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Case report

Case report: Etizolam and its major metabolites in two unnatural death cases

Takuma Nakamae [a,c,*], Tatsuo Shinozuka [b,c], Chizuko Sasaki [c], Akira Ogamo [a], Chikako Murakami-Hashimoto [c], Wataru Irie [c], Masaru Terada [d], Shigeki Nakamura [c], Masataka Furukawa [c], Katsuyoshi Kurihara [c]

[a] Department of Pharmaceutical Science, International University of Health and Welfare, 2600-1 Kitakanemaru, Ohtawara, Tochigi 324-8501, Japan
[b] Department of Phathophysiology, Yokohama College of Pharmacy, 601 Matanocho, Totsuka-ku, Yokohama, Kanagawa 245-0066, Japan
[c] Department of Legal Medicine, Kitasato University, Kitasato1-15-1, Sagamihara, Kanagawa 228-8555, Japan
[d] Department of Legal Medicine, Toho University School of Medicine, 5-21-16 Omorinishi, Ota-ku, Tokyo 143-8540, Japan

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ABSTRACT

A simultaneous analytical method for etizolam and its main metabolites (α-hydroxyetizolam and 8-hydroxyetizolam) in whole blood was developed using solid-phase extraction, TMS derivatization and ion trap gas chromatography tandem mass spectrometry (GC–MS/MS). Separation of etizolam, TMS derivatives of α-hydroxyetizolam and 8-hydroxyetizolam and fludiazepam as internal standard was performed within about 17 min. The inter-day precision evaluated at the concentration of 50 ng/mL etizolam, α-hydroxyetizolam and 8-hydroxyetizolam was evaluated 8.6, 6.4 and 8.0% respectively. Linearity occurred over the range in 5–50 ng/mL. This method is satisfactory for clinical and forensic purposes.

This method was applied to two unnatural death cases suspected to involve etizolam. Etizolam and its two metabolites were detected in these cases.

1. Introduction

Benzodiazepines are used for the treatment of a wide spectrum of clinical disorders. Etizolam, 6-(*o*-chlorophenyl)-8-ethyl-1-methyl-1-methyl-4H-s-triazolo [3,4-*c*]-thieno [2,3-*e*]-[1,4]diazepine, was introduced in 1983 under the trade name Depas® in Japan. Now, etizolam is used in Japan, Korea and Italy and is not approved for use in other countries. Etizolam is one of the most widely prescribed drugs for the treatment of anxiety and has strong muscle relaxing properties [1,2]. Its relative safety and low abuse potential has caused etizolam to be one of the most prescribed benzodiazepines in Japan [3]. In clinical studies oral administration of a single 2 mg dose resulted in average peak plasma concentration of 25 ng/mL [4]. In studies of patients receiving 1 mg/day dividing twice chronically, the peak plasma concentration of etizolam is the same concentration [5]. Characteristics of etizolam are low dose usage and short time acting ($T_{1/2}$: 6 h) and strong muscular relaxing property [5]. In recent years, the number of abusers, suicide and murder cases after ingestion of etizolam and accident caused by its muscular relaxing property have increased and have become a serious problem in Japan [6,7]. In these cases sensitive method to detect low dose etizolam used is necessary.

Etizolam is frequently seen taken with other drugs and prescribed only in Japan and a few countries [8]. Case report of etizolam is a few and lethal and poisoning level is not reported. Toxicological knowledge of etizolam is not enough. Toxicological analysis of etizolam is required. The metabolites often have the activity on the benzodiazepine drug, and etizolam is metabolized to α-hydroxyetizolam and 8-hydroxyetizolam [9]. These metabolites show longer half life than unchanged etizolam [5]. Both metabolites have activity, and especially 8-hydroxyetizolam show higher activity than etizolam itself in mouse [4]. For toxicological estimation of etizolam, analysis of its metabolites is required.

We reported here a sensitive method for the analysis of etizolam and its two major metabolites in forensic whole blood sample, by using Oasis® HLB cartridge and ion trap GC–MS/MS. Several methods have been described for determination of etizolam using GC, GC–MS, high performance liquid chromatography (HPLC) [10–12]. However analytical method for metabolites and its application to actual forensic sample have not been reported. Although LC–MS and LC–MS/MS have been utilized

* Corresponding author at: Department of Pharmaceutical Science, International University of Health and Welfare, 2600-1 Kitakanemaru, Ohtawara, Tochigi 324-8501, Japan. Tel.: +81 287 24 3273.
E-mail address: tak-n@iuhw.ac.jp (T. Nakamae).

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frequently used in analysis of metabolite, they are very expensive. Ion trap GC–MS/MS permits high sensitive and selective MS/MS analysis with low cost.

The purpose of this study was to develop an analytical method for etizolam and its two metabolites to obtain toxicological information of etizolam applied to two unnatural death cases suspected of taking etizolam.

2. Experimental

2.1. Specimens

Blank blood sample was obtained from laboratory personnel, through verbal consent.

Whole blood samples used in case studies were obtained from heart, and stored in −20 °C until analysis. In each case, drug screening on Triage® drug of abuse (DOA) with urine sample obtained was performed.

2.2. Chemical

Etizolam, α-hydroxyetizolam and 8-hydroxyetizolam were gifts from Mitsubishi pharma corporation (Osaka, Japan). Fludiazepam was gifts from Dainippon Sumitomo Pharma (Osaka, Japan). Dichrolomethane (HPLC grade), methanol (HPLC grade), were purchased from Wako (Osaka, Japan). *N*,*O*-bis(trimethylsilyl)trifluoroacetamide with 1% trimethylchlorosilane (BSTFA/1% TMCS) was purchased from Sigma–Aldrich (St. Louis, MO, USA). Chemicals for the 1/15 M phosphate buffer were purchased from Mitsubishi Kagaku Iatron.

2.3. Standard solution

All drug stock solutions were prepared by dissolving 10 mg in 10 mL of methanol (1 mg/mL), stored at −20 °C and diluted with methanol necessarily.

2.4. Extraction and derivatization

To 1 mL of specimen (whole blood), 4 mL of distilled water, and 10 ng fludiazepam (internal standard) was added. The mixture was centrifuged (3000 rpm, R.T.: 15 min) and supernatant was mixed with 3 mL of 1/15 M phosphate buffer (pH 7.4).

Oasis® HLB cartridge was washed with 5 mL of dichloromethane, activated with 5 mL of methanol, and conditioned with 5 mL of distilled water. Then the sample was loaded. The cartridge was washed with 3 mL of distilled water. Finally analyte was eluted with 5 mL of dichloromethane. Aqueous layer was discarded with a pipette and the organic layer was dehydrated by solid Na_2SO_4 and evaporated to dryness at 40 °C. The residue was dissolved with 70 μL of dichloromethane and 70 μL of BSTFA 1% TMCS was added and incubated at 70 °C for 30 min and allowed to cool down to room temperature prior to GC–MS/MS analysis.

2.5. Instrument and ion preparation and analysis

GC–MS/MS analysis was performed using Varian Saturn 2200 apparatus consisting of CP-3800 gas chromatograph equipped with an ion trap mass spectrometer and 8400 auto sampler. The chromatographic separation was carried out on a fused silica capillary column (30 m × 0.25 mm i.d., film thickness 0.25 μm, DB-5MS, J&W Scientific, USA). Helium was used as GC carrier gas at constant 1.0 mL/min. Injector temperature was 300 °C. The column temperature was programmed at 150 °C (1 min)–150 °C–270 °C (50 °C/min)–285 °C (1 °C/min)–300 °C (10 °C/min), transfer line was maintained at 280 °C, manifold at 45 °C respectively. Injection mode was splitless, and splitless time was 1.0 min. Ionizing mode was electron ionization (EI). The ion trap and manifold temperatures were 200 °C and 45 °C. The mass spectrometry technique was optimized to have best sensitivity to etizolam and its two metabolites (Table 1). Tuning of mass spectrometer was automatically performed.

2.6. Calibration curve and method validation

Standard calibration curves were obtained by preparing authentic spiked blank blood (1 mL) containing final 5, 10, 20, 30, 40, and 50 ng/mL of etizolam and its two metabolites. Calibration curves were obtained by plotting the peak area ratio of analyte to internal standard in each target ion chromatogram. Recovery was established at 50 ng/mL, by comparing the analyte peak areas of extracted samples with same amounts of the analyte extracted from water. The inter-day precision was determined by analyzing 50 ng/mL on the same day. The same procedure was repeated on different days to determine the intra-day precision. The results are expressed as the relative standard deviation (R.S.D.).

3. Result

3.1. Analyses of etizolam and its two metabolites

For quantification, the tandem mass spectra for etizolam: *m*/*z* total of 313, 301, and 266, TMS derivative of α-hydroxyetizolam: *m*/*z* 386, TMS derivative of 8-hydroxy-etizolam: *m*/*z* 341 and fludiazepam: *m*/*z* 239 were used (Fig. 1). The calibration curves for etizolam, α-hydroxyetizolam and 8-hydroxyetizolam were linear in concentration range 5–50 ng/mL with correlation coefficients of 0.9955, 0.9860 and 0.9820 respectively (Fig. 2). The inter-day precision evaluated at the concentration of 50 ng/mL of etizolam, α-hydroxyetizolam and 8-hydroxyetizolam was evaluated 8.6, 6.4 and 8.0% respectively (Table 2). The intra-day precision evaluated at the concentration of 50 ng/mL of etizolam, α-hydroxyetizolam and 8-hydroxyetizolam was evaluated 9.3, 5.9 and 8.3% respectively. Separation of etizolam, TMS derivatives of α-hydroxyetizolam and 8-hydroxyetizolam and fludiazepam as internal standard was performed within about 17 min (Fig. 3).

Table 1
Parameter for the GC–MS/MS methods.

Segment mode	Time range (min)	Compounds	Retention time (min)	Ionization mode	Excitation storage level (*m*/*z*)	Excitation amplitude (V)
2. MS/MS	5.7–6.5	Fludiazepam (I.S.)	6.0	Non-resonant	120.8	94.00
4. MS/MS	13.0–15.0	Etizolam	13.6	Resonant	150.9	00.45
5. MS/MS	15.0–17.5	α-OH etizolam	15.4	Resonant	183.2	00.77
		8-OH etizolam	15.7	Resonant	183.2	00.77