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Short Communication

Procedure for verification of flunitrazepam and nitrazepam intake by gas chromatographic–mass spectrometric analysis of urine*

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Introduction

Benzodiazepines (BZ) are the most frequently prescribed sedative and hypnotic drugs in the world. These drugs have clearly been shown to produce dependence and can potentially be abused [1, 2]. It is effective to diagnose BZ abuse biochemically by analysing BZ and their metabolites in plasma and in urine [3]. Nitrazepam and flunitrazepam are two BZ derivatives which possess muscle-relaxed properties as well as central sedative effects and are excreted in urine mainly as 7-amino-nitrazepam (7-AN) and 7-aminoflunitrazepam (7-AF) (Fig. 1), respectively [4, 5].

Several methods, such as LC, GC, TLC and GC–MS have been developed to determine nitrazepam and flunitrazepam and their metabolites in body fluid [6–9]. However, most of those methods were applied for measurement in plasma. Few methods have been described to specifically determine 7-AN and 7-AF in urine. Therefore, we undertook to develop method using GC–MS for the determination of 7-AN and 7-AF in human urine, and to use it together with screening by immunoassay to demonstrate that this method is selective and sensitive to detect nitrazepam and flunitrazepam intake.

Materials and Methods

Reagents

7-AN, 7-AF and 7-Amino-1-methyl-clon-

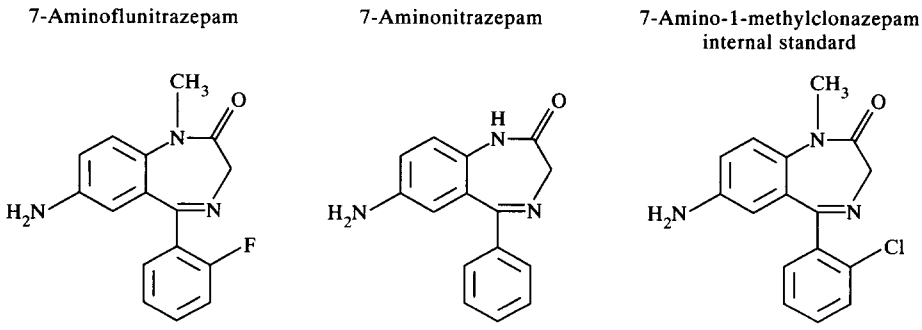

Figure 1
Chemical structures.

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azepam (internal standard) were obtained from Roche (Rocheprodukter AB, Stockholm, Sweden). *E. coli* β-glucuronidase was obtained from Boehringer–Mannheim (Mannheim, Germany) as a solution in 50% glycerol. All solvents used were LC grade or better and obtained from commercial sources.

Extraction procedure

Urine (1 ml) was added to a glass tube containing 5 μmol l^{-1} of 7-amino-1-methyl-clonazepam (25 μl of 200 μmol l^{-1}) and 100 μl β-glucuronidase (4 U). The mixture was incubated at ambient temperature (20–22°C) for 20 min. Thereafter, 1.0 ml of borate buffer (0.6 mol l^{-1}, pH 9.5) was added. Solid-phase extraction was performed with Bond Elut C_2 cartridges (Varian Co. Harbor City, CA, USA) which were conditioned before use with 2 ml of methanol and 2 ml water. The sample was applied with an approximate flow rate of 2 ml min^{-1}. The cartridge was washed sequentially with 2 ml of water and 2 ml of CH_3OH–H_2O (20:80), and dried by aspiration for 1–2 min. The cartridge was eluted with 1 ml of methanol which was evaporated to dryness under nitrogen in a heating block (40°C). The residue was dissolved in 50 μl ethyl acetate.

GC–MS

The GC–MS system consisted of an Incos 50 mass spectrometer (Finnigan Co., San Jose, CA, USA) connected via a heated transfer line (300°C) to a HP5890 gas chromatography (Hewlett–Packard). An aliquot (2 μl) of the final extract was introduced via a "moving needle" injector onto an Rtx®-200 fused silica capillary column (30 m × 0.25 mm, i.d., film thickness of 0.25 μm, Restek Co., Bellefonte, PA, USA). The GC–MS operating conditions were as follow: The injection temperature, 300°C; carrier gas, helium; column temperature, 300°C; ionization energy, 70 eV; ion source temperature, 200°C. The mass spectrometer operated in the selected ion monitoring mode and the following mass numbers were monitored: *m/z* 283 (7-AF), 251 (7-AN), and 299 (internal standard).

Quantitative analysis was achieved by comparing peak area ratios *m/z* 283/299 and 251/299 of unknowns with reference to the calibration curves.

Preparation of calibrators

Urine samples were prepared to contain 7-AN and 7-AF at concentration of 0.2, 0.5, 1, 2, 5, 10, 15, 20 μmol l^{-1}. These samples were extracted as described above and used as calibration samples.

Extraction recovery

Samples containing 7-AN, 7-AF and 7-amino-1-methyl-clonazepam were prepared in urine at 5 and 0.5 μmol l^{-1} and were compared with methanolic solution at the equivalent concentrations. The analytes were measured by LC analysis. The LC apparatus consisted of an LDC-ConstaMetric 3000 pump, LDC-SpectroMonitor 3000 UV detector set at 245 nm. Separation was achieved using a Nucleosil C18 (5 μM, 150 × 4.6 mm i.d.) column. The mobile phase was a mixture of phosphate buffer (pH, 2.1) containing 3 mM sodium dodecyl sulphate–acetonitrile (63:27 v/v). The flow rate was set at 2.0 ml min^{-1}.

Results and Discussion

Chromatograms and mass spectra

Representative selected ion monitoring GC–MS chromatograms of a urine standard containing 500 nmol l^{-1} of 7-AF and 500 nmol l^{-1} of 7-AN as well as internal standard (7-amino-1-methyl-clonazepam) are given in Fig. 2. The retention times for 7-AF, 7-AN and internal standard were about 4.1, 4.4 and 5.1 min, respectively. Major ions and their relative

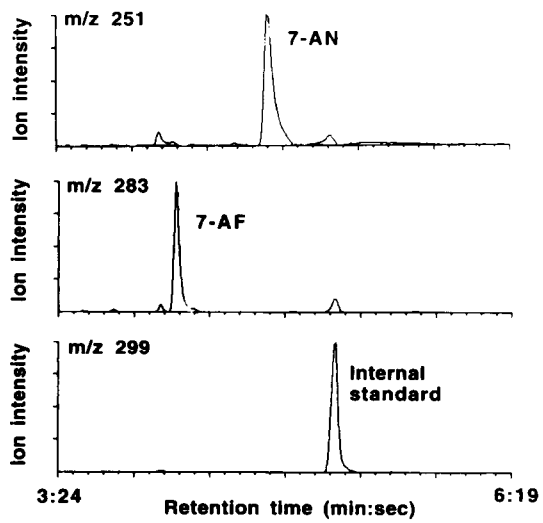

Figure 2
Chromatograms obtained from the GC–MS analysis of a blank urine spiked with 500 nmol l^{-1} of 7-aminoflunitrazepam and 7-aminonitrazepam as well as 5000 nmol l^{-1} of 7-amino-1-methyl-clonazepam (i.s.).

abundance of 7-AF, 7-AN and internal standard are shown in Table 1. Chromatograms obtained from blank human urine demonstrated that no endogenous substances interfered with these compounds (not shown).

Quantitative analysis and linearity

The applied lower limit of quantitation for 7-AN and 7-AF was 0.2 μmol l^{-1} based on the analysis of 1 ml sample. The calibration was linear over the concentration range from 0.2 μmol l^{-1} to 20 μmol l^{-1}. The linear repression equations are following: for 7-AN: $Y = 0.22\ X + 0.025$ ($r^2 = 0.997$); for 7-AF: $Y = 0.44\ X - 0.34$ ($r^2 = 0.997$). The limit of detection ($S/N = 3$) was about 0.01 μmol l^{-1} for 7AF and 0.04 μmol l^{-1} for 7AN. The variability in the quantification of 7-AN and 7-AF is shown in Table 2. These results indicate that the present method possesses a high enough sensitivity to both compounds.

Extraction recovery

Nowadays solid-phase extraction has become an effective approach for isolating the analytes of interest in biological samples. Several studies on the solid-phase extraction of BZs have been reported [10, 11]. When comparing several solid-phase extraction columns, such as CN, C_{18}, C_2, Certify, Certify II, SPEC column, as well as several solvent extraction procedures (Table 3), we found that C_2 solid-phase extraction column is the best one. The recoveries of 7-AF, 7-AN and 7-amino-1-methyl-clonazepam were 97, 97 and 91% at 5 μmol l^{-1} and 96, 94, 86% at 0.5 μmol l^{-1} by using the C_2 cartridge and the present extraction procedure.

Application to clinical samples

A total number of 229 clinical routine urine samples were analysed by the present method and by the fluorescence polarization immunoassay (FPIA) screening method [12, 13], respectively. Among 49 samples which gave immunoresponse below but close to the cut-off limit (corresponding to 100 ng ml^{-1} of nordiazepam), 2 and 22% frequency of positives were found for 7-AN and 7-AF when using our GC–MS method. This result indicates a higher sensitivity for the present method compared with the FPIA method. In 180 samples which were positive in the FPIA screening method, the frequency of positives for 7-AN and 7-AF

Table 1
Mass spectral data (electron ionization) of 7-aminonitrazepam (7-AN), 7-aminoflunitrazepam (7-AF) and 7-amino-1-methyl-clonazepam (7-AMC)

Compound	Molecular ions (relative abundance)	Other abundant ions
7-AF	283 (100%)	255 (80%), 254 (58%), 282 (41%), 207 (38%), 133 (28%), 264 (27%), 240 (15%), 284%(14%)
7-AN	251 (100%)	222 (97%), 223 (75%), 105 (23%), 250 (21%), 195 (17%), 251 (15%), 119 (11%), 111 (11%)
7-AMC (i.s.)	299 (100%)	270 (54%), 264 (45%), 271 (44%), 301 (33%), 300 (31%), 272 (23%), 236 (21%), 256 (13%)

Table 2
Validation of the method

Compound	Concentration (μmol l^{-1})	Mean (μmol l^{-1})	SD	RSD (%)	*n*
Intraday					
7-AN*	5.00	4.95	0.21	4.2	9
	0.50	0.52	0.07	13.5	10
7-AF†	5.00	4.79	0.25	5.2	9
	0.50	0.52	0.05	9.6	10
Interday					
7-AN	5.00	5.55	0.34	6.1	7
7-AF	5.00	5.24	0.34	6.5	7

*7-Aminonitrazepam.
†7-Aminoflunitrazepam.

Table 3
Extraction recovery

Extraction*	Recovery (%) 7-AN	7-AF
C2 cartridge	97	97
CN cartridge	12	25
C18 cartridge	56	90
SPEC C18 cartridge	60	58
Bond Elut Certify	High background/interference	
Bond Elut Certify II	High background/interference	
Solvent extraction	64	63

*Extraction procedure: for C2, Bond Elut Certify and Bond Elut Certify II (Varian Co. USA) extraction procedure was the reported procedure. Other solid-phase extractions were performed as above with modification of washing step. For CN cartridge (Varian Co. USA), using 2 ml 10 mmol l^{-1} borate buffer (pH, 9.5)–methanol (95:5) and 2 ml water. For C18 cartridge (Varian Co. USA), using 2 ml CH_3CH–H_2O (20:80) and 2 ml H_2O, eluated with 1 ml CH_3CN instead of 1 ml methanol. For SPEC cartridge (ANSYS Inc. CA, USA), using 2 ml H_2O and 2 ml CH_3CN–H_2O (15:85). Solvent extraction procedure: 1 ml urine was added to 0.5 ml borate buffer (0.6 mol l^{-1}, pH 9.5) and extracted with 4 ml ethyl acetate–haptane (85:15), the organic layer was evaporated to dryness at 40°C, then dissolved in 150 µl mobile phase.

were 9 and 39%, respectively determined by the present method, indicating a frequent use of these two compounds.

Conclusion

In conclusion, a method with adequate sensitivity and specificity suitable for the identification and quantitation of flunitrazepam and nitrazepam metabolites in clinical urine samples has been developed.

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