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Case Report Open Access

# Flunitrazepam Product Detected in the Stomach of an Autopsy Case Buried Under the Ground for Two Years

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## Abstract

Flunitrazepam (FNZ) is a short-intermediate acting benzodiazepine derivative, and easily reduced to 7-amino FNZ. We autopsied the buried cadaver of homicide victim two years after the strangulation following ingestion of FNZ. The whole body of the cadaver had become moderately decomposed and adipocere without any obvious dehiscence. There was a fracture of the lamina thyroid cartilage. Gas chromatography/mass spectrometry (GC/MS) was performed on bone marrow sample and the stomach contents. Only 7-amino FNZ could be detected in the stomach contents. The detection of 7-amino FNZ brought important information about the ante-mortem circumstances. To our knowledge, such detection of FNZ product in a cadaver after a long period of time has not been reported previously.

**Keywords** Chromatography; Stomach; Benzodiazepine; Post-mortem; Ante-mortem; Bone marrow

## Introduction

Flunitrazepam (FNZ) is prescribed for the treatment of severe insomnia, and is often cited as a date rape drug because of its high potency, strong effects and the ability to cause strong amnesia during its duration of action [1].

FNZ is extensively metabolized in the liver to desmethyl FNZ (nor-FNZ), which has some activity, and to 7-amino FNZ by reduction of the 7-nitrogen group, which is inactive; other metabolites include 3-hydroxyl FNZ, 3-hydroxy-7-acetamido FNZ, and 7-amino-1-desmethyl FNZ [2]. The detection of FNZ was difficult in blood in the report describing fatal cases of FNZ, but 7-amino FNZ was detected [3,4]. If FNZ or 7-amino FNZ metabolite is proved in the post mortem victim, even in its stomach contents, it will bring useful information to the diagnosis. To our knowledge, such detection of FNZ metabolite has not been reported before in the cadaver over months or years after death.

We performed an autopsy of a victim who was assumed to have ingested FNZ before the death, and buried under the ground for two years. Verification of ingestion of FNZ was necessary, although it was assumed that decomposition of FNZ in the body had occurred. This is a report of an autopsy case in which a FNZ product was detected in the body regardless of the long post mortem period.

## Case History

Man of in his forties was involved in a quarrel. According to the confession of the criminals, the man was made to fall asleep by ingestion of several broken tablets of FNZ that had been mixed into a glass of tomato juice beforehand by criminals and then he was murdered by strangulation. The whole body of the cadaver was covered with concrete of 2 cm-2.5 cm thickness and was buried at 1.2 m depth from the surface of the earth. Due to the confession of the murderers, the corpse was found at that place approximately two years after death. An autopsy was performed on the day following finding of the body.

## Autopsy findings

The whole body of the cadaver had become moderately decomposed and adipocerous. There were no obvious injuries such as dehiscence on the skin or muscles by the naked eyes. Primary organs such as the lungs, heart, liver and kidneys had also become decomposed and adipocerous, and they barely retained their shape. Histological findings could not be identified due to the decomposition, and therefore the existence of diseases was unknown. Among the cervical organs, there was a fracture of the lamina thyroid cartilage running from the midline to the left internal lamina. There were no other fractures in the cervical organs including the body of the hyoid bone or in other parts of the body.

## Methods of Analysis

Bone marrow of femur and the stomach content of the cadaver were used for the following analysis. The bone marrow had become dry and was adhered to the inner wall of the bone. The stomach contents had remained in 9 g and turned into a kind of reddish-brown mud.

FNZ was kindly gifted from F. Hoffmann-La Roche, Ltd. (Basel, Swiss). 7-amino FNZ was purchased from Lipomed Inc. (Cambridge, MA). Diazepam was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan) and used as the internal standard (IS) in quantitative analysis. Other reagents and chemicals were analytical grade and purchased from Nacalai Tesque, Inc. (Kyoto, Japan) unless otherwise mentioned. Each standard was diluted or dissolved in methanol solution.

FNZ was not detectable up to 0.3 μg/ml in quantitative analysis, which did not reach the clinical nor toxic level of blood FNZ. 7-amino FNZ was detectable to 0.03 μg/ml or less. Therefore, FNZ was not examined but 7-amino FNZ was analyzed in the present samples. A modified method of three-step extraction [5] was applied for the analysis using GC/MS as follows.

For the qualitative analysis, approximately 0.5 g of bone marrow of the femur and the stomach contents was weighed precisely. The weighed samples were put into a 10 ml centrifuging glass tube. 10 mg of sodium fluoride was added as an inhibitor of reductase in each sample. The sample was homogenized in 2 ml of 0.2 M carbonate buffer (pH 10.3). A 4 ml volume of tert-butyl methyl ether was added and the preparation was shaken for 10 min and centrifuged at 850 g at

room temperature for 20 min. The upper ether layer was transferred into a new 10 ml centrifuge tube and a 1 ml volume of 2 N hydrochloric acid was added. To remove lipids, the acid layer was washed with 4 ml of tert-butyl methyl ether in the same manner as above. The upper organic layer was discarded and the lower aqueous layer was transferred into a new 10 ml glass tube containing two drops of 0.04% bromothymol blue solution as a pH indicator and the mixture was made weakly alkaline (ca. pH 8 ) by adding sodium hydroxide solution until the indicator turned pale blue. To the mixture were then added 0.4 ml of carbonate buffer (0.2 M, pH 10.3) and 2 ml of tert - butyl methyl ether again. After mixing and centrifuging, the upper ether layer was moved into a new tube. The layer was dried with sodium sulfate and evaporated under a gentle stream of nitrogen. The residue was dissolved in 20 ml of ethyl acetate, and subjected to TFA derivatization by adding 20 ml of trifluoroacetic anhydride (Wako Pure Chemical Industries, Ltd., Osaka, Japan) and incubation at 60°C for 20 min. The reagent was dried under nitrogen gas until slight moisture remained in the tube and was then diluted with 20 µL of acetone. A 2 µl aliquot of the solution was injected into the gas chromatograph-mass spectrometer. The GC/MS apparatus was a GC mate (Jeol, Tokyo, Japan) equipped with a capillary column (J&W DB-1, 0.32 mm i.d. × 15 m length, 1.0 µm film thickness) (Agilent Technology, Santa Clara, CA). The column temperature was programmed to increase from 100°C to 200°C at a rate of 30°C/min, and then increase to 290°C at a rate of 10°C/min to be maintained at the final temperature for 5 min. The injection port and ion source were kept at 260°C. The scan mode was used for the qualitative analysis. For the quantitative analysis, the deuterium compound of 7-amino FNZ (7-amino FNZ-d3, Lipomed AG, Arlesheim, Swiss) was tested as an internal standard at first. The molecular difference between 7-amino FNZ and the d3 compound was so small that their target ions overlapped with each other by their mutual side ions appearing at the proximate retention time. Sufficient calibration curve couldn't be obtained by the use of the deuterium compound, and then diazepam was used as IS instead. The method of standard addition was applied for the quantitative analysis, because there was no adequate blank sample for it. Approximately 0.2 g of the stomach contents of this case was weighed precisely, put into a 10 ml centrifuging glass tube as a test tube and spiked 25 ng of diazepam in 0.2 g of sample. Test tubes were prepared to the number of plotting points in a calibration curve. The single ion-monitoring (SIM) mode was set at m/z 379, 360, 351 for the trifluoroacetate product of 7-amino FNZ; and m/z 256 for IS. The relative ratio of 7-amino FNZ area to IS area was plotted against the concentration of 7-amino FNZ in a correlative graph using the peak of 7-amino FNZ at m/z 379 and the peak of IS at m/z 256. The intercept of the horizontal axis was read as the concentration of 7-amino FNZ in the sample.

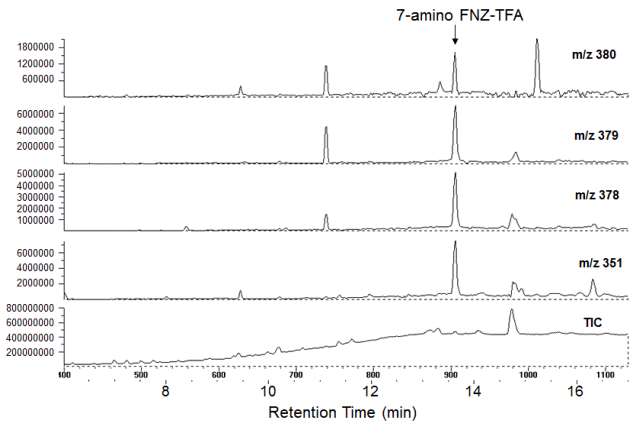


**Figure 1:** Mass chromatogram of the stomach contents.

## Results of Analysis

The peak of the trifluoroacetate product of 7-amino FNZ appeared at 13 min 38 s, and the characteristic peak of 7-amino FNZ was observed at m/z 379, 378, 360, 352, 351 and 350 in authentic standard solution in scan mode. The peak of 7-amino FNZ was detected in the stomach contents (Figure 1) but not in the bone marrow. The spectrum of 7-amino FNZ in the stomach contents in qualitative scan mode (Figure 2) was consistent with that of the authentic standard.

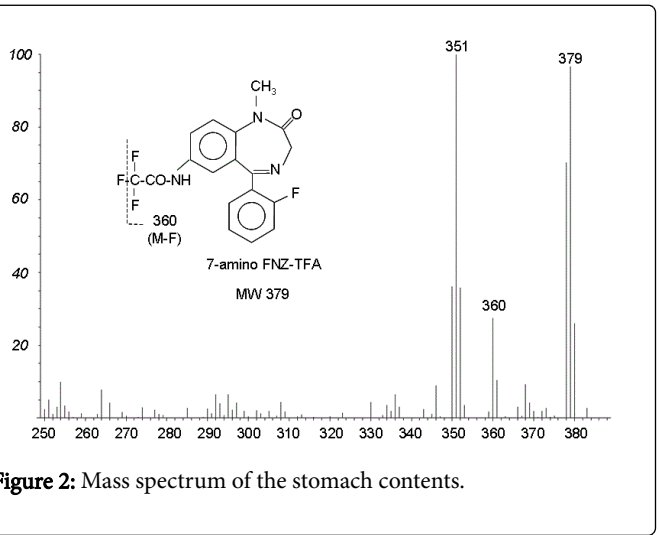


**Figure 2:** Mass spectrum of the stomach contents.

There were no other remarkable overlapping peaks in the stomach contents in the quantitative analysis. The linearity of the calibration curve for the 7-amino FNZ quantification was 0.999 in the range of 0 µg/ml to 0.30 µg/ml blood (Figure 3). The concentration of 7-amino FNZ in the stomach contents was read as 0.14 µg/g into the calibration curve. The total amount of 7-amino FNZ was estimated as 1.26 µg in 9 g of the stomach contents.

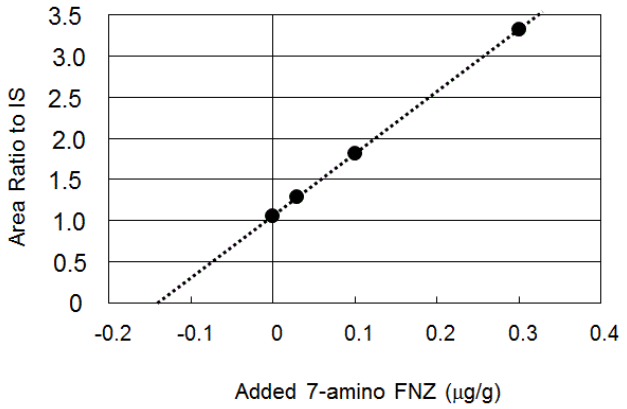


**Figure 3:** Calibration curve of 7-amino FNZ in the method of standard addition.

## Discussion

FNZ is quickly absorbed after oral administration by the small intestine within 20-30 min [6], peaked plasma concentrations in about 1h with an elimination half-life of 13-19 h [6]. The clinical effect varies between 4 and 8 h depending on the dosage given [6]. FNZ is excreted in the urine almost entirely as metabolites, both free and conjugated with glucuronic acid, with less than 1% as unchanged drug [2]. Seven subjects given the 2 mg of FNZ were positive up to 240 h for 7-amino FNZ in their urine [6].

In the present analysis, three-step liquid-liquid extraction and GC/MS apparatus were used. The recent minimum limit of detection of FNZ and 7-amino FNZ in urine was lower than those of the present analysis by the use of solid column extraction and LC-MS-MS system [6]. If their methods could be examined in the present case, the sensitivity in the samples might be improved. However, the present samples were so old that the many degenerated ingredient such as lipids and putrefactive substances could disturb the analysis, so their method was not applied.

The total amount of 7-amino FNZ in the stomach was calculated as 1.26 μg, which corresponds to a small particle of the clinical dose of FNZ. The dose of FNZ ingestion seems to be difficult to assume for the decomposition, but the FNZ ingestion itself was demonstrated in the present case. The degenerations of toxic chemicals and histological structure in the cadaver made the findings obscure during the long elapsed time. While it was difficult to diagnose definitely from the autopsy findings, strangulation was one of the most speculated causes of death in this case and the post mortem interval was estimated as not far from two years.

Post mortem and ante-mortem variation of the levels of FNZ and its metabolite was also reported [3,4,7-9]. In the report describing 44 fatal cases of FNZ [3], FNZ was not detected in their blood, but the concentration range of 7-amino FNZ was between 0.16 μg/ml and 0.64 μg/ml (mean, 0.31 μg/ml) up to 43 h to 158 h after the death. Post mortem and ante-mortem variation of the levels of FNZ and its metabolite was also reported by Robertson and Drummer [7]. In case 1 of the study, the blood FNZ level was 0.07 μg/ml at the ante-mortem term, but at 55 h after his death, FNZ was undetectable and the concentration of 7-amino FNZ was 0.3 μg/ml. In case 2 in the study by Robertson and Drummer [7], FNZ was not detected at the ante-mortem period nor at 158 h post mortem, but 7-amino FNZ was detectable at both sampling times. In the suicidal case of with FNZ, FNZ was 0.065 μg/ml and 7-amino FNZ was 0.34 μg/ml in a post mortem heart blood and 7-amino FNZ was detected in gastric contents, too [8].

The change in the content of FNZ and its metabolite were studied after the FNZ addition to blood [10] or medium [11]. When FNZ was incubated for 8 h in bacterially-contaminated post mortem blood at 22°C [10], only 4% of FNZ remained, and the remaining 96% was converted rapidly to 7-amino FNZ. No other metabolites were detected in the incubation. When FNZ was added to blood under sterile conditions, 75% of FNZ remained on the 28th day of incubation. In contrast to FNZ, 90% of 7-amino FNZ remained after incubation in bacterially contaminated post mortem blood for 45 h and 88% of 7-amino FNZ remained after 28 days' incubation under sterile conditions [10]. Samples incubated with Bacteroides fragilis, Clostridium perfringens and mixed culture resulted in nearly complete conversion of FNZ [11]. Increased 7-amino FNZ concentrations accounted for the majority of the conversion [11]. Therefore, it seems that FNZ is immediately converted to 7-amino FNZ both in bacterially-contaminated blood and medium, and that the level of 7-amino FNZ decreases slowly either in the presence or absence of bacteria.

If the results can apply to the present case, the concentration of FNZ in the bone marrow was so low at the ante-mortem period that 7-amino FNZ was not detectable when it was analyzed approximately two years later. On the other hand, the concentration of FNZ in the stomach contents was presumed to be so high at the ante-mortem period that FNZ was immediately converted to 7-amino FNZ by bacteria, etc. and decomposed slowly during the post mortem interval, so that 7-amino FNZ could be detected even after long time had passed from the death. The possible acidic condition in the post mortem stomach might supply the protective circumstance for the benzodiazepine. This preservative tendency would be enhanced underground where the cadaver was buried, although we couldn't find the enough data applicable to the progress of 7-amino FNZ in the similar condition.

In conclusion, while it is difficult to detect FNZ or FNZ product from the old cadaver, the major FNZ product, 7-amino FNZ was detected in the stomach of a cadaver approximately two years later from death. The data was useful to construct the ante-mortem circumstance of the victim. To our knowledge, such detection of FNZ product in a cadaver after a long period of time has not been reported previously.

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