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SENSITIVE DETERMINATION OF DIAZEPAM AND N-DESMETHYLDIAZEPAM IN HUMAN MATERIAL USING CAPILLARY GAS CHROMATOGRAPHY–MASS SPECTROMETRY

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SUMMARY

A reliable and sensitive capillary gas chromatographic–mass spectrometric method was developed for the detection and determination of diazepam and its major metabolite, N-desmethyldiazepam, in human material. Medazepam served as the internal standard. Quantitative determination was achieved using mass fragmentography with selected ions of *m/z* 256 for diazepam and *m/z* 242 for N-desmethyldiazepam and medazepam. The limit of detection was 1 ng/g and the recoveries were 98.54 ± 3.95% for diazepam and 98.66 ± 6.48% for N-desmethyldiazepam. The calibration graph was linear over the concentration range from 1.0 ng/g to 1.0 μg/g for diazepam and N-desmethyldiazepam. Using this method, trace amounts of diazepam and N-desmethyldiazepam were detected in the tissues of an autopsied individual.

INTRODUCTION

Diazepam, a benzodiazepine tranquillizer, is widely prescribed as an antianxiety, antispasmodic and antiepileptic drug, and has received much attention in toxicological analyses for forensic purposes. Reports on the determination of diazepam and its metabolites by high-performance liquid chromatography (HPLC) [1–5], gas chromatography (GC) with electron-capture detection (ECD) [6–8] and gas chromatography–mass spectrometry [9] have been published. Most of these studies were focused on the determination of these substances in biological fluids for clinical purposes and the sensitivity of the methods used did not make feasible detection of minute amounts of the drug in biological materials. We have therefore devised a more sensitive and reliable method.

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EXPERIMENTAL

Reagents

Diazepam and N-desmethyldiazepam were provided by Takeda Chemical Industries (Osaka, Japan) and medazepam by Shionogi Pharmaceutical Industries (Osaka, Japan). A buffer solution of pH 9 was prepared by mixing 21.3 ml of 0.1 *M* sodium hydroxide solution with 50 ml of each of 0.1 *M* boric acid and 0.1 *M* potassium chloride solution and diluting to 100 ml with distilled water. Ethyl acetate and *tert.*-butyl methyl ether were of analytical-reagent grade and were purified by distillation. Bromothymol blue indicator solution was purchased from Ishizu Seiyaku (Osaka, Japan). A Shimadzu CBP1 capillary column was purchased from Shimadzu (Kyoto, Japan). Other chemicals used were analytical-reagent grade.

Biological samples

Human tissue and blood samples were obtained at autopsy and kept at −20°C until analysis. Outdated whole blood was obtained from a blood bank and was used to provide control samples.

Standard solutions of diazepam, N-desmethyldiazepam and internal standard (I.S.)

Diazepam (1 mg) was dissolved in *n*-butyl acetate and the volume was adjusted to 10 ml with *n*-butyl acetate to give a concentration of 100 ng/μl. This solution was further diluted to give concentrations of 10 and 1 ng/μl. Standard solutions of N-desmethyldiazepam and the I.S. were prepared in the same manner.

Extraction procedure

The method of extracting medazepam, diazepam and nitrazepam reported by Greaves [10] was modified. Approximately 1.0 g of whole blood or tissues was weighed and homogenized in a mixture of 5 ml of borate buffer (pH 9.0) and 1 μl of I.S. solution (100 ng of medazepam) in a 30-ml centrifuge tube. 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 solvent layer was transferred into a 30-ml centrifuge tube containing 2.0 ml of 2 *M* hydrochloric acid. The mixture was then shaken for 10 min and centrifuged at 850 *g* for 20 min. To the acid layer was added *tert.*-butyl methyl ether (5 ml) and the mixture was shaken for 10 min and centrifuged at 850 *g* for 20 min to remove lipid materials from the acid phase. The aqueous layer was transferred into a 10-ml centrifuge tube containing 2 drops of bromothymol blue solution (0.04%) as indicator and the mixture was made alkaline by adding 2 *M* sodium hydroxide solution until the indicator turned blue (pH ≈8). To the solution were 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 sulphate and evaporated. The residue was dissolved in 40 μl of ethyl acetate and a 2-μl aliquot of the solution was injected into the gas chromatograph–mass spectrometer. The extraction procedure is summarized in Fig. 1.

Sample (1 g)
| 1 Add borate buffer (5 ml, pH 9.0) and I.S. (100 ng)
| 2 Homogenize
| 3 Add *tert.*-butyl methyl ether (10 ml)
| 4 Shake (10 min) and centrifuge (850 *g*, 20 min)
|
Organic layer
| 1 Add 2 *M* hydrochloric acid (2 ml)
| 2 Shake and centrifuge
|
Aqueous layer
| 1 Add *tert.*-butyl methyl ether (5 ml)
| 2 Shake and centrifuge
|
Aqueous layer
| 1 Add bromothymol blue indicator (2 drops)
| 2 Add 2 *M* sodium hydroxide solution until the indicator turns blue (pH ≈8)
| 3 Add *tert.*-butyl methyl ether (2 ml)
| 4 Shake and centrifuge
|
Organic layer
| 1 Dry with sodium sulphate
| 2 Evaporate and dissolve in ethyl acetate (40 µl)
|
Ethyl acetate (2 µl)
|
|
GC–MS

Fig. 1. Extraction procedure for diazepam and N-desmethyldiazepam.

Preparation of calibration graphs

Whole blood samples were prepared to contain diazepam and N-desmethyldiazepam at concentrations of 1, 5, 10, 50, 100 and 1000 ng/g, each containing 100 ng/g medazepam. These samples were extracted in the same manner as described above. Calibration graphs were obtained by plotting the peak-area ratio of diazepam (or N-desmethyldiazepam) to medazepam versus the amount of diazepam (or N-desmethyldiazepam), measured by a data system built into the gas chromatograph–mass spectrometer.

GC–MS conditions

The apparatus used was a Shimadzu QP-1000 gas chromatograph–mass spectrometer equipped with a multiple-ion detector. The fused-silica capillary column (10 m×0.2 mm I.D., 0.25 µm film thickness) was coated with Shimadzu CBP1 bonded methylsilicone stationary phase. The carrier gas was helium at an inlet pressure of 1.2 kg/cm^2. Sample introduction was via an all-glass falling-needle injector. The operating temperatures were as follows: column, 200–300 °C (25 °C/min); injection port, 300 °C; separator, 300 °C; ion source, 300 °C. The ionization energy was 70 eV. Multiple-ion detectors were set to the ions at m/z 242 and 256.

RESULTS AND DISCUSSION

Extraction procedure

A single extraction yielded many interfering peaks on the gas chromatogram, especially in tissue analysis. This problem was overcome by back-extraction and selective ion monitoring in GC–MS.

We substituted *tert.*-butyl methyl ether for diethyl ether as the extraction solvent. Its advantages are that all undesirable effects due to oxidation products such as peroxide present in diethyl ether were prevented and time was saved.

Determination of diazepam and N-desmethyldiazepam by GC–MS

The mass spectra of diazepam, N-desmethyldiazepam and medazepam obtained by GC–MS are shown in Fig. 2. Diazepam showed a base ion at m/z 256 and a molecular ion at m/z 284, N-desmethyldiazepam a base ion at m/z 242 and a molecular ion at m/z 270 and medazepam a base ion at m/z 242 and a molecular ion at m/z 270. These major ions were checked on each examination. The ions at m/z 256 for diazepam and m/z 242 for N-desmethyldiazepam and medazepam were selected as the appropriate ions for mass fragmentography. The mass frag-

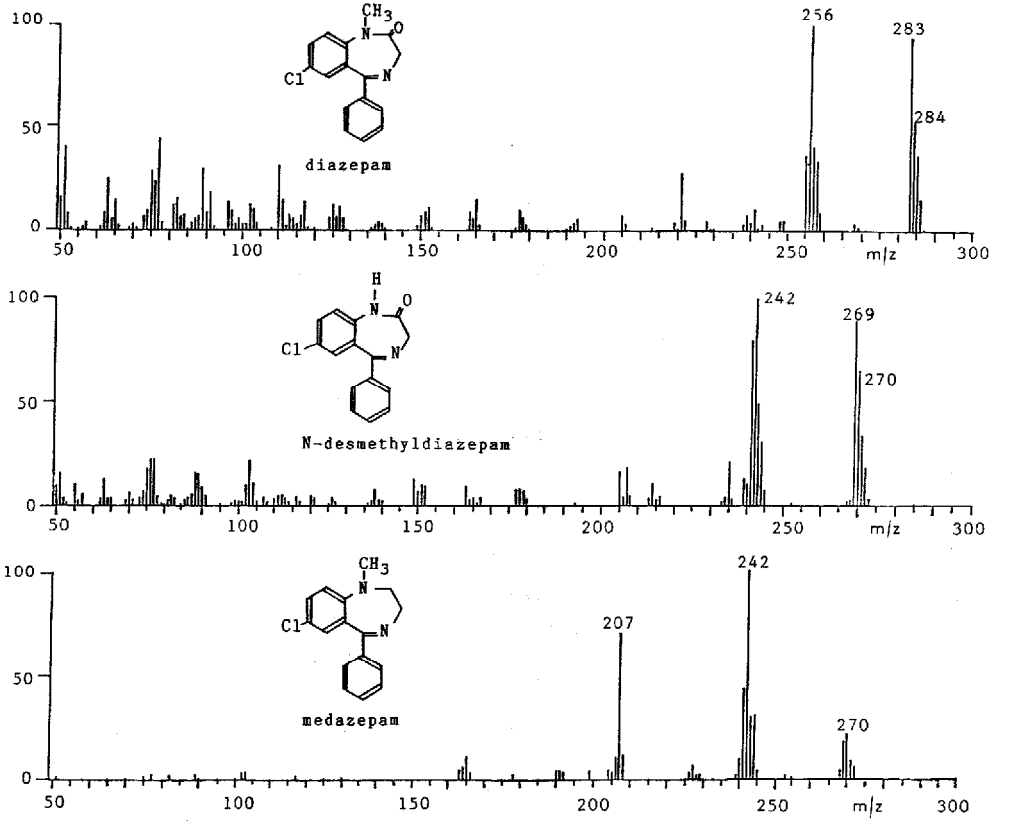

Fig. 2. Mass spectra of diazepam, N-desmethyldiazepam and medazepam.

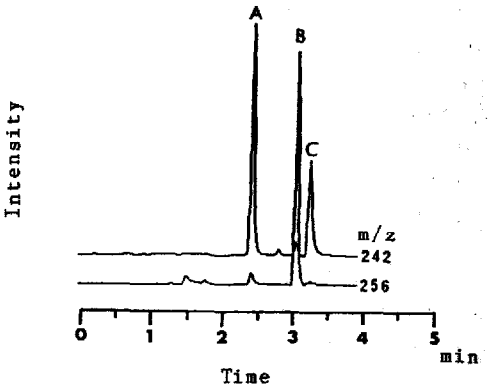

Fig. 3. Mass fragmentograms of an extract from blood containing 100 ng/g each of (B) diazepam, (C) N-desmethyldiazepam and (A) I.S.

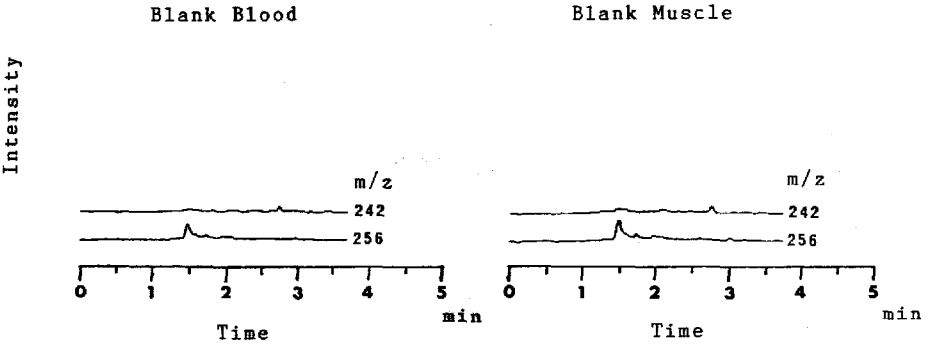

Fig. 4. Mass fragmentograms of an extract from drug-free whole blood and skeletal muscle.

TABLE I

PRECISION DATA FOR DIAZEPAM AND N-DESMETHYLDIAZEPAM IN WHOLE BLOOD

Drug	Within-day ($n=5$)		Between-day ($n=5$)	
	Concentration (mean ± S.D.) (ng/g)	C.V. (%)	Concentration (mean ± S.D.) (ng/g)	C.V. (%)
Diazepam	10.062 ± 1.140	11.3	9.366 ± 0.812	8.7
	52.220 ± 4.998	9.6	50.993 ± 5.703	11.2
	98.544 ± 3.948	4.0	97.460 ± 6.036	6.2
N-Desmethyldiazepam	10.027 ± 0.952	9.5	10.666 ± 0.786	6.7
	51.752 ± 1.701	3.3	48.109 ± 4.528	9.4
	98.658 ± 6.478	6.6	100.313 ± 7.361	7.3

mentogram of the extract from a blood sample in which 100 ng each of diazepam, N-desmethyldiazepam and I.S. were present is shown in Fig. 3. Each peak was clearly separated on the mass fragmentogram. No or few interfering peaks appeared in the mass fragmentograms of the diazepam-free human tissues and fluids, as shown in Fig. 4. Owing to the lower activity of the fused-silica capillary column compared with a packed column, the detection of trace amounts of N-desmethyldiazepam was feasible with no derivatization [8] of the drug or deactivation [6,7] of the column.

The calibration graphs for diazepam and N-desmethyldiazepam were linear in the concentration range from 1 ng/g to at least 1 μg/g, with correlation coefficients of 0.997 and 0.996, respectively. The limit of detection was about 50 pg for diazepam and 200 pg for N-desmethyldiazepam, as pure substances. The calculated recoveries at a concentration of 100 ng/g were $98.54 \pm 3.95\%$ for diazepam and $98.66 \pm 6.48\%$ for N-desmethyldiazepam.

Within-day and between-day precisions were obtained using three different concentrations (10, 50 and 100 ng/g) by adding diazepam and N-desmethyldiazepam to the blank blood. The coefficients of variation (C.V.) for these compounds ranged from 3.3 to 11.3% for the within-day and from 6.2 to 11.2% for the between-day precision (see Table I).

Practical application

A toxicological examination was required on an 80-year-old man who was apparently murdered. The victim was allegedly given a number of tablets, including diazepam, by the suspects, under the pretence that the tablets would stimulate sexual potency.

No drug was found in the blood by routine techniques involving the use of GC and GC–MS with a packed column system. As evidence of the presence of diazepam was the key to the solution of the case, we used the method described here to examine the body tissues. Diazepam was indeed present in the body of the victim, at concentrations of 8.0, 24.9 and 35.7 ng/g, and N-desmethyldiazepam at concentrations of 9.3, 18.9 and 90.6 ng/g in the blood, skeletal muscle and liver, respectively. These findings led to prosecution of the suspects.

CONCLUSION

A sensitive and specific GC–MS assay was developed for the trace determination of diazepam and its major metabolite, N-desmethyldiazepam, that is applicable to body tissues and fluids obtained at autopsy. This method made feasible the detection of 1 ng/g drug.

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