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原　　報

DETECTION OF BENZODIAZEPINES IN HUMAN URINE BY DIRECT IMMERSION SOLID PHASE MICRO EXTRACTION AND GAS CHROMATOGRAPHY

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直浸固相ミクロ抽出／ガスクロマトグラフィーによる尿中ベンゾジアゼピン系薬剤の検出

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Summary

Direct immersion (DI)-solid phase micro extraction (SPME) has been applied to 13 benzodiazepines spiked to human urine. Urine was mixed with distilled water and sodium chloride in a vial, and an SPME fiber was directly immersed in the sample solution during its stirring for 30 min at room temperature. Immediately after the fiber was pulled out of the vial, the fiber needle was injected into the port of a gas chromatograph with a flame ionization detector. Medazepam, fludiazepam, diazepam, midazolam, flunitrazepam, prazepam, nimetazepam and flurazepam could be extracted under the present DI-SPME conditions, and their recoveries were 1.55−17.0%. Their calibration curves showed linearity up to 1000 ng/ml urine. The detection limit of nimetazepam was 150 ng/ml; those for medazepam, fludiazepam, diazepam, midazolam, flunitrazepam, prazepam and flurazepam were 10−20 ng/ml.

Key words: Solid phase micro extraction (SPME); Direct immersion method; Gas chromatography; Benzodiazepines; Medazepam; Fludiazepam; Diazepam

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Introduction

Benzodiazepines are widely used as sedative or hypnotic drugs, and poisoning cases with them are commonly encountered [1, 2]. Nowadays, the clean-up of benzodiazepines from biological samples is often being made by solid-phase extraction [3−5]. Solid phase micro extraction (SPME) is a new, simple and solvent-free extraction method first introduced by Arthur and Pawliszyn in 1990 [6]. The SPME fiber is either immersed directly in sample solution or exposed in the headspace in a sealed vial to adsorb compounds to be analyzed. The analytes can be thermally desorbed from the fiber in an injector of a gas chromatography (GC) instrument. Recently, some reports have appeared for extraction of cocaine, local anesthetics and amphetamines from biological fluids by direct immersion (DI)-SPME [7−9]. In this study, we present that benzodiazepines can be extracted from human urine by DI-SPME.

Experimental

Materials

Thirteen benzodiazepines were used in this study. Diazepam was obtained from Yamanouchi Pharmaceutical Co., Ltd., Tokyo; fludiazepam, prazepam and nimetazepam from Sumitomo Pharmaceuticals Co., Ltd., Osaka; flurazepam and midazolam from Hoffmann-La Roche Ltd., Basel, Switzerland; medazepam from Shionogi & Co., Ltd., Osaka; flunitrazepam from Eisai Co., Ltd., Tokyo; etizolam from Yoshitomi Pharmaceutical Ind., Ltd., Osaka; estazolam and alprazolam from Takeda Chem. Ind. Co., Ltd., Osaka; triazolam from Pharmacia & Upjohn Inc., London, UK; brotizolam from Dainippon Pharmaceutical Co., Ltd., Osaka. SPME devices and their 65 μm polydimethylsiloxane/divinylbenzene coated SPME fiber assemblies were purchased from Supelco Inc., Bellefonte, PA, USA; an Rtx-5 Amine (5 % diphenyl/95 % dimethyl polysiloxane, crossbond) fused silica capillary column (30 m × 0.32 mm ID, film thickness 1.0 μm) from Restek Corp., Bellefonte, PA, USA. Other common chemicals used were of the highest purity commercially available. Urine was obtained from a healthy subject.

Extraction procedure

One milliliter of urine, with or without addition of 1 μg each of 13 benzodiazepines, was placed in a 2 ml-conical vial containing a small triangle-shaped magnetic stirring bar. To the sample solution, 1 ml of distilled water and 0.5 g of sodium chloride were added. The vial was sealed with a silicone septum cap, and the contents were mixed for 10 s. The septum piercing needle of the SPME fiber holder was passed through the septum, and the SPME fiber was directly immersed into the sample solution for 30 min with stirring. The fiber was retracted into the needle, pulled out of the vial and then immediately inserted into the GC port for

desorption of the benzodiazepines at 300℃ for 2 min.

GC conditions

GC was carried out on an HP-5890 Series II gas chromatograph equipped with flame ionization detection (Hewlett-Packard, Palo Alto, CA, USA) and an Rtx-5 Amine fused silica capillary column. Column temperature was set at 150℃ for 2 min, programmed from 150 to 260℃ at 20℃/min, and then from 260 to 300℃ at 5℃/min. Injector and detector temperature was 300℃ and helium flow rate 3 ml/min. The samples were injected in the splitless mode, and the splitter was opened after 2 min.

Results and discussion

Gas chromatograms for non-extracted authentic compounds (200 ng on column) dissolved in methanol, a DI-SPME extract and its background are shown in Fig. 1. Estazolam, alprazolam, etizolam, triazolam and brotizolam could not be extracted under the present DI-SPME conditions. The eight compounds detected were completely separated on the chromatograms with the Rtx-5 Amine column. Impurity peaks appeared around 260−280℃, but they did not interfere with peaks of the drugs. We had also tested various types of columns, such as Rtx-1, Rtx-35 and Rtx-1701; the Rtx-5 Amine column gave better results than the above columns in separation and tailing.

The recoveries were calculated by comparing the peak areas for the DI-SPME extracts with

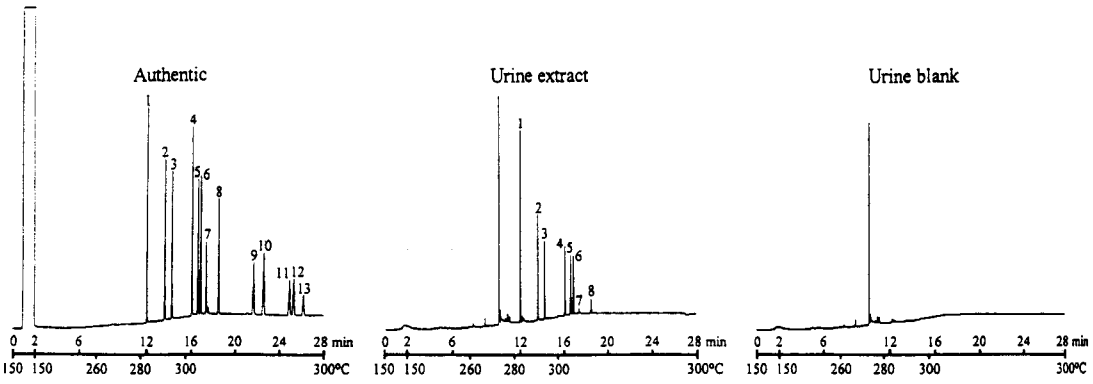

Fig. 1. Gas chromatograms for non-extracted authentic drugs dissolved in methanol (200 ng each on column), an extract of human urine (1 μg/ml for each drug), and its background obtained by DI-SPME/GC. 1: medazepam; 2: fludiazepam; 3: diazepam; 4: midazolam; 5: flunitrazepam; 6: prazepam; 7: nimetazepam; 8: flurazepam; 9: estazolam; 10: alprazolam; 11: etizolam; 12: triazolam; 13: brotizolam.

Table 1. Recoveries for benzodiazepines extracted from human urine by DI-SPME

Compound[a]	Recovery (%)
Medazepam	17.0
Fludiazepam	12.3
Diazepam	10.3
Midazolam	6.50
Flunitrazepam	8.29
Prazepam	8.09
Nimetazepam	1.55
Flurazepam	2.29
Estazolam	ND[b]
Alprazolam	ND
Etizolam	ND
Triazolam	ND
Brotizolam	ND

[a] One microgram of each drug was added to 1 ml of urine.

[b] Not detectable.

those for the non-extracted authentic compounds dissolved in methanol (Table 1). The absolute calibration curves for nimetazepam and flurazepam showed linearity in the range of 200−1000 ng/ml and 100−1000 ng/ml, respectively; those for medazepam, fludiazepam, diazepam, midazolam, flunitrazepam and prazepam were linear in the range of 40−1000 ng/ml. The detection limits were 10 ng/ml for medazepam, fludiazepam and diazepam; 15 ng/ml for midazolam, flunitrazepam and prazepam; 150 ng/ml for nimetazepam; and 20 ng/ml for flurazepam.

Sodium chloride was added to the sample solution in this study. This treatment made recoveries more than two times higher than those without the salt. We also tested other salts, such as sodium bicarbonate, potassium carbonate and ammonium sulfate; the recoveries obtained with ammonium sulfate and potassium carbonate were less than 60 and 7 % of those with sodium chloride, respectively. When sodium bicarbonate was added to the sample solution, the recoveries for medazepam, flunitrazepam, prazepam and flurazepam were 130−220 % of those with sodium chloride; but those for fludiazepam, diazepam, midazolam and nimetazepam were only about 60 % of those obtained with sodium chloride.

In the present study, we have been able to extract eight benzodiazepines from human urine by DI-SPME. To our knowledge, this is the first report to employ SPME for extraction of benzodiazepines. SPME is a simple method which requires no solvents, and cleaner extract is

obtained than that by liquid-liquid or solid-phase extraction. Further studies on SPME are now under way in our laboratories.

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