TECHNICAL NOTE

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Rapid Isolation of Benzodiazepines with Sep-Pak® C_{18} Cartridges

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ABSTRACT: A rapid method for isolation of various benzodiazepines from human samples with Sep-Pak® C_{18} cartridges before wide-bore capillary gas chromatography is described. The drugs dissolved in alkaline samples were directly applied to the cartridges and eluted with hexane/isopropanol (9:1). The recoveries were excellent for all drugs in urine samples, but were somewhat lower for some drugs in plasma samples. The latter problem could be easily circumvented by using a deproteinization process before their application to the cartridge. We can recommend the Sep-Pak C_{18} cartridges for isolation of benzodiazepines because of their simplicity and rapidity.

KEYWORDS: toxicology, benzodiazepines, Sep-Pak® C_{18} cartridges, chromatographic analysis, wide-bore capillary gas chromatography

Benzodiazepines are now the bestsellers in pharmaceutical markets in the world and used as antianxietics, hypnotics, and antiepileptics. The drugs are most frequently encountered in forensic chemistry and clinical toxicology.

In this paper, we demonstrate a simple and rapid isolation method using Sep-Pak® C_{18} cartridges for benzodiazepines before wide-bore capillary gas chromatography (GC).

Materials and Methods

Materials

Twenty-two benzodiazepines were used in our experiments. Diazepam and chlordiazepoxide were obtained from Yamanouchi Pharmaceutical Co., Ltd., Tokyo; fludiazepam, prazepam, nimetazepam, and clonazepam from Sumitomo Pharmaceutical Co., Ltd., Osaka; flurazepam from Hoffmann-La Roche & Co., AG, Basel, Switzerland; lorazepam from Wyeth Labs., Philadelphia; dipotassium clorazepate from Dainippon Pharmaceutical Co., Ltd., Osaka; medazepam and nitrazepam from Shionogi & Co., Ltd., Osaka; flunitrazepam

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and bromazepam from Eisai Co., Ltd., Tokyo; clotiazepam and etizolam from Yoshitomi Pharmaceutical Industries Ltd., Osaka; tofisopam from Mochida Pharmaceutical Co., Ltd., Tokyo; oxazolam, mexazolam, and haloxazolam from Sankyo Co., Ltd., Tokyo; estazolam and alprazolam from Takeda Chem. Ind. Co., Ltd., Osaka; and triazolam from The Upjohn Co., Kalamazoo, Michigan. Sep-Pak C_{18} cartridges were purchased from Waters Associates, Milford, Massachusetts; fused silica wide-bore capillary columns (SPB-1, 15-m by 0.53-mm inside diameter (ID), film thickness 1.5 μm; and HP-17, 10-m by 0.53-mm ID, film thickness 2.0 μm) from Supelco, Inc., Bellefonte, Pennsylvania and Hewlett-Packard Co., Palo Alto, California, respectively. Other common chemicals used were of the highest purity commercially available.

The urine and plasma obtained from healthy subjects were also used.

Isolation of Benzodiazepines

Isolation of benzodiazepines from biological impurities with use of Sep-Pak C_{18} cartridges was made essentially according to the method of Narasimhachari [*1*], which had been used for tricyclic antidepressants.

To 1 mL of urine or plasma containing benzodiazepines was added 1 mL of carbonate buffer solution (pH 9.8, 5 g of sodium carbonate (Na_2CO_3) and 5 g of sodium bicarbonate ($NaHCO_3$) dissolved in 100 mL of water). After activating a Sep-Pak C_{18} cartridge by passing 20 mL of acetonitrile and then 20 mL of water, the sample solution was poured into the cartridge at a flow rate not greater than 5 mL/min. The sample tube was rinsed with 2 mL of water, which was also poured into the same cartridge. The cartridge was washed with 10 mL of water; finally 10 mL of hexane/isopropanol (9:1) was passed through it to elute benzodiazepines and collected in a 15-mL glass test tube. The eluate consisted of a major amount of an organic layer and a small amount of an aqueous layer; the latter was discarded by aspiration with a Pasteur pipet. The organic layer was evaporated to dryness under a stream of nitrogen and the residue dissolved in 100 μL of methanol. The 1-μL aliquot of it was subjected to GC analysis. The Sep-Pak cartridge could be reused several times by repeating washings with 20 mL of acetonitrile and 20 mL of water.

When the recovery of a benzodiazepine added to plasma was unsatisfactory, a deproteinization process was used before loading on the Sep-Pak cartridge. To 1 mL of plasma containing benzodiazepines was added 9 mL of 0.4*N* perchloric acid solution. After mixing on a Vortex-type mixer, it was centrifuged at 3000 rpm for 5 min. The pH of the clear supernatant was brought to 9 to 10 by adding 1*N* NaOH solution; the resulting solution was poured into the cartridge and the following procedure was exactly the same as described above.

GC Conditions

GC was carried out on a Shimadzu GC-4CM instrument with the use of two different fused silica wide-bore capillary columns (SPB-1, 15-m by 0.53-mm ID, film thickness 1.5 μm; and HP-17, 10-m by 0.53-mm ID, film thickness 2.0 μm), with flame ionization detection. The GC conditions were: injection temperature 260°C and column temperature 180 to 250°C (5°C/min) for the SPB-1 column; injection temperature 290°C and column temperature 210 to 280°C (5°C/min) for the HP-17 column; and nitrogen flow rate 20 mL/min for both columns.

Results

Figures 1 and 2 show gas chromatograms obtained with nonpolar (SPB-1) and intermediately polar (HP-17) wide-bore capillary columns, respectively, for 22 benzodiazepines, which had been added to urine or plasma and directly extracted with Sep-Pak C_{18} cartridges with-

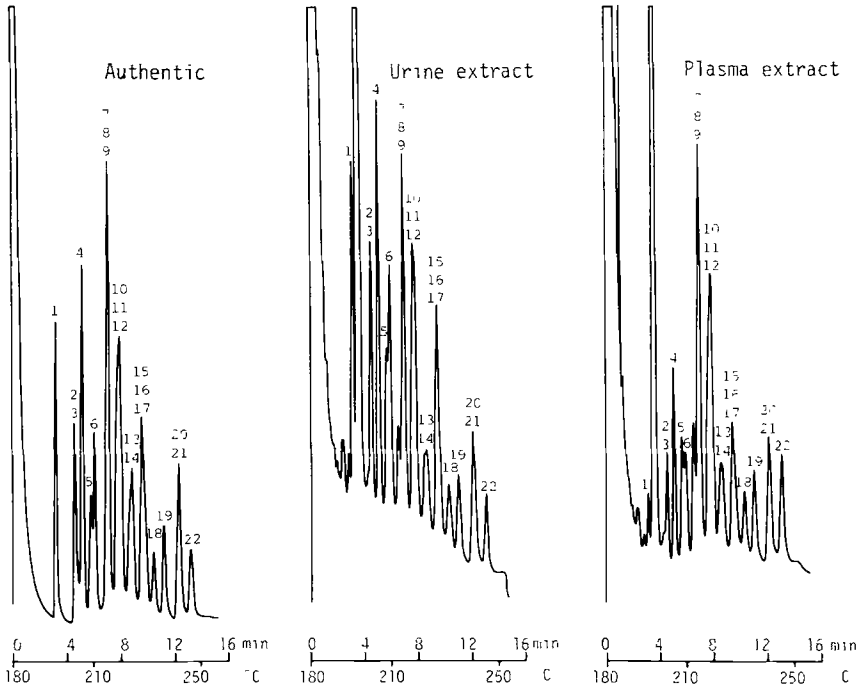

FIG. 1—*Wide-bore capillary GC (SPB-1 column) for benzodiazepines isolated from human urine and plasma by use of Sep-Pak C_{18} cartridges.* (1) *medazepam,* (2) *fludiazepam,* (3) *lorazepam,* (4) *diazepam,* (5) *dipotassium clorazepate,* (6) *clotiazepam,* (7) *oxazolam,* (8) *flunitrazepam,* (9) *bromazepam,* (10) *prazepam,* (11) *haloxazolam,* (12) *nimetazepam,* (13) *mexazolam,* (14) *nitrazepam,* (15) *chlordiazepoxide,* (16) *flurazepam,* (17) *clonazepam,* (18) *estazolam,* (19) *alprazolam,* (20) *etizolam,* (21) *triazolam, and* (22) *tofisopam. GC was carried out with a fused silica wide-bore capillary column (SPB-1, 15-m by 0.53-mm ID, film thickness 1.5 μm). Its conditions were: column temperature 180 to 250°C (5°C/min) and nitrogen flow rate 20 mL/min. The mixture of 22 benzodiazepines, 10 μg of each, was added to 1 mL of urine or plasma.*

out the deproteinization process. A big impurity peak appeared in each chromatogram with the SPB-1 column for urine and plasma extracts (Fig. 1), but not in the ones with the HP-17 column (Fig. 2). The impurity peak did not overlap any peak of benzodiazepines tested.

The recoveries were excellent for all drugs mixed in urine, but were somewhat lower for some drugs in plasma samples (Figs. 1 and 2); medazepam especially in the plasma sample showed only 10 to 20% recovery. The efforts to improve the recoveries by adding sodium chloride (NaCl) (final 10%) to the plasma solutions or by changing their pH before loading on the Sep-Pak cartridges were unsuccessful. Thus, we placed a deproteinization with perchloric acid at the initial step as described before. As a result, the recovery of medazepam added to plasma was much improved up to 97%. Other benzodiazepines, such as fludiazepam, lorazepam, and diazepam, which had showed relatively low recoveries (50 to 60%) for plasma extracts (Figs. 1 and 2), also gave much improved recoveries of more than 90%.

Discussion

To our knowledge, this is the first trial to use Sep-Pak C_{18} cartridges for the cleanup of benzodiazepines present in biological samples. In the previous reports, benzodiazepines were isolated by extraction and washings with organic solvents [2–7] and with Amberlite®

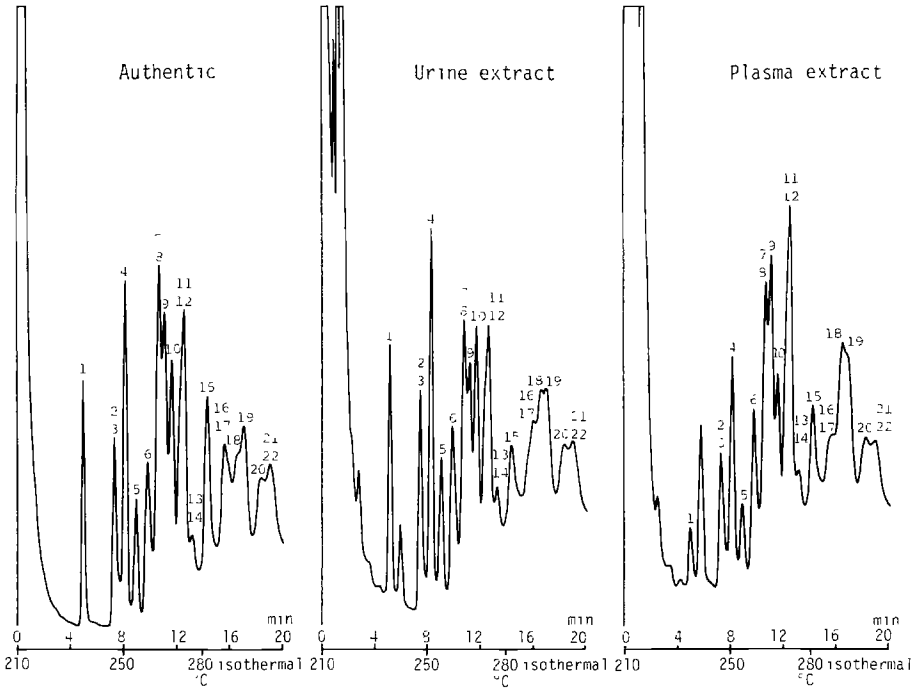

FIG. 2—*Wide-bore capillary GC (HP-17 column) for benzodiazepines isolated from human urine and plasma by use of Sep-Pak C_{18} cartridges.* (1) *medazepam,* (2) *fludiazepam,* (3) *lorazepam,* (4) *diazepam,* (5) *clotiazepam,* (6) *dipotassium clorazepate,* (7) *oxazolam,* (8) *prazepam,* (9) *flunitrazepam,* (10) *flurazepam,* (11) *nimetazepam,* (12) *bromazepam,* (13) *haloxazolam,* (14) *mexazolam,* (15) *nitrazepam,* (16) *chlordiazepoxide.* (17) *clonazepam,* (18) *estazolam,* (19) *alprazolam,* (20) *etizolam.* (21) *triazolam. and* (22) *tofisopam. GC was carried out with a fused silica wide-bore capillary column (HP-17, 10-m by 0.53-mm ID, film thickness 2.0 μm). Its conditions were. column temperature 210 to 280°C (5°C/min) and nitrogen flow rate 20 mL/min. The mixture of 22 benzodiazepines, 10 μg of each, was added to 1 mL of urine or plasma.*

XAD-2 resin [*8*]. These extraction methods are much more complicated and time-consuming than the present Sep-Pak method.

The recovery of medazepam added to plasma was low when the plasma solution was directly loaded on a Sep-Pak cartridge without deproteinization (Figs. 1 and 2). This is probably due to firm binding of the drug to plasma proteins under alkaline conditions, which probably results in its poor transfer to the C_{18} phase. This problem was easily circumvented by placing a deproteinization step to liberate drugs from proteins before application to the cartridges. Although most benzodiazepines can be directly applied to the Sep-Pak cartridges with no problems in recovery even for plasma samples with high protein contents, a deproteinization process should be adopted when low recovery of a benzodiazepine is noted.

We have demonstrated wide-bore capillary GC for 22 benzodiazepines (Figs. 1 and 2). The coexistence of many benzodiazepines in a sample is, of course, rare, but our results on gas chromatograms can be used for selection of an appropriate internal standard benzodiazepine against a benzodiazepine to be analysed. The wide-bore capillary GC or GC/mass spectrometry is recommended for benzodiazepine analyses because the drugs are relatively stable during passage through a wide-bore capillary column as a result of much faster flow inside the column and thus much shorter exposure to heat [*9*]; they are easily decomposed in narrow-bore capillary or packed columns [*10*].

The present isolation method for benzodiazepines with use of Sep-Pak C_{18} cartridges seems very useful because of its simplicity and rapidity. This method can probably be used also for cleanup before high-performance liquid chromatography and thin-layer chromatography.

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