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SCREENING OF BENZODIAZEPINES IN URINE BY THE IMMUNOASSAY AND QUANTITATION BY GC-NPD METHOD

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ABSTRACT: *We developed a simple method to determine benzodiazepines in biological samples using electron capture detectors and nitrogen-phosphorous detectors. The extraction of 13 benzodiazepines in urine at pH 9.5 with toluene and its analysis in GC/NPD showed the peaks in 9-16 min. In this retention time range, the biological background was fairly low and the drugs could be identified in low concentrations. The benzodiazepines in urine samples were screened by the fluorescence polarization immunoassay and positive samples were confirmed by the GC/NPD method. The results showed that benzodiazepines in addition to its metabolites were easily identified as oxazepam, nordiazepam or medazepam by the GC/NPD method. This procedure is simple and sensitive and can be used in the screening of benzodiazepines for quick identification and quantitation of individual benzodiazepines after a preliminary screening by the immunoassay method.*
Key words: *Benzodiazepine assay, GC-NDP method, Fluorescence polarization immunoassay*

INTRODUCTION

Extensive studies are reported on the detection of benzodiazepines in biological fluids. For the screening of benzodiazepines in accidental poisoning or drug addict cases, currently, the immunoassay methods are widely used (Poklis, 1981; Slightom *et al.*, 1982; Jolley *et al.*, 1981). The fluorescence polarization immunoassay (FPIA) and the enzyme multiplied immunoassay techniques (EMIT) are very simple and sensitive methods, but they cannot be used for the identification of individual drugs.

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For chromatographic identification, TLC (Meola *et al.*, 1981; Kaisha and Tadrus, 1978), GLC (Chiarotti *et al.*, 1986; Baselt *et al.*, 1977) and HPLC (Brodie *et al.*, 1978; Peat and Kopjak, 1979) are employed, but sensitivity is generally lower than the immunoassays. To increase the sensitivity, electron capture detectors (ECD) (Zingales, 1971; Pacifici and Placidi, 1977) and nitrogen-phosphorous detectors (NPD) (Dhar and Kult, 1979; Vandemark and Adams, 1977) are also examined. These methods are useful for pharmacolinetic studies of individual benzodiazepines or identification in overdose cases, but they are not used in the screening of benzodiazepines. Also, the acid hydrolysis of benzodiazepines to benzophenones is used for screening (Hailey, 1974). But it is quite complicated since different kinds of benzophenones are formed.

This paper describes a simple and sensitive chromatographic method for the extraction and identification of benzodiazepines using the GLC system with a nitrogen-phosphorus detector.

MATERIALS AND METHODS

Standard and Chemicals

Pure chemical standards of clobazam [7-chloro-1-methyl-5-phenyl-1H-1,5-benzodiazepin-2,4 (3H, 5H)-dione], fludiazepam [7-chloro-5-(2-fluorophenyl) 1,3-dihydro-1-methyl-2H-1,4-benzodiazepin-2-one], oxazepam [7-chloro-1,3-dihydro-3-hydroxy-5-phenyl-2H-1,4-benzodiazepin-2-one], pinzepam [7-chloro-1,3-dihydro-5-phenyl-1-(2-propyl)-2H-1,4-benzodiazepin-2-one], flunitrazepam [5-(2-fluorophenyl)-1,3-dihydro-1-methyl-7-nitro-2H-1,4-benzodiazepin-2-one], diazepam [7-chloro-1,3-dihydro-1-methyl-5-phenyl-2H-1,4-benzodiazepin-2-one], nordiazepam [7-chloro-1,3-dihydro-5-phenyl-1 (2H)-1,4-benzodiazepin-2-one], chlordiazepoxide [7-chloro-N-methyl-5-phenyl-3H-1,4-benzodiazepin-2-amine-4-oxide], lorazepam [7-chloro-5-(2-chlorophenyl)-1,3-dihydro-3-hydroxy-2H-1,4-benzodiazepin-2-one], bromazepam [7-bromo-1,3-dihydro-5-(2-pyridinyl)-2H-1, 4-benzodiazepin-2-one, flurazepam [7-chloro-1-[2-(diethylamino) ethyl]-5-(2-fluorophenyl)-1,3-dihydro-2H-1,4-benzodiazepin-2-one], nitrazepam [1,3-dihydro-7-nitro-5-phenyl-2H-1,4-benzodiazepin-2-one], medazepam [7-chloro-2,3-dihydro-1-methyl-5-phenyl-1H-1,4-benzodiazepine], Prazepam [7-chloro-1-(cyclopropylmethyl)-1,3-dihydro-5-phenyl-2H-1,4-benzodiazepin-2-one] were obtained from commercial products by methanolic extraction.

Other solvents and chemicals were of analytical grade and used without purification.

Instrumentations

A Hewlett-Packard 5890 gas chromatograph equipped with a nitrogenphosphorous detector (NPD) was used for the determination of drugs in urine. The chromatographic column was a cross-linked 5% phenylmethyl silicon capollary (length 16 m, I.D. 0.2 mm, film thickness 0.33 μm). Helium at 0.75 ml/min was used as a carrier gas. The auxiliary gases were helium at 50 ml/min and He/Air at 5.4/6.0 ml/min. Samples were injected in the split mode (split ratio, 1 : 13).

Injector temperature was 280℃ and detector temperature was 300℃. Oven temperature was started at 150℃ and ramped 10℃/min to 250℃ and held for 3 min, then ramped 25℃/min.

Extraction Procedure for Benzodiazepines

Urine (5 m*l*) was adjusted to a pH value of 9.5± 0.1 with a sodium carbonate buffer. The sample was extracted with 8 m*l* of toluene by mechanical shaking for 20 minutes (Pacifici and Placidi, 1977). The two phases were separated by spinning for 5 minutes. The organic phase was transferred to a 20 m*l* glass stoppered tube, and the solvent was evaporated to dryness in a vacuo. The dry residue was dissolved in 40 μ*l* of methanol. The solution was analysed in GC/NPD.

Calibration of Diazepam

Diazepam was spiked into the blank urine to give concentrations of 5, 10, 20, 50 and 100 ng/m*l*. The internal standard fludiazepam was also spiked to each sample at a concentration of 20 ng/m*l*.

Screening of Benzodiazepines and Its Metabolites by the TDx Analyzer

The analyzer (Abbott, Labs, Chicago, U.S.A.) and the following reagents were obtained from Abbott Labs.

Calibrators (Nordiazepam): 6 levels
Controls: 2 levels
Bar-code reagent pack:
Drug antiserum: sheep antiserum in buffer
Fluorescein tracer (F*): Fluorescein labeled drug
Pretreatment solution: Protein and NaN_3 in buffer
Buffer (Wash solution): Protein and NaN_3 in buffer

The urine analysis was performed according to the instruction manual using a TDx analyzer. Approximately 100 μ*l* of each sample was applied to the sample cup in the carousel.

Prior to the assay, the analyzers were calibratred by determining the polarization intensity of the pipet check solution. The assay parameters of benzodiazepines were stored according to the manual and the standard curve for each group of drugs was established using 6 levels of nordiazepam solution. To validate the assay values in the sample, two levels of controls were included in each run of the assay.

RESULT AND DISCUSSION

Detection of Benzodiazepines

Chromatograms of blank urine and extracts of spiked drugs are presented in Fig. 1. In blank urine, there were no interfering peaks in the retention time of 9-16 min where the benzodiazepines examined showed their peaks. the retention times of the benzodiazepine drugs are listed in Table 1.

The extraction yield of this procedure was over 85% at 80 ng/m*l*. Only chlorodiazepoxide had a slightly lower yield than the others as shown in Table 2.

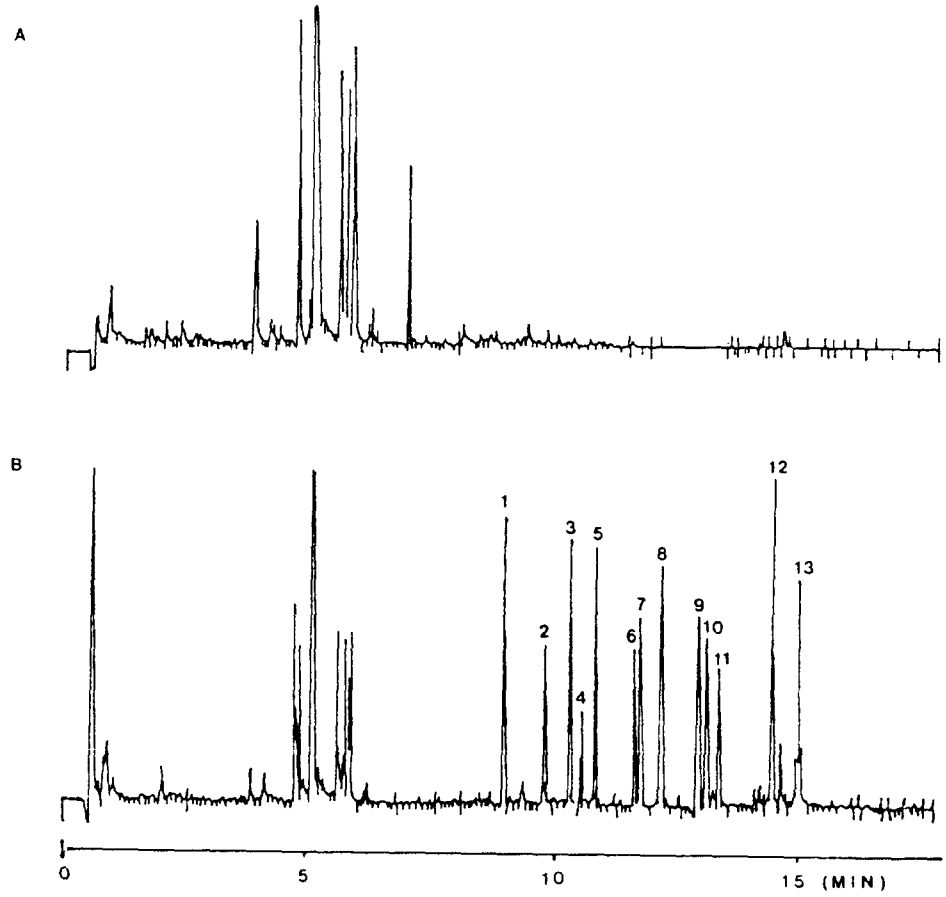

Fig. 1. Gas chromatograms of the extract from (A) blank urine and (B) urine spiked with 80 ng/ml of each benzodiazepine drugs; 1. medazepam, 2. oxazepam, 3. fludiazepam, 4. lorazepam, 5. diazepam, 6. chlordiazepoxide, 7. pinazepam, 8. clobazam, 9. flunitrazepam, 10. brmazepam, 11. prazepam, 12. flurazepam, 13. clonazepam.

Table 1. Retention times of benzodiazepine

Drugs	Retention time (min.)
Medazepam	9.04
Oxazepam	9.87
Fludiazepam	10.38
Lorazepam	10.62
Diazepane	10.90
Chlordiazepoxide	11.75
Pinazepam	11.86
Clobazam	12.31
Flunitrazepam	13.09
Bromazepam	13.26
Prazepam	13.51
Flurazepam	14.61
Clonazepam	15.17

To extract the conjugated metabolites of benzodiazepines, glucuronidase-hydrolyzed urine samples were examined but the biological backgrounds of blank urine samples were quite complicated.

The detection limit of this method was ca 1 ng/ml for most drugs except oxazepam, lorazepam, bromazepam and clonazepam.

In TDx analysis, the threshold concentration for positive samples is considered to be 80 ng/ml.

Screening of Benzodiazepine Positive Samples

Eleven benzodiazepine positive samples from the Olympic Games were analyzed using this method. These samples showed TDx benzodiazepine assay values higher than 200 ng/ml. Eight of these were confirmed to contain diazepam, nordiazepam, oxazepam and medazepam as shown in Table 3. Each sample was further confirmed by GC/MS.

Table 2. Extraction yield of benzodiazepines (n=3)

Drugs	Extraction yield (%)	RSD (%)
Medazepam	93.6	1.0
Qxazepam	96.4	4.2
Fludiazepam	96.9	1.1
Lorazepam	86.6	4.4
Diazepam	97.8	2.6
Chlordiazepoxide	74.6	0.7
Pinazepam	94.4	0.8
Chlobazam	94.3	0.8
Flunitrazepam	90.2	1.1
Bromazepam	106.2	2.3
Prazepam	87.5	1.3
Flurazepam	101.2	1.1
Clonazepam	103.6	1.8

Table 3. GC-MS findings of TDx benzodiazepine-positive sample

Case	TDx value (ng/ml)	GC/MS Confirmation
1	592	Unknown
2	518	Nordiazepam, diazepam, oxazepam
3	407	Oxazepam
4	332	Oxazepam
5	294	Nordiazepam
6	267	Nordiazepam
7	252	Nordiazepam, medazepam
8	251	Nordiazepam
9	232	Nordiazepam, diazepam
10	229	Unknown
11	214	Unknown

Quantitation of Diazepam

The linearity of diazepam calculated from the calibration graph was good ($r=0.999$, $n=3$). The reproducibility of the method was tested with five urine samples each containing 30 ng/ml of diazepam. The amount found was 31.15 ± 1.3 ng/ml and the precision was 4.1%.

Four diazepam positive samples from athletes were quantitatively analyzed by this method. The concentrations of the samples ranged from 10 ng/ml to 50 ng/ml and the relative standard deviation (RSD) values were lower than 4% (Table 4). One typical chromatogram is shown in Fig. 2.

Table 4. Concentration of diazepam in urine analyzed by GC/NPD

Samples	Concentration of diazepam (ng/ml)	RSD (%)
1	44.5 ± 2.4	0.2
2	12.6 ± 0.1	0.5
3	48.6 ± 0.6	1.2
4	12.1 ± 0.5	3.7

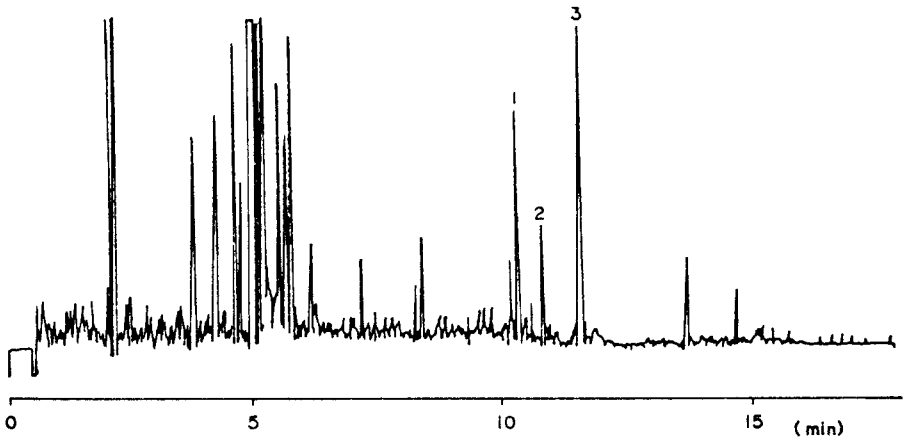

Fig. 2. Gas chromatogram of benzodiazepine positive urine which was pre-screened by TDx.; 1. fludiazepam (ISTD), 2. diazepam and 3. nordiazepam.

CONCLUSION

The extraction of 13 benzodiazepines in urine at pH 9.5 with toluene and its analysis in GC/NPD showed the peaks in 9-16 min. In this retention time range, the biological background was low and the drugs could be identified in low concentration. When benzodiazepine positive samples were examined by this method, diazepam in addition to its metabolite nordiazepam, was easily identified.

This procedure is simple and sensitive and can be used in the screening of benzodiazepines or for quick identification and quantitation of individual benzodiazepines after preliminary screening by the immunoassay method.

REFERENCES

Baselt, R.C., Stewart, C.B. and Franch, S.J. (1977): Toxicological determination of benzodiazepines in biological fluids and tissues by flame-ionization gas chromatography, *J. Anal. Toxicol.*, **1**, 10-13.

Brodie, R.R., Chasseaud, L.F. and Taylor, T. (1978): High-performance liquid chromatographic determination of benzodiazepines in human plasma, *J. Chromatogr.*, **150**, 361-366.

Chiarotti, M., de Giovanni, N. and Fiori, A. (1986): Analysis of benzodiazepines I. Chromatographic identification, *J. Chromatogr.*, **358**, 169-178.

Dhar, A. and Kult, H. (1979): Monitoring diazepam and desmethyldiazepam concentrations in plasma by gas-liquid chromatography, with use of a nitrogen-sensitive detector, *Clin. Chem.*, **25**, 137-140.

Hailey, D.M. (1974): Chromatography of the 1,4-benzodiarepines, *J. Chromatogr.*, **98**, 527-68.

Jolley, M.E., Stroups, S.D., Schwenzer, K.S., Wang, C.J., Lu-Steffes, M., Hill, H.D., Popelka, S.R., Holen, J.T. and Kelso, D.M. (1981): Fluorescence polarization immunoassay, III. An automated system for therapeutic drug determination, *Clin. Chem.*, **27**, 1575-1579.

Kaisha, K.K. and Tadrus, R. (1978): Thin-layer detection of diazepam and/or chlordiazepoxide alone or in combination with major drugs of abuse in drug abuse urine screening programs, *J. Chromatogr.*, **154**., 211-218.

Meola, J.M., Rosano, T.G. and Swift, T. (1981): Thin-layer chromatography, with fluorescence detection, of benzodiazepines and tricyclic antidepressants in serum from emergency-room patients, *Clin. Chem.*, **27**, 1254-1255.

Pacifici, G.M. and Placidi, G.F. (1977): Rapid and sensitive electrocapture gas chromatographic method for the determination of pinazepam and its emtabolite sin human plasma, urine and milk. *J. Chromatogr.*, **135**., 133-139.

Peat, M.A. and Kopjak, L. (1979): The screening and quantitation of diazepma, flurazepam, chlordizaepoxide and their metabolites in blood and plasma by electron-capture gas chromatography and High Pressure liquid chromatography, *J. Forensic Sciences*, **24**, 46-54.

Poklis, A. (1981): An Evaluation of EMIT-dau benzodiazepine metabolite assay for urine drug screening, *J. Anal. Toxicol.*, **5**, 174-176.

Slightom, E.L., Cagle, J.C., McCurdy, H.H. and castagna (1982): Direct and indirect homogeneous enzyme immunoassay of benzodiazepines in biological fluids and tissues, *J. Anal. Toxicol.*, **6**, 22-25.

Vandemark, F.L. and Adams, R.F. (1977): Determination of some benzodiazepines in serum utilizing gas chromatography and nitrogen detector, *Chromatogr. Newsletter*, **5**, 4-7.

Zingales, I.A. (1971): Determination of chlordiazepoxide plasma concentrations by election capture gas-liquid chromatography, *J. Chromatogr.*, **61**, 237-252.