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Simultaneous determination of triazolam and its metabolites in human plasma by liquid chromatography–tandem mass spectrometry

Ryh-Nan Pan[a], Chang-Ching Lin[b], Pei-Wei Huang[b], Cheng-Huei Hsiong[b], Li-Heng Pao[b,*]

[a] Department of Disaster Management, Taiwan Police College, Taipei, Taiwan, ROC
[b] School of Pharmacy, National Defense Medical Center, Taipei, Taiwan, ROC

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ABSTRACT

A sensitive and selective liquid chromatography–tandem mass spectrometry (LC–MS/MS) method for the determination of triazolam and its metabolites, alpha-hydroxytriazolam (α-OHTRZ) and 4-hydroxytriazolam (4-OHTRZ), was developed and validated. Triazolam-D4 was used as the internal standard (IS). This analysis was carried out on a Thermo® C_{18} column and the mobile phase was composed of acetonitrile:H_2O:formic acid (35:65:0.2, v/v/v). Detection was performed on a triple-quadrupole tandem mass spectrometer using positive ion mode electrospray ionization (ESI) and quantification was performed by multiple reaction monitoring (MRM) mode. The MS/MS ion transitions monitored were m/z 343.1 → 308.3, 359.0 → 308.3, 359.0 → 111.2 and 347.0 → 312.0 for triazolam, α-OHTRZ, 4-OHTRZ and triazolam-D4, respectively. LLOQ of the analytical method was 0.05 ng/mL for triazolam and 0.1 ng/mL for α-OHTRZ and 4-OHTRZ. The within- and between-run precisions were less than 15.26% and accuracy was −8.08% to 13.33%. The method proved to be accurate and specific, and was applied to the pharmacokinetic study of triazolam in healthy Chinese volunteers.

1. Introduction

Triazolam, structurally related to alprazolam, is a triazolobenzodiazepine with sedative and hypnotic effects. It was approved for the use of short-term management of insomnia characterized by difficulty in falling asleep, frequent nocturnal awakenings, and/or early morning awakenings [1–3]. Triazolam is metabolized to two major metabolites, alpha-hydroxytriazolam (α-OHTRZ) and 4-hydroxytriazolam (4-OHTRZ) by the human CYP3A4 [4].

Triazolam is widely reported to be abused and is alleged to be involved in date-rape cases [5]. Besides, several studies have shown triazolam–drug interactions caused by CYP 3A4 [6–8]. In order to improve the detection ability of abused drugs like triazolam and study the pharmacokinetics of triazolam to reduce drug–drug interaction, a selective and sensitive analytical method must be developed. There have already been several methods to analyze triazolam and its metabolites in human biological samples, i.e., gas chromatography (GC) [9,10], GC–mass spectrometry (GC–MS) [11–13] and high-performance liquid chromatography (HPLC) [14–17]. Recently, LC–MS [18–22] and LC–MS/MS [23] analytical methods were also developed. The LC–MS/MS method developed by Lee et al. is convenient and validated, but it can only determine triazolam level in human plasma species. To our knowledge, no previous study has reported simultaneous determination of triazolam and its metabolites in human plasma by using LC–MS/MS.

In the present study, a fully validated, accurate and sensitive LC–MS/MS method was established to simultaneously determine triazolam and its two major metabolites, α-OHTRZ and 4-OHTRZ, in human plasma. This validated method was also applied to a pharmacokinetic study of triazolam in healthy Chinese subjects.

2. Experimental

2.1. Chemicals and reagents

The standard of triazolam, α-OHTRZ were purchased from Cerillant Co. (TX, USA). 4-OHTRZ was purchased from Biomol (PA, USA). Triazolam-D4 was purchased from Sigma–Aldrich (MO, USA). Acetonitrile (ACN) and methanol (MeOH), both of HPLC grade, were obtained from Tedia Company, Inc. (OH, USA) and Merck KgaA (Dermstadt, Germany), respectively. All the other chemicals were of analytical grade and commercially available. Drug-free human plasma used in this study was obtained from healthy Chinese volunteers and stored at −80 °C until the assay. Water was prepared

* Corresponding author at: School of Pharmacy, National Defense Medical Center, 9F, 161 Minchuan East Road, Section 6, Taipei 114, Taiwan, ROC. Tel.: +886 2 87923100x18843; fax: +886 2 87924859.
E-mail address: paolh@ndmctsgh.edu.tw (L.-H. Pao).

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using a Milli-Q water purification system (Millipore, Bedford, MA, USA). The Oasis® HLB (hydrophilic–lipophilic balance) extraction cartridges was purchased from Waters (MA, USA).

2.2. Instruments

2.2.1. Liquid chromatography

The HPLC system consisted of a pump (SHIMADZU LC-10AD*VP*, Japan) and an autosampler (SHIMADZU SIL-10AD*VP*, Japan). Chromatography separation was performed with a Thermo® C_{18} column (2.1 mm × 50 mm, i.d., 3 μm particle size, MA, USA). The mobile phase consisting of acetonitrile:H_2O:formic acid (35:65:0.2, v/v/v) was used. The flow rate was set at 0.25 mL/min and the autosampler was conditioned at 4 °C. The run time was 4 min.

2.2.2. Mass spectrometry

An Applied Biosystems-Sciex API 3000 (Foster City, CA, USA) triple-quadrupole mass spectrometer equipped with an electrospray ionization (ESI) interface was used to perform this study. Ionization was conducted in the positive mode. Ion source temperature was maintained at 425 °C and ionspray voltage was 5500 V. High purity nitrogen gas was used as collision-induced dissociation (CAD) gas (setting 12), curtain gas (setting 12) and nebulizer gas (setting 11). Multiple reaction monitoring (MRM) mode was used for the quantification. The selected transitions of *m*/*z* were 343.1 → 308.3 for triazolam, 359.0 → 331.0 for α-OHTRZ, 359.0 → 111.2 for 4-OHTRZ and 347.0 → 312.0 for triazolam-D4.

2.3. Preparation of standards and quality control samples

Stock solution concentrations of triazolam, α-OHTRZ and triazolam-D4 as the internal standard (IS) were all 100 μg/mL and dissolved in MeOH when purchased. Stock solution concentration of 4-OHTRZ was prepared with dimethyl sulfoxide (DMSO) to 100 μg/mL. Standard working solutions were serially diluted from stock solutions with MeOH:H_2O (50:50, v/v).

One hundred microliters of 50 ng/mL IS solution was added into 900 μL of blank plasma with 100-μL standard working solution to give calibration standards. Plasma calibration concentrations of triazolam were 0.05, 0.1, 0.25, 0.5, 1, 2.5, 5 and 10 ng/mL, and those for both α-OHTRZ and 4-OHTRZ were 0.1, 0.25, 0.5, 0.75, 1, 2.5, 5 and 10 ng/mL.

Quality control (QC) samples were prepared with blank plasma at LLOQ, low, medium and high concentrations, which were set at 0.05, 0.15, 4 and 8 ng/mL for triazolam and 0.1, 0.3, 4 and 8 ng/mL for α-OHTRZ and 4-OHTRZ.

2.4. Sample preparation

Plasma samples were prepared by solid-phase extraction. The samples were stored as 1-mL aliquots at −80 °C; the aliquots were thawed at room temperature before analysis. One hundred microliters of 2 M ammonium acetate (pH 5.0), 25 μL of β-glucuronidase and 100 μL aliquot of internal standard solution were added to 1 mL of plasma sample; then the sample was incubated in water bath at 50 °C for 30 min. Solid-phase extraction (SPE) was conducted by using Oasis® HLB extraction cartridges. The cartridges were conditioned sequentially with 1 mL of MeOH and 1 mL of H_2O, and 1 mL of plasma sample was then loaded. The loaded cartridges were washed with 1 mL of MeOH:H_2O:NH_4OH (5:95:2, v/v/v) twice, and subsequently the analyte was eluted with 2% formic acid in MeOH. The elution was then evaporated to dryness under N_2 stream and reconstituted with 100 μL of HPLC mobile phase and vortexed for 30 s. Finally, the solvent was transferred to the autosampler vials and 15 μL was injected into LC–MS/MS system.

2.5. Method validation

The method validation assays were carried out by following the currently accepted US Food and Drug Administration (FDA) bioanalytical method validation guidance [24]. The following parameters were determined for the validation of the analytical method developed for triazolam, α-OHTRZ and 4-OHTRZ in human plasma: selectivity, linearity, lower limit of quantification (LLOQ), precision, accuracy, extraction recovery, matrix effect and stability.

2.5.1. Selectivity

Selectivity was evaluated by comparing chromatograms of six blank plasma samples from six different sources to make sure there were no significant interfering peaks at retention time at LLOQ of the analytes.

2.5.2. Linearity and lower limit of quantification

A line ($y = ax + b$) was fitted through the standard curve ranged by a weighted linear regression (weight = $1/x^2$) of peak area ratio of triazolam, α-OHTRZ or 4-OHTRZ to IS (y) versus actual concentration of the analyte (x).

LLOQ, defined in the presented study, is the lowest plasma concentration in the calibration curve that can be measured by precision and accuracy. The precision and accuracy were evaluated by the relative standard deviation (R.S.D.) and relative error (R.E.), respectively. The acceptable value of R.S.D. was below 20% and R.E. was within ±20%.

2.5.3. Accuracy and precision

The within-run precision and accuracy were determined by analyzing QC samples ($n = 6$) as described above. The between-run precision and accuracy were also carried out by analyzing QC samples in six batches on different days. The precision was presented as R.S.D. and accuracy as R.E.

2.5.4. Extraction recovery and matrix effect

The used concentrations for the evaluation of recovery and matrix effect were set at 0.15, 4 and 8 ng/mL for triazolam, α-OHTRZ and 4-OHTRZ. One hundred microliters of 50 ng/mL IS was spiked with each experimented concentration.

The extraction recoveries were determined by comparing the response ratio of extracted plasma standards with those of extracted blank plasma spiked with corresponding concentrations. The response was defined as the peak area of analyte divided by the peak area of IS.

Six different sources of blank plasma were used to assess the matrix effect. The absolute and relative matrix effect was previously defined by Matuszewski et al. [25]. The absolute matrix effect was evaluated by comparing the peak areas of analytes added to extracted blank plasma with those of extracted water. The R.S.D. of the mean peak areas of analytes in the extracted blank plasma indicated the relative matrix effect.

2.5.5. Stability

2.5.5.1. Working solution stability. Standard working solutions were prepared at 10 and 100 ng/mL for triazolam, α-OHTRZ and 4-OHTRZ. The working solutions were either immediately diluted with 1 mL of mobile phase containing 100 μL of 50 ng/mL IS or kept at ambient temperature (25 °C) for 4 h then diluted with the aforementioned mobile phase. The samples were then analyzed. The peak area ratio of the analytes of interest to IS obtained from

Table 1
Mass spectrometry parameters of triazolam, α-OHTRZ, 4-OHTRZ and triazolam-D4

	Triazolam	α-OHTRZ	4-OHTRZ	Triazolam-D4
Declustering potential (V)	62	52	48	62
Focusing potential (V)	350	194	333	350
Entrance potential (V)	10	9	13	10
Collision energy (V)	37	39	59	37
Collision cell exit potential (V)	20	20	21	20

4-h stored samples was compared with the values of initial samples ($n = 3$).

2.5.5.2. Freeze and thaw stability. The stability test was carried out by analyzing unextracted triazolam, α-OHTRZ and 4-OHTRZ QC samples processing three freeze-thaw cycles. The concentrations of QC samples were set at 0.15, 4 and 8 ng/mL. The results were presented by comparing the experimental concentrations with initial values ($n = 3$).

2.5.5.3. Autosampler stability. 0.15, 4 and 8 ng/mL of triazolam, α-OHTRZ and 4-OHTRZ were prepared as low, medium and high concentration of plasma QC samples. QC samples were placed in autosampler for 12 h, and the concentrations of the analytes were determined and compared with the initial QC samples to access autosampler stability ($n = 3$).

2.5.5.4. Long-term stability. The concentrations of plasma QC samples for triazolam were set at 0.15, 4 and 8 ng/mL. For α-OHTRZ and 4-OHTRZ, 0.3, 4 and 8 ng/mL were used. QC samples were stored at −80 °C for 3 weeks. Samples were then processed and analyzed. The results were evaluated by comparing the obtained concentrations with initial values ($n = 3$).

2.6. Pilot pharmacokinetic study

The presented analytical method was applied to a pilot pharmacokinetic study of triazolam approved by the Ethics Committee. All volunteers gave written informed consent to participate in the study. Three Chinese healthy male volunteers were selected and underwent an overnight fast before starting the treatment. A single dose of triazolam (Trialam® 0.25 mg/tab) was administered orally by the subjects. Blood samples were collected pre-dose and 0.33, 0.66, 1, 1.5, 2, 3, 4, 6, 8, 12, 24 h post-dose. Plasma was obtained by centrifuging blood samples at 3000 rpm for 5 min. All the samples were stored at −80 °C before analyzing.

3. Results and discussion

3.1. Method development

LC–MS/MS is a powerful technique, and is now widely used in biological analysis. Since phosphate buffer is normally forbidden for a LC–MS/MS method, the chromatographic conditions for the present work, especially the composition of mobile phase, were optimized to achieve good resolution and symmetric peak shapes of analytes as well as short run time. It was found that acetonitrile:H_2O:formic acid (35:65:0.2, v/v/v) could achieve our purpose and was finally adopted for the present work.

The most intensive product ion was observed at m/z 308.3 for triazolam, 331.0 for α-OHTRZ, 111.2 for 4-OHTRZ and 312.0 for the internal standard. Therefore, the precursor-to-product ion transitions m/z 343.1 → 308.3, m/z 359.0 → 308.3, m/z 359.0 → 111.2 and m/z 347.0 → 312.0 in the MRM mode were selected for triazolam, α-OHTRZ, 4-OHTRZ and triazolam-D4, respectively. The parameters of mass spectrometry were optimized and are shown in Table 1.

SPE protocol was followed by the recommended instruction of Oasis® HLB extraction procedures with some modifications. Different proportions of MeOH (5%, 10% and 15%) in the clean-up step were tested. Finally, 5% of MeOH was used due to the lower recovery of the other two compositions.

3.2. Selectivity

Representative chromatograms of drug-free plasma are shown in Fig. 1(A), and those of plasma samples spiked with triazolam, α-OHTRZ, 4-OHTRZ at LLOQ and trazolam-D4 at 5 ng/mL are shown in Fig. 1(B). Chromatograms corresponding to 40 min after administrating of 0.25 mg triazolam tablet for the real sample are shown in Fig. 1(C). The retention times were 2.44, 1.70, 1.73 and 2.41 min for triazolam, α-OHTRZ, 4-OHTRZ and triazolam-D4, respectively. No significant interfering peaks due to endogenous compounds or reagent were observed at the retention times of the three analytes of interest and IS in the chromatogram of blank plasma.

3.3. Method validation

3.3.1. Linearity and LLOQ

The standard calibration curves of triazolam and its two metabolites were linear over the concentration range of 0.05–10 ng/mL for plasma. The equation of calibration curves were weighted by $1/x^2$. Typical equations of calibration curves were as follows: $y = 2.48 \times 10^{-1}x - 1.79 \times 10^{-3}$ ($r = 0.9975$) for triazolam,

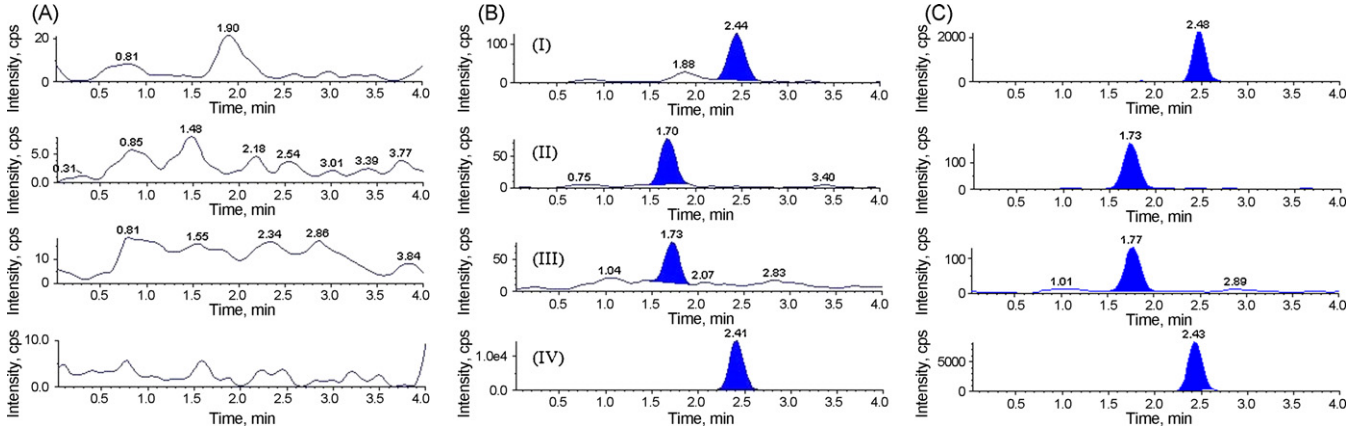

Fig. 1. MRM chromatograms of (A) blank human plasma; (B) blank human plasma sample spiked with triazolam, α-OHTRZ, 4-OHTRZ at LLOQ and triazolam-D4 at 5 ng/mL; (C) real samples at 40 min after administrating of 0.25 mg triazolam tablet; peak I, triazolam; peak II, α-OHTRZ; peak III, 4-OHTRZ; peak IV, triazolam-D4.

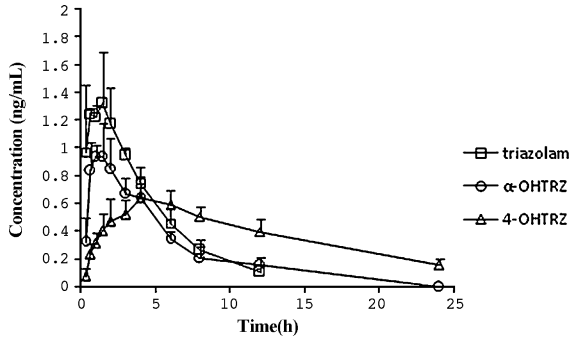

Fig. 2. Mean plasma concentration–time profile of triazolam, α-OHTRZ and 4-OHTRZ in three healthy Chinese volunteers taken a single dose of 0.25 mg triazolam orally.

$y = 7.89 \times 10^{-2}x + 5.05 \times 10^{-4}$ ($r = 0.9984$) for α-OHTRZ and $y = 7.88 \times 10^{-2}x - 2.32 \times 10^{-4}$ ($r = 0.9990$) for 4-OHTRZ. Precision and accuracy of LLOQ are shown in Table 2. R.E. was within the acceptable range (±20%) and R.S.D. was lower than 20%.

3.3.2. Accuracy and precision

The data of accuracy and precision are shown in Table 2. As the results showed, the precisions (R.S.D.) and accuracies (R.E.) of QC samples for within- and between-run were all in the acceptable range (R.S.D. < 15% and R.E. within ±15%), which indicated the analytical method was precise and accurate within the analytical range.

3.3.3. Extraction recovery and matrix effect

The extraction recoveries of triazolam in plasma were 95.3%, 94.0% and 98.6% for low (0.15 ng/mL), medium (4 ng/mL) and high (8 ng/mL) concentrations, respectively. That of α-OHTRZ were 94.3%, 94.8% and 102.7%; 4-OHTRZ were 106.8%, 97.3% and 101.2%.

The absolute and relative matrix effects are shown in Table 3. The results indicated that significant ion enhancement occurred for α-OHTRZ and 4-OHTRZ at low concentration, where diminished with a concentration dependent manner at medium and high concentrations. The absolute matrix effect of IS showed the same pattern with the analytes of interest. The relative matrix effects were all less than 10.7%, which indicated the impact from extracted plasma matrix was consistent and limited.

3.4. Stability

The data are shown in Table 4. Working solution, freeze and thaw, autosampler and long-term stability tests were all evaluated by the R.E. Triazolam and its two metabolites showed ideal stability for all the tests. The results indicated that this analytical method was proved to be applicable for routine analysis.

3.5. Pilot pharmacokinetic study

This validated LC–MS/MS method was applied to a pilot pharmacokinetic study and yield satisfactory results for determination of triazolam, α-OHTRZ and 4-OHTRZ in human plasma samples following a single oral dose of 0.25 mg triazolam in three healthy Chinese volunteers. Mean plasma concentration–time profiles of triazolam, α-OHTRZ and 4-OHTRZ are shown in Fig. 2, and the related pharmacokinetic parameters are listed in Table 5. C_{max}, t_{max} and $AUC_{0\to t}$ of triazolam obtained in the pilot pharmacokinetic study were similar to the previous reported values ($C_{max} = 1.3 \pm 0.4$ ng/mL; $t_{max} = 1.4 \pm 1.0$ h; $AUC_{0\to 24} = 6.3 \pm 2.7$ ng h/mL) [26].

Table 2
Precision and accuracy for the determination of triazoiam, α-OHTRZ and 4-OHTRZ in human plasma ($n = 6$)

	Triazolam				α-OHTRZ				4-OHTRZ			
	0.05[a]	0.15[a]	4[a]	8[a]	0.10[a]	0.30[a]	4[a]	8[a]	0.10[a]	0.30[a]	4[a]	8[a]
Within-run												
Found concentration (ng/mL)	0.05 ± 0.01	0.16 ± 0.01	4.53 ± 0.08	9.00 ± 0.19	0.10 ± 0.01	0.32 ± 0.02	4.04 ± 0.20	7.78 ± 0.27	0.10 ± 0.01	0.31 ± 0.01	3.89 ± 0.17	7.65 ± 0.18
R.S.D. (%)	10.44	3.16	1.68	2.13	13.70	5.98	4.91	3.44	8.40	3.61	4.48	2.36
R.E. (%)	−1.00	6.44	13.33	12.44	−0.83	7.83	0.92	−1.60	0.33	3.50	−2.79	−4.33
Between-run												
Found concentration (ng/mL)	0.05 ± 0.01	0.16 ± 0.01	4.03 ± 0.25	8.10 ± 0.58	0.10 ± 0.00	0.31 ± 0.04	3.68 ± 0.28	7.44 ± 0.72	0.10 ± 0.02	0.28 ± 0.03	3.77 ± 0.23	7.56 ± 0.69
R.S.D. (%)	11.59	6.64	6.30	7.18	1.68	13.65	7.50	9.70	15.26	11.56	5.97	9.07
R.E. (%)	3.33	4.22	0.83	1.23	0.02	1.89	−8.08	−7.04	4.50	−6.44	−5.67	−5.48

[a] Added concentration (ng/mL).

Table 3
Absolute and relative matrix effect of triazolam, α-OHTRZ, 4-OHTRZ and triazolam-D4

Added concentration (ng/mL)	Triazolam	α-OHTRZ	4-OHTRZ	Triazolam-D4[a]
0.15	115.3 (10.6)	137.0 (10.7)	140.1 (7.9)	113.0 (8.2)
4	89.6 (3.6)	110.4 (2.6)	104.8 (3.1)	86.1 (3.6)
8	83.0 (6.8)	95.3 (7.8)	93.1 (9.0)	80.2 (6.4)

The results are expressed as absolute matrix effect percentage (relative matrix effect percentage). Six different sources of blank plasma were used in the experiment.

[a] 100 μL of 50 ng/mL triazolam-D4 was spiked with the analytes of interest at 0.15 (low), 4 (medium) and 8 (high) ng/mL.

Table 4
Summary of stability tests of triazolam, α-OHTRZ and 4-OHTRZ

Stability	Triazolam	α-OHTRZ	4-OHTRZ
Working solution[a]	84.9–108.5	91.4–96.8	90.5–90.7
Freeze and thaw[b]	92.6–97.7	90.4–104.0	100.3–110.7
Autosampler[c]	93.8–102.2	104.0–113.2	101.4–109.7
Long-term[c]	95.3–101.9	86.7–92.1	94.2–98.9

Results are expressed as R.E. (%).

[a] 10 and 100 ng/mL for the three analytes were tested ($n = 3$).

[b] 0.15, 4 and 8 ng/mL for the three analytes were tested ($n = 3$).

[c] 0.15, 4 and 8 ng/mL for triazolam; 0.3, 4 and 8 ng/mL for α-OHTRZ and 4-OHTRZ were tested ($n = 3$).

Table 5
Mean pharmacokinetic parameters of triazolam, α-OHTRZ and 4-OHTRZ after a single oral dose of 0.25 mg triazolam tablet to three healthy Chinese volunteers

Parameters	Triazolam	α-OHTRZ	4-OHTRZ
T_{max} (h)	1.22 ± 0.48	2.33 ± 1.44	5.33 ± 1.15
C_{max} (ng/mL)	1.44 ± 0.21	1.00 ± 0.14	0.68 ± 0.05
$AUC_{0\rightarrow t}$ (ng h/mL)	6.77 ± 1.05	5.13 ± 0.79	9.07 ± 1.23
$AUC_{0\rightarrow\infty}$ (ng h/mL)	7.20 ± 1.26	6.42 ± 1.38	11.28 ± 2.06
$t_{1/2}$ (h)	2.78 ± 0.33	5.53 ± 1.16	9.45 ± 1.47

Results are expressed as mean ± S.D.

4. Conclusion

The presented study describes and validates a LC–MS/MS method for the determination of triazolam, α-OHTRZ and 4-OHTRZ in human plasma. The method proved to be linear in the concentration range studied as well as accurate, precise and selective. Also, clinical human plasma samples were successfully analyzed by this method, which indicated that it could applied to pharmacokinetic, bioavailability and bioequivalent studies.

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