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CASE REPORT

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Detection of triazolam in skeletal remains buried for 4 years

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Abstract Analyses of the hypnotic triazolam from the remains of two human skeletons buried underground for 4 years were made for purposes of confirmation. The bone marrow and mummified muscle were digested with 2 M sodium hydroxide, efficiently extracted using a 3-step solvent extraction procedure, and selectively analyzed by gas chromatography/mass spectrometry with the negative ion chemical ionization mode. Estazolam was the internal standard used. Triazolam was detected in all the samples; the concentrations were 0.36 ng/g in the bone marrow of one victim, and 0.37 and 5.5 ng/g in the bone marrow and mummified muscle of the other victim. This method should prove useful for determination of triazolam in extensively decomposed bodies.

Key words Triazolam · GC/MS/NICI · Bone marrow · Putrefied tissue · Toxicology

Introduction

Toxicological analysis of triazolam, a triazolobenzodiazepine hypnotic, is often required since this drug is used for both suicidal and criminal purposes. As dosage of this drug is as low as 0.125 mg in one tablet, and the half-life is short (1.8–3.9 h), the detection and quantitation of minute amounts of triazolam in human tissues are difficult especially in cases of extensive putrefaction [1, 2].

We studied two homicides where the victims were buried alive after ingestion of triazolam for sedation. We describe here the sensitive and specific analysis of triazolam from the remains, to confirm the confessions of the murderers.

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Case report

The bodies of two men who had been missing since July 1992 were found buried in a forest in June 1996. One body was completely skeletonized and the other was skeletonized with the exception of the buttocks where a small amount of mummified muscle remained. As the suspected murderers testified that they had buried the victims alive after sedation with triazolam, toxicological examinations were carried out on the samples of femoral bone marrow and mummified muscle for purposes of confirmation.

Materials and methods

Reagents

Triazolam was provided by Upjohn, Kalamazoo, MI, USA and estazolam was from Takeda Chemical Industries, Osaka, Japan. Hexane and tert.-butyl methyl ether were of analytical grade and were purified by distillation. Other chemicals used were of analytical grade.

Extraction procedure

A method we previously reported was modified so that trace amounts of triazolam in decomposed tissues could be analyzed [1, 3]. Bone marrow or mummified muscle samples (1 g) were mixed with 1 μl of IS solution (10 ng of estazolam) in a 30-ml centrifuge tube, and digested with 5 ml of 2 M sodium hydroxide at 100° C for 30 min. After cooling the mixture, a 10-ml volume of tert.-butyl methyl ether was added, and the preparation was shaken for 10 min and centrifuged at 850 g for 20 min. The organic phase was transferred to a 10-ml centrifuge tube, and the aqueous phase was re-extracted with 5 ml tert.-butyl methyl ether. The combined organic phase was evaporated to dryness under a stream of nitrogen and reconstituted in 3 ml of 0.1 M disodium citrate solution (pH 5). This solution was then washed twice with 3 ml of hexane for 10 min and centrifuged at 850 g for 20 min. The aqueous layer was transferred to a 10-ml centrifuge tube containing 2 drops of bromothymol blue solution (0.04%) as an indicator and the mixture was made alkaline by adding 0.2 M sodium hydroxide until the indicator turned blue (pH ca.9). To the solution we added 2 ml of tert.-butyl methyl ether and the preparation was shaken for 10 min. After centrifugation, the solvent layer was dried with sodium sul-

fate and evaporated. The residue was dissolved in 10 µl tert.-butyl methyl ether and a 3 µl aliquot of the solution was injected into a gas chromatograph-mass spectrometer.

GC/MS conditions

Analyses were performed on a Hewlett-Packard 5989A GC/MS system operated in the negative ion chemical ionization (NICI) mode. The chromatographic column was a HP-1 capillary column (13 m × 0.2-mm i.d., 0.33-µm film thickness) from Hewlett-Packard. Helium was used as a carrier gas with a flow rate of 1 ml/min and the reagent gas was methane. The splitless injection mode was selected with a valve off-time of 2 min. The GC/MS conditions were as follows: the initial temperature of 100° C was held for 2 min, then the temperature was programmed 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 mass spectrometer monitored the ions of m/z306 and 308 for triazolam, and m/z294 and 296 for estazolam.

Preparation of calibration curves

To quantify triazolam present in bone marrow and mummified tissue, the calibration curves were prepared using drug-free bone marrow and thigh muscle. Bone marrow and muscle samples were prepared to contain triazolam at concentrations of 0.2, 0.5, 1, 5 and 10 ng/g, each containing 10 ng/g estazolam and extracted in the same manner. The standard curves were obtained by plotting the peak area ratio of triazolam to estazolam versus the amount of triazolam.

Results

Figure 1 shows the selected ion monitoring (SIM) chromatograms of extracts from bone marrow and muscle each containing 5 ng triazolam and 10 ng IS. Each peak

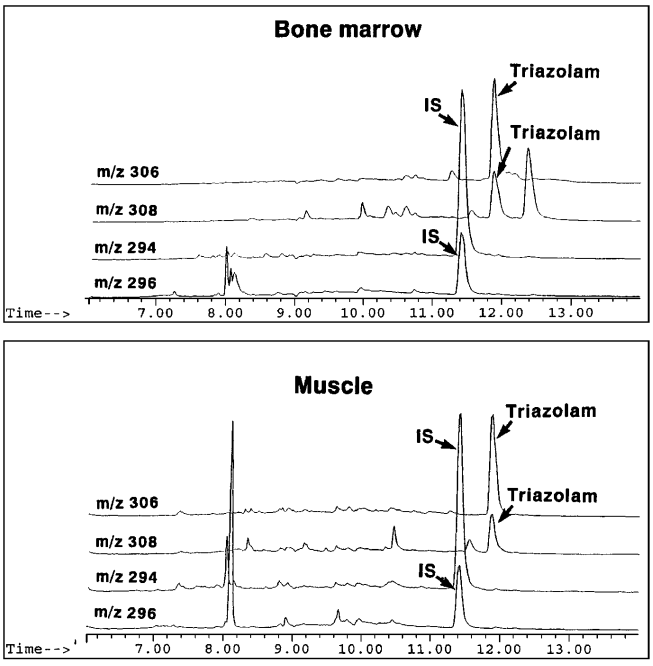

Fig. 1 SIM chromatograms of the extracts from bone marrow and thigh muscle each containing 5 ng triazolam and 10 ng IS

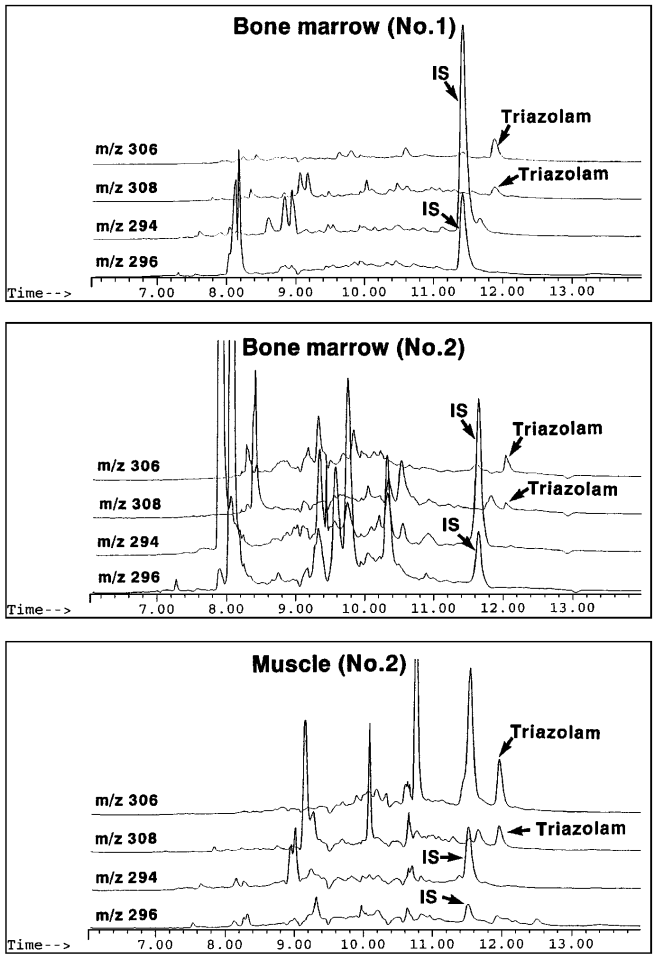

Fig. 2 SIM chromatograms of extracts from bone marrow and mummified muscle obtained from the two victims

was clearly separated on the chromatograms, and there were no interfering peaks on the chromatograms of triazolam-free human tissues. Calibration curves in bone marrow and muscle were linear in the concentration range from 0.2 to 10 ng/g, with correlation coefficients over 0.99.

Figure 2 shows SIM chromatograms of extracts from bone marrow and mummified muscle from the victims. Although many peaks appeared on the chromatograms presumably originating from putrefied tissues, the peaks of triazolam were clearly evident for each sample. The concentrations of triazolam were 0.36 ng/g in the bone marrow of one victim, and 0.37 and 5.5 ng/g in the bone marrow and mummified muscle of the other.

Discussion

The method previously established for the analyses of triazolam in human solid tissues [1] was first used for the mummified tissue. The drug was extracted using a 3-step solvent extraction procedure, purified on a silica gel column, and analyzed by gas chromatography with a nitro-

gen phosphorus detector. However, many interfering peaks appeared on the chromatogram. Inoue et al. [2] successfully detected triazolam in putrefied human muscle by GC/MS with electron impact (EI) mode after purifying the extract obtained by 3-step solvent extraction procedure with high-performance liquid chromatography (HPLC). Although fewer interfering peaks were observed on the chromatogram, the lower limit of detection of 10 ng/g was not sufficiently sensitive to detect triazolam in our sample.

Compared to the EI mode, the NICI mode is highly sensitive to some benzodiazepines and also affords a high degree of selectivity against endogenenous components such as lipids and most other classes of drugs [4–9]. Cairns et al. [10] reported the sensitive and specific determination of triazolam in hemolyzed whole blood and liver digests by GC/MS with NICI mode. When we applied the above HPLC fraction to GC/MS with NICI mode, the peak of triazolam was clearly evident. As the amount of triazolam in the bone marrow samples was near the detection limit of GC/MS with NICI mode, direct subjection of the extracts to GC/MS was attempted in order to avoid contamination during the HPLC step of purification. Our extraction procedure [1] was modified so that even a trace amount of triazolam in skeletonizing remains could be selectively determined by GC/MS with NICI mode. Fewer interfering peaks were observed on the chromatogram by digesting samples with 2 M sodium hydroxide and by washing the aqueous layer with hexane twice in the 3-step solvent extraction procedure.

In human bone marrow, several drugs including methamphetamine, paracetamol, aminopyrine and cyclobarbital can be identified [11–14] but this seems to be the first report on the presence of triazolam in human bone marrow and mummified tissue.

Since the concentration of triazolam in bone marrow of one victim (0.36 ng/g) was similar to that in the other victim (0.37 ng/g), they were probably given the same dosage of triazolam. Although the drug level at the time of death is not known owing to the lack of studies on postmortem changes of triazolam concentrations in the body buried underground for several years, detection of triazolam in skeletonized remains proved useful to confirm the statements of the suspects.

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