SIMULTANEOUS DETERMINATION OF TRIAZOLAM AND ITS METABOLITES IN HUMAN BLOOD AND URINE BY GC/MS

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ABSTRACT: A sensitive and selective method has been developed for simultaneous determination of triazolam and its major metabolites, α-hydroxytriazolam and 4-hydroxytriazolam in human whole blood and urine. The drugs were effectively extracted using a 3-step solvent extraction procedure followed by tert-butyldimethylsilyl derivatization, and subjected to GC/MS in the negative ion chemical ionization mode.

KEY WORDS: Triazolam; Hydroxytriazolam; GC/MS.

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INTRODUCTION

Toxicological analysis of triazolam, a triazolobenzodiazepine hypnotic, is often required, since this drug is used for both suicidal and criminal purposes. As the dosage of this drug is as low as 0.125 mg in one tablet, and the half-life is very short (1.8–3.9 h), the detection and quantitation of low levels of triazolam in human body fluids and tissues are sometimes difficult.

Triazolam is readily metabolized to α-hydroxytriazolam and 4-hydroxytriazolam (Figure 1) after its oral administration in humans; and most of these metabolites are excreted into urine. Therefore, detection of these metabolites in human blood and urine is important to prove that triazolam has been ingested. Although highly sensitive methods were developed for analysis of triazolam in human biological materials using gas chromatography (GC) [5, 8], and GC-mass spectrometry (MS) [2, 6], there are no reports on the simultaneous determination of triazolam and its metabolites by GC or GC/MS to our knowledge. Inoue and Suzuki [2] developed a high performance liquid chromatography (HPLC) method for the determination of triazolam and its metabolites in human urine; this method requires 10 ml urine and the detection limits were about 5 ng/ml. Senda et al. [9] reported a more sensitive method to quantify triazolam and its metabolites using liquid chromatography tmospheric pressure chemical ionization MS. Since GC/MS methods seem more preferable for the simultaneous analysis of these drugs, we have established a detailed procedure for such a method in this study.

MATERIALS AND METHODS

Materials

Triazolam, α-hydroxytriazolam and 4-hydroxytriazolam were provided by Upjohn (Kalamazoo, MI, USA) and estazolam was from Takeda (Osaka). N-(tert-Butyldimethylsilyl)-N-methyltrifluoro-acetamide (MTBSTFA) used for tert-butyldimethylsilyl (tBDS) derivatization was purchased from GL Sciences (Tokyo). Hexane and tert-butyl methyl ether were of analytical grade and were purified by distillation. β-glucuronidase purified from *E. coli* bacteria was purchased from Sigma (St. Louis, MO, USA). Other common chemicals used were of analytical grade.

Triazolam, α-hydroxytriazolam and 4-hydroxytriazolam (5 mg each) were separately dissolved in 5 ml methanol to give a concentration of 1000 ng/μl and further diluted to concentrations of 100, 10, 1 and 0.1 ng/μl. The internal standard (IS) solution containing estazolam at a concentration of 5 ng/μl was prepared in the same manner. Whole blood and urine used for control samples were obtained from healthy volunteers and were kept at –20°C until analysis.

Extraction and derivatization

A sample of whole blood (1 g) was mixed with 5 ml 0.2 M borate buffer (pH 9) and 2 μl of IS solution (10 ng of estazolam) in a 30 ml centrifuge tube. To the mixture, 10 ml tert-butyl methyl ether was added, shaken for 10 min and then centrifuged at 850 g for 20 min. The organic phase was transferred to a 10 ml centrifuge tube, evaporated to dryness under a stream of nitrogen and dissolved in 3 ml of 0.1 M disodium citrate solution (pH 5). The solution was then washed twice by shaking with 3 ml of hexane for 10 min and centrifuged at 850 g for 20 min. To the aqueous layer, 2 ml of tert butyl methyl ether was added and shaken for 10 min. After centrifugation, the organic layer was evaporated to dryness under a stream of nitrogen. The residue was dissolved in 50 μl acetonitrile, and 10 μl of MTBSTFA was added to the solution for tBDS derivatization by heating at 60°C for 1 h. The mixture was evaporated to dryness under a stream of nitrogen. The residue was reconstituted in 20 μl acetonitrile and a 3 μl aliquot of the solution was injected into a GC/MS instrument. For urine samples, 1 ml was mixed with 1 ml 1 M phosphate buffer (pH 6.8) in a 30 ml centrifuge tube and incubated at 37°C for 2 h with 2 mg β-glucuronidase. The solution was mixed with 5 ml 0.2 M borate buffer (pH 9.0), 10 ml tert-butyl methyl ether and 2 μl IS solution (10 ng estazolam), shaken for 10 min and centrifuged at 850 g for 15 min. The organic layer was subjected to the same procedure as that used for blood analysis.

GC/MS conditions

Analyses were performed on a Hewlett-Packard 5989A GC/MS system operated in the electron impact (EI) and negative ion chemical ionization (NICI) modes. The column used was an HP-1 capillary column (12 m x 0.2-mm i.d., 0.33-μm film

thickness) obtained from Hewlett-Packard (Wilmington, DE, USA). Helium was used as carrier gas with a flow rate at 1 ml/min. The reagent gas in NICI mode was methane. The splitless injection was used with a valve off time of 2 min. The GC conditions were as follows. The initial temperature of 100°C was held for 2 min; then the temperature was programmed up to 300°C at a rate of 20°C/min and maintained for 2 min. The temperatures of the injection port and transfer line were maintained at 280°C. The MS conditions were: repeller voltage 7.0 V and ionization current 300 μA; in the EI mode, quadrupole temperature 100°C, ion source temperature 250°C, electron energy 70 eV; in the NICI mode, quadrupole temperature 100°C, ion source temperature 200°C, electron energy 230 eV and ion source pressure 1.5 Torr. Selected ion monitoring (SIM) was made at m/z 308 for triazolam, m/z 438 and 436 for α-hydroxytriazolam, and m/z 342 and 340 for 4-hydroxytriazolam and m/z 296 for estazolam (IS).

Preparation of calibration curves

Whole blood and urine samples were prepared to contain triazolam, α-hydroxytriazolam and 4-hydroxytriazolam at concentrations of 0.5, 1, 5, 10, 50 and 100 ng/ml, each containing 10 ng/ml estazolam. These samples were extracted in the same manner as described above. The standard curves were obtained by plotting the peak area ratios of triazolam (or metabolites) to IS versus the amounts of triazolam (or metabolites).

RESULTS AND DISCUSSION

Extraction procedure

The 3-step solvent extraction procedure previously established for the analysis of triazolam in human tissues Kudo et al. [5] was used to extract the metabolites, α-hydroxytriazolam and 4-hydroxytriazolam. The metabolites were extracted with tert-butyl methyl ether under slight alkaline condition (pH 9), back-extracted to the citrate buffer solution (pH 5), and re-extracted with tert-butyl methyl ether after alkalizing the aqueous solution with sodium hydroxide. As 4-hydroxytriazolam was unstable under alkaline conditions at the re-extraction step, the recovery rates were not constant. This problem was overcome by extracting the metabolite from the 0.1 M disodium citrate solution (pH 5.0) directly with tert-butyl methyl ether. Two washes of the aqueous layer with hexane significantly reduced interfering peaks appearing on the chromatograms.

Derivatization and GC/MS analysis

Derivatization methods, including trimethylsilylation (TMS) [1], methylation [4] and propylation-propionylation [7], were tested to make the benzodiazepine metabolites volatile for GC/MS analysis. Since we found no reports on the simultaneous

derivatization of both α-hydroxytriazolam and 4-hydroxytriazolam, we first tried methylation, using the tetrabutylammonium hydroxide-dimethyl sulfoxide reagent and iodomethane; 4-hydroxytriazolam was completely methylated, but the target methyl derivative was not obtained from α-hydroxytriazolam. We then used tBDS, as this derivative is more stable than the TMS derivative.

As shown in Figure 2, characteristic ions for tBDS derivatives, $[M-57]^+$, were observed for both compounds in the EI mode at m/z 415. In the NICI mode, the spectra showed characteristic peaks at m/z 436 and 438 $[M-cl-l]^-$ for α-hydroxytriazolam and m/z 340 and 342 $[M-tBDSOH]^-$. We found that NICI mode was highly sensitive for detection of triazolam with clean backgrounds and therefore adopted the mode for determination of small amounts of triazolam and its metabolites in human whole blood and urine.

Determination of triazolam and its metabolites in whole blood and urine by GC/MS

Figure 3 shows SIM chromatograms of tBDS-derivatized extracts from whole blood and urine samples spiked with 10 ng each of IS, triazolam, 4-hydroxytriazolam and α-hydroxytriazolam. Each peak was clearly separated with retention times of 11.3, 11.8,

Fig. 1. Structures of triazolam, α-hydroxytriazolam, 4-hydroxytriazolam and estazolam (IS).

12.6 and 13.3 min, respectively. There were no interfering peaks on the chromatograms of blank whole blood and urine. The calibration curves for triazolam and its metabolites were linear in the concentration range of 0.5 to 100 ng/g with correlation coefficients of > 0.99. The lower limits of detection for all compounds were 0.1 ng/g for whole blood samples and 0.2 ng/g for urine samples, which are sufficiently sensitive for the analyses

of samples even after the ingestion of a single triazolam tablet [9]. The absolute recoveries for triazolam, α-hydroxytriazolam and 4-hydroxytriazolam at a concentration of 10 ng/g were 58, 71 and 31%, respectively.

Within-day precision of this method in whole blood and urine samples was examined at concentrations of 10 ng/g sample. The coefficients of variation (C.V.) were 6.0 and 3.1% for triazolam in whole blood and urine, 2.7 and 10.8% for α-hydroxytriazolam and 6.4 and 7.8% for 4-hydroxytriazolam, respectively (n = 5).

Since GC/MS is more commonly used than HPLC/MS in laboratories of toxicology, our method should be useful to prove that triazolam has been ingested.

Fig. 2. EI and NICI mass spectra of tBDS derivatives of α-hydroxytriazolam and 4-hydroxytriazolam.

CASE STUDY

A 5-year-old boy was found unconsciousness in the garden of his home. He remained unconscious and was sent to our hospital the following morning. Poisoning was suspected, and drug screening by Triage[R] was done; the presence of benzodiazepines

was apparent. Since ingestion of triazolam, prescribed for the patient uncle, a schizophrenic, was suspected, triazolam and its metabolites in serum and urine of the boy collected 1 day after the incident were analyzed using our methods. Although only a trace level of triazolam and of α-hydroxytriazolam were detected in the serum, α-hydroxy-

Fig. 3. SIM chromatograms of tBDS-derivatized extracts from whole blood and urine spiked with 10 ng each of IS, triazolam, 4-hydroxytriazolam and α-hydroxytriazolam.

triazolam and 4-hydroxytriazolam were clearly evident in urine samples with the concentrations of 405.6 and 27.7 ng/ml, respectively. Therefore ingestion of triazolam by the patient was proved.

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