C in the presence of EDTA-2Na as an anticoagulant. The plasma was decanted and stored at −40 °C until use.

2.2. Sample procedure

Stock standard solutions of triazolam, and lorazepam as an internal standard (IS) were prepared separately by dissolving appropriate amounts of each compound in acetic acid to give a concentration of 1 mg/mL. Working standard solutions of compounds were prepared by serial dilution of stock standard solutions using the initial HPLC mobile phase (distilled water containing 10 mM ammonium acetate and 0.1% formic acid, pH 3.56).

To a 1 mL plasma sample containing triazolam and IS were added 3 mL of distilled water containing 13.3 mM ammonium acetate and 0.13% formic acid. After centrifugation of the mixture at 4600 × *g* for 30 min, the supernatant fraction was filtered through a syringe filter, and an aliquot (100 μL) of clear supernatant was directly injected into the HPLC–MS.

2.3. HPLC

The HPLC system was an Agilent 1100 series (Palo Alto, CA, USA) equipped with a G1315A diode array detector (DAD) (Agilent) set at 260 nm, and a Model 7725i manual injector equipped with a 200 μL sample loop (Rheodyne, Cotati, CA, USA). The mobile phase was degassed on-line using a G1322A Vacuum Membrane Degasser (Agilent). Chromatographic separations were performed with a Shodex MSpak GF-310 4B column (50 mm × 4.6 mm i.d., 6 μm particle size, Showadenko, Tokyo, Japan). The flow rate was 0.2 mL/min. The mobile phases were distilled water containing 10 mM ammonium acetate (pH 3.56) with 0.1% formic acid (solvent A), and 100% acetonitrile (solvent B). Gradient runs were programmed as follows: 100% solvent A and 0% solvent B for 5 min, changing to 40% solvent A/60% solvent B for 30 min, then to 30% solvent A/70% solvent B for 5 min. An in-line filter (SUMIPAX, 0.2-μm pore size, Sumika Chemical Analysis Service, Osaka, Japan) was installed between the manual injector and the MSpak GF column in the present experiments. The column was maintained at 30 °C.

2.4. MS/MS with ESI

A PE SCIEX API 2000 (Applied Biosystems Sciex, Ontario, Canada) triple-stage quadrupole mass spectrometer interfaced to a TurboIonSpray source was used for mass analysis and detection. Ionization of analytes was carried out using the following settings of the electrospray ionization (ESI) in the positive ion mode: TurboIonSpray temperature, 400 °C; ion source voltage, 5000 V; nebulizer gas (high-purity air), 30 psi; curtain gas (nitrogen), 30 psi; turbospray (high-purity air), 60 psi; orifice and ring voltages, 71 and 360 V for triazolam, respectively and 41 and 200 V for the IS, respectively.

MS/MS analysis was performed using nitrogen as the collision gas (setting, 7). Quantitation was performed using selected reaction monitoring (SRM) of precursor → product ion transitions at *m/z* 343 → 343 (collision energy, −20 eV) for triazolam and *m/z* 321 → 275 (collision energy, −39 eV) for IS.

Mass calibration was performed by infusing a 10^{-4} M polypropylene glycol solution into the ionspray source. Full-scan data were obtained with a mass range of *m/z* 50–500, a dwell-time of 500 ms, and a step-size of 0.1 amu. Peak widths of precursor ions were maintained at 0.6–0.75 u in SRM. Data acquisition, peak integration and calculation were interfaced to a computer workstation running the Analyst^{TM} software, version 1.0.

2.5. Methods for recovery, quantification and linearity studies

Recoveries were calculated by comparing chromatographic peak areas obtained from the spiked plasma with those obtained by direct HPLC injection of non-extracted authentic compounds dissolved in the mobile phase, and determined at four different concentrations of triazolam. The regression equation for triazolam spiked to human plasma depended on the peak area ratios with the IS (1 μg). The concentration of calibrators ranged from 0.25 to 20 ng/mL for triazolam (seven calibrators: 0.25, 0.5, 1.0, 2.0, 4.0, 10 and 20 ng/mL).

Intra- and inter-day precisions were assessed by performing duplicate determinations of triazolam in plasma to construct calibration curves. Intra-day precision was determined by analyzing a spiked sample at three concentrations in triplicate on the same day ($n = 3$). The same procedure was repeated on different days ($n = 5$) to determine the inter-day precision. Accuracy was expressed as the percentage of extraction efficiency and precision was given by the intra- and inter-day relative standard deviations. The detection limit was determined at a signal-to-noise ratio of 3.

2.6. Administration of triazolam to volunteers

A therapeutic oral dose of triazolam (0.5 mg) was administered orally to two volunteers, a 38-year-old male (75 kg body weight) and a 37-year-old female (58 kg body weight). Informed consent was obtained from the subjects. Whole blood samples were obtained 45 min after drug administration, and were

centrifuged at 1700 × *g* for 10 min. Plasma samples were stored at −40 °C until analysis.

3. Results and discussion

3.1. HPLC separation

Separation by the MSpak GF column is made basically according to size exclusion chromatography associated with the slight effects of partition and adsorption, and the column is suited to eliminate proteins, nucleic acids and polysaccharides from biological samples, because their molecular size is too large to enter the pores of the stationary phase; whereas drugs with small molecular size can enter the pores and be retained at the polyvinyl alcohol phase. This principle has allowed direct injection of crude biological samples [23,24]. This polymer support is chemically and structurally stable, and can be used in a wide pH range from 2 to 9. Another advantage of the present polymer support is that both water and various organic solvents can be used for elution. Expansion or constriction of the stationary phase associated with solvent replacement is very small owing to a highly cross-linked hard gel of polyvinyl alcohol, compared with conventional polymer columns.

The left panel of Fig. 1 shows a chromatogram of blank plasma obtained by HPLC-DAD detection; most of the matrix peaks in the plasma were eluted about 25 min. The right panel of Fig. 1 shows a chromatogram obtained by HPLC-DAD detection for a monitored plasma sample in the presence of test compounds; it was obtained from 30 μg each of triazolam and the IS spiked to a 1 mL sample. Triazolam and the IS were separated and could be determined simultaneously by the MSpak GF column without pretreatment and without a column-switching technique.

Direct injection methods are generally dirtier than extraction methods. We had made various preliminary experiments for optimization of drug separation by HPLC/MS/MS with direct injection. The plasma sample was diluted 4 times with a mobile phase, and 100 μL of the diluted sample were introduced into HPLC/MS/MS after filtration with a syringe microfilter (0.2 μm pore size). In this study, the SUMIPAX HPLC in-line filter was used for protection of the MSpak GF column and HPLC–MS/MS system. By use of the in-line filter, the MSpak GF column demonstrated excellent stability in our system; one column can be used repeatedly for at least more than 500 injections of plasma samples with good precision. As shown in Fig. 1, several matrix peaks in plasma were eluted within 25 min. From these results, the mobile phase containing them was discarded up to 27 min, and then introduced into the MS by an injector-switching valve for preservation of the ionization intensity. This proved to be an easy and practical solution to ensure cleanliness of the ion source and to reduce maintenance of the mass spectrometer.

3.2. Mass spectra

Characteristic ions of triazolam and IS obtained by HPLC/MS and HPLC/MS/MS are shown in Table 1. As the most abundant peak, triazolam and the IS showed protonated molecules at *m/z* 343 and 321, respectively, in the full-scan mode (mass range: *m/z* 50–500). Triazolam and the IS also revealed ions at *m/z* 308 and 304, corresponding to $[(M+H)-Cl]^+$ and $[(M+H)-H_2O+H]^+$, respectively.

In the HPLC/MS experiments, spectra showed protonated molecules constituting the base peaks for triazolam and the IS. Therefore, these protonated molecules were subjected to product ion formation by HPLC/MS/MS. Triazolam gave a predominant fragment ion at *m/z* 343 (base peak) corresponding to $[M+H]^+$, and the ion at *m/z* 315 was due to the loss of an HCN molecule corresponding to $[(M+H)-HCN-H]^+$. Another ion at *m/z* 308 for triazolam was attributable to $[(M+H)-Cl]^+$. Moreover, ions at *m/z* 239 and 138 could correspond to $[(M+H)-104]^+$ and $[C_6H_4+HCN+Cl]^+$, respectively. Protonated IS showed ions at *m/z* 321, 304 and 275 (base peak), corresponding to $[M+H]^+$, $[(M+H)-H_2O+H]^+$ and $[(M+H)-HCN-H_2O-H]^+$, respectively.

3.3. SRM

Quantification of triazolam and the IS in plasma samples was made by selective reaction monitoring using each base peak of the product ions of HPLC–MS/MS. The ion set (precursor to product) was selected at *m/z* 343 → 343 for triazolam, and *m/z* 321 → 275 for the IS. In preliminary experiments, we tested the sensitivity of the different ion sets (precursor to product) for SRMs of triazolam in human plasma samples; the intensity of the ion set at *m/z* 343 → 343 was 8–10 times higher

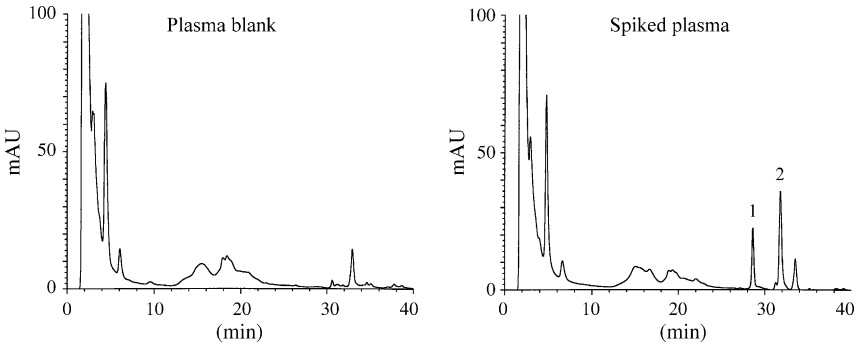
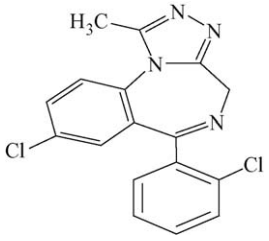
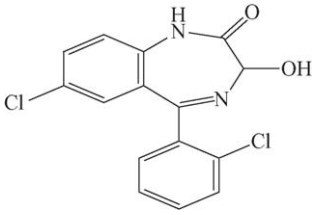


Fig. 1. HPLC-DAD chromatograms with MSpak GF column at 260 nm for triazolam and IS in human plasma. Amounts of triazolam and IS (30 μg each) were spiked into 1 mL of plasma. Peak 1, triazolam; peak 2, IS.

Table 1
Structures of triazolam and lorazepam (IS) and their characteristic ions obtained by HPLC/MS and HPLC/MS/MS

Structure	Compound	MW[a]	MS		MS/MS		
			m/z (relative intensity, %)	Assignation	Precursor ion *m/z* (relative intensity, %)	Product ion *m/z*	Assignation
	Triazolam	342	343 (100) 308 (7)	$[M+H]^+$ $[(M+H)-Cl]^+$	343	343 (100) 315 (17) 308 (32) 239 (8) 138 (5)	$[M+H]^+$ $[(M+H)-HCN-H]^+$ $[(M+H)-Cl]^+$ $[(M+H)-104]^+$ $[C_6H_4+HCN+Cl]^+$
	Lorazepam	320	321 (100) 304 (9)	$[M+H]^+$ $[(M+H)-H_2O+H]^+$	321	321 (3) 304 (7) 275 (100) 228 (11) 163 (3)	$[M+H]^+$ $[(M+H)-H_2O+H]^+$ $[(M+H)-HCN-H_2O-H]^+$ $[(M+H)-93]^+$ $[(M+H)-C_6H_4-HCN-H_2O-Cl-2H]^+$

[a] MW = molecular weight.

than the ion set at *m/z* 343 → 308. Selection of the same ion for precursor and product ions at *m/z* 343 was very effective for the disappearance of the interference peak without loss of sensitivity by the present method for triazolam. It seems that interference originated from plasma at *m/z* 343 when it easily dissociated in the collision cell by the setting conditions, but the molecule of triazolam was not significantly affected. A similar phenomenon was seen in the determination of morphine using HPLC–MS/MS [25,26]. Therefore, the use of the ion set at *m/z* 343 → 343 for triazolam was recommended in the present SRM. Fig. 2 shows chromatograms with SRMs obtained from triazolam and the IS spiked into human plasma samples. Distinct peaks appeared for each compound on the chromatograms. The blank chromatograms gave small impurity peaks and no interfering peaks appeared around the test peaks (data not shown). These observations obviously showed that SRMs of HPLC/MS/MS provided high sensitivity and selectivity for the determination of triazolam in plasma samples.

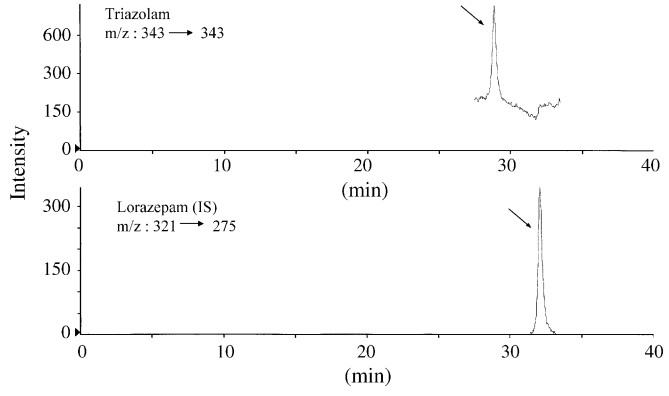


Fig. 2. SRM of HPLC/MS/MS for triazolam and IS from human plasma. A mixture of triazolam (1 ng) and IS (1 μg) was spiked into 1 mL of plasma. Arrows show locations where drug peaks should appear.

3.4. Method performance

We have demonstrated the quantitative analysis of triazolam with lorazepam as the IS. The best way to select an IS is to use a stable isotope-labeled analyte. Since such an IS for triazolam is not commercially available, an alternative approach was used. The chromatographic retention, recovery and ionization properties of the IS should match those of triazolam. In preliminary trials, other benzodiazepines were assessed for the selection of the IS, and lorazepam was found to fulfill these criteria sufficiently. Moreover, the coexistence of both triazolam and lorazepam in a sample is rare clinically. Therefore, lorazepam was chosen as the IS in the quantitative analysis of triazolam from human plasma.

The recoveries of triazolam from plasma samples using the present method are shown in Table 2. The recoveries of triazolam were 86.4–92.7% from plasma and were considered satisfactory for our protocol.

Table 2
Recoveries of triazolam in human plasma samples using by the present method[a]

Amount added (ng/mL)	Amount found (ng/mL)	Recovery (%)
0.5	0.43 ± 0.04	86.4
1.0	0.88 ± 0.07	87.8
2.0	1.81 ± 0.13	90.6
5.0	4.63 ± 0.39	92.7

[a] Triazolam was spiked into 1 mL of human plasma. The results are mean ± S.D. of six experiments.

Table 3
Intra- and inter-day precision and accuracy for triazolam in human plasma samples obtained using by the present method

Amount added (ng/mL)	Intra-day[a]			Inter-day[b]		
	Amount detected (ng/mL)	Precision (%)	Accuracy (%)	Amount detected (ng/mL)	Precision (%)	Accuracy (%)
0.5	0.47 ± 0.04[c]	8.5	94.0	0.44 ± 0.05	11.4	88.0
1.0	0.95 ± 0.08	8.4	95.0	0.97 ± 0.12	12.4	97.0
5.0	5.07 ± 0.26	5.1	101.4	4.82 ± 0.47	9.8	96.4

[a] Intra-day precisions were calculated from measurements of three spiked samples on same day.
[b] Spiked plasma were kept at 4 °C and analyzed on five separate days, with one sample each day.
[c] Values are mean ± S.D.

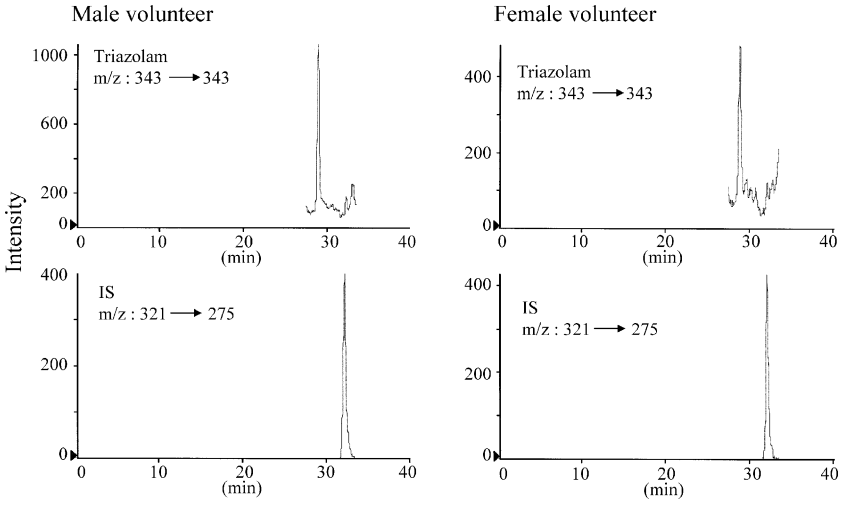


Fig. 3. SRM chromatograms obtained by present method from plasma of male and female volunteers 45 min after oral administration of triazolam. Amount of lorazepam used as IS was 1 μg for 1 mL of plasma.

The linearity of the regression equation for triazolam was investigated using spiked plasma samples. The regression equation was obtained by plotting the ratio of the triazolam peak area to the IS against the respective concentrations. The equation and r-value were: $y = 0.3481x + 0.0878$, and 0.9994 in the range of 0.25–20 ng/mL. The limit of detection under optimal conditions for triazolam was 0.1 ng/mL. The lower limit of quantification was determined to be 0.25 ng/mL and the upper limit of quantification was determined to be 20 ng/mL. Intra- and inter-day precision and accuracy data are presented in Table 3. Intra-day precision was less than 8.5% ($n = 3$) and accuracy ranged from 94.0 to 101.4% when determined at concentrations of 0.5, 1.0 and 5.0 ng/mL. Inter-day precision was less than 12.4% ($n = 5$) and accuracy ranged from 88.0 to 97.0% when determined at concentrations of 0.5, 1.0 and 5.0 ng/mL.

3.5. Stability

The stock standard solutions containing 1 mg/mL of triazolam and the IS prepared in acetic acid were stable for at least 3 months when kept at 4 °C. The working standard solutions of the compounds were investigated over a period of 2 months, and no significant changes were observed. The plasma samples containing triazolam and the IS were stored at a temperature of 4 °C for four weeks and no effects on the quantification were observed.

3.6. Actual measurements of triazolam in human plasma after oral administration

In addition to spiked human plasma, the present method was applied to samples of human plasma after oral administration of triazolam. Typical SRM chromatograms obtained from the male and female volunteers are shown in Fig. 3 Lorazepam as the IS was added to the samples in the initial step. The drug concentration in the plasma of the male volunteer was 5.8 ng/mL at 45 min after administration, and that of the female volunteer was 2.3 ng/mL. These concentrations in plasma were within therapeutic levels, reported to be 2–20 ng/mL [27].

4. Conclusion

To our knowledge, this is the first study reporting the use of a new polymer HPLC column (MSpak GF) and HPLC/MS/MS for simple determination of triazolam using direct injection of plasma samples without an extraction procedure or a column-switching technique. In view of its simplicity, sensitivity, and excellent quantitativeness, the present method is recommendable for determination of triazolam from human plasma in therapeutic drug monitoring, in addition to detection of its high levels in clinical toxicology and forensic toxicology. Trials to detect drugs belonging to other classes, such as phenothiazines, butyrophenones and barbiturates, in human

samples using the present technique are now under way in our laboratory.

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