INTRODUCTION

Dietary supplements have recently been gaining popularity with increasing health awareness among the Japanese. The presence of therapeutic medicinal ingredients intentionally added to supplements has been reported.1–5 Such supplements are worrying and, without prior knowledge of the addition of drugs, potential hazards to health cannot easily be avoided. For surveillance programs, the establishment of a more effective screening procedure for medicinal ingredients in supplements is required.6–8

Benzodiazepines (BZDs) are most frequently prescribed as sedatives and hypnotics and patients are easily subject to intoxication due to accidental overdose or misuse. A large number of analytical methods have been published for the determination of BZDs. Among these various chromatographic techniques, GC/MS,9,10 HPLC,11,12 and LC/MS13 are commonly used for analyzing BZDs. However, they are not suitable to analyze BZDs in adulterated dietary supplements when considering single oral doses of marketed BZDs and minimizing sample preparation for periodic inspection. There is a need for the development of a new screening method that determines and identifies most common BZDs using simple, low-cost instrumentation.

The present paper describes an HPLC method for the simultaneous analysis of seven BZDs: oxazolam (OXO), nitrazepam (NIT), oxazepam (OXE), tofisopam (TOF), triazolam (TRI), clotiazepam (CLO), and diazepam (DIA) as adulterants in dietary supplements. Finally, an DIA-adulterated capsule-type supplement was identified and determined using a combination of three different analytical methods: HPLC/photodiode array, thin-layer chromatography (TLC), and LC/MS. DIA was present at a concentration of 1.9 mg/capsule. The procedure described here can provide a broad analysis in a single run within 17 min and is available for the screening of seven BZDs in adulterated supplements with minimal sample preparation.

Key words —— dietary supplement, benzodiazepine, diazepam, HPLC
determined using a combination of three different analytical methods: HPLC/photodiode array, thin-layer chromatography (TLC), and LC/MS.

**MATERIALS AND METHODS**

**Samples** — Fourteen supplements (six of the tablet type, eight of the capsule type) were collected in Nagoya (Japan) markets in 2003. One capsule-type supplement was offered to an inhabitant of Hiroshima prefecture (Japan), who received it from a traveler to China.

**Chemicals and Instrumentation** — OXO, NIT, TOF, TRI, CLO, and DIA were of Japanese Pharmacopoeia (14th ed.) quality. OXE was of Japanese Pharmaceutical Codex (2002) quality. 1-Heptanesulfonic acid sodium salt (HSA) and HPLC-grade acetonitrile were purchased from Wako Pure Chemical Industries (Osaka, Japan). All other reagents and organic solvents were of analytical grade. HPLC/photodiode array analyses were carried out using a Shimadzu CLASS-VP Series (Kyoto, Japan). LC/MS with electrospray-ionization (ESI) interface analysis was carried out using a Micromass LCT mass spectrometer (Manchester, U.K.) to the effluent from an Agilent HP1100 LC system (Palo Alto, CA, U.S.A.).

**Sample Preparation** — A powdered sample of a single intake was transferred to a 100-ml volumetric flask. The volume was adjusted with methanol, and the solution was sonicated for 60 min in an ultrasonic bath. Portions of these solutions were then filtered through a 0.5-µm PTFE filter (Toyo Roshi Kaisha, Tokyo, Japan). An aliquot (10 µl) of each solution was injected onto the column.

**HPLC Operating Conditions** — HPLC analysis was performed with a column of Wakosil 5C18 (5 µm, 4.6 × 150 mm, Wako Pure Chemical Industries), at a flow rate of 1.0 ml/min. The mobile phase consisted of HSA 5 mM dissolved in 1000 ml of water/acetonitrile (13 : 7, v/v), adjusted with phosphoric acid to pH 2.4. The column temperature was 40°C and the eluent was monitored using a photodiode array detector (220–340 nm). The sample solution (10 µl) was applied to quantitative analysis at 225 nm using a detector range of 0.08 aufs.

**Calibration Curves of Standard Solutions** — Standard stock solutions of OXO (0.2 mg/ml), NIT (0.05 mg/ml), OXE (0.05 mg/ml), TOF (0.12 mg/ml), TRI (0.01 mg/ml), CLO (0.1 mg/ml), and DIA (0.04 mg/ml) were prepared by dissolving appropriate amounts of the respective BZDs in methanol.

**RESULTS AND DISCUSSION**

**Optimization of the Chromatographic System**

The seven BZDs were insufficiently separated on the reverse-phase column when the mobile phase consisted of acetonitrile/water. They showed reduced resolution and it hampered their accurate determination. The addition of HSA, the hydrophobic ion-pair reagent, is particularly useful for the separation and detection of several drugs, such as nabumetone and mosapride, because it provides appropriate capacity factors for complexes formed with the counterion. When the effects of HSA concentration were examined, as shown in Fig. 1A, the capacity factor of the analyte was enhanced by increasing the HSA concentration from 1.25 to 7.5 mM. A concentration of 5 mM was selected due to its optimum peak shape and resolution. The effects of mobile
phase pH from 2.0 to 3.6 on the analyte retention were examined, in which seven BZDs were eluted within 20 min. As shown in Fig. 1B, CLO and DIA were strongly retained on the column. A pH value of 2.4 in the mobile phase was selected due to its optimum peak resolution and elution of the compounds within 17 min. Utilizing chromatographic conditions, the capacity factors were 1.7 (OXO), 3.5 (NIT), 4.1 (OXE), 5.1 (TOF), 6.1 (TRI), 7.2 (CLO), and 8.1 (DIA), respectively. When six other BZDs (ethyl lofazepate, flutazolam, haloxazolam, lormetazepam, midazolam, prazepam) were examined under these HPLC conditions, ethyl lofazepate, haloxazolam, lormetazepam, midazolam, and prazepam were eluted over 20 min, and flutazolam displayed a broad peak.

An HPLC system equipped with a photodiode array detector was used because it provided a more definite identification than the retention index alone for effective screening. Figure 2 shows the UV spectra of the seven monitored BZD standards. The detector wavelength for BZDs in quantitative analysis was 225 nm, which showed higher sensitivity.

Analytical Performance Characteristics

The calibration curves of BZDs in the concentration (µg/ml) ranges of 10–80 (OXO), 2.5–20 (NIT, OXE), 6–50 (TOF), 0.5–50 (TRI), 5–40 (CLO), and 2–16 (DIA) showed good linearity with a detection wavelength of 225 nm at a sensitivity of 0.08 aufs. The correlation coefficients were better than 0.999 in all cases. The intercepts with the y-axis were not markedly different from the origin. The instrumental detection limits (µg/ml), based on a signal-to-noise ratio of about 3 for standard solutions of BZDs, were 2.0 (OXO), 0.5 (NIT, OXE, TOF), 0.4 (TRI), 1.0 (CLO), and 0.5 (DIA), respectively. The relative standard deviations (RSD) after six identical repeated analyses at each concentration were < 0.8%.

The accuracy and precision of the HPLC analysis of the seven BZDs were determined in recovery experiments. Commercial supplement placebos were spiked at concentration levels (µg/ml) of 19.4 (OXO), 5.2 (NIT), 5.1 (OXE), 11.6 (TOF), 1.4 (TRI), 9.7 (CLO), and 4.4 (DIA), respectively, and analyzed. Recoveries and standard deviations (S.D.) are presented in Table 1. The accuracy, calculated as the recovery of each BZD, was in the range of 98.0% to 104.8%. The precision, calculated as RSD, was < 2.5%. Figure 3 shows chromatograms obtained in the recovery experiment using a drug-free tablet-type
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Table 1. Accuracy and Precision of the Method for HPLC Analysis of Seven Benzodiazepines in Commercial Dietary Supplements (n = 5)

<table>
<thead>
<tr>
<th>Type</th>
<th>Drug</th>
<th>OXO</th>
<th>NIT</th>
<th>OXE</th>
<th>TOF</th>
<th>TRI</th>
<th>CLO</th>
<th>DIA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tablet</td>
<td></td>
<td>104.3 ± 2.3</td>
<td>102.8 ± 1.9</td>
<td>100.1 ± 1.1</td>
<td>102.1 ± 1.2</td>
<td>99.8 ± 1.4</td>
<td>100.1 ± 0.9</td>
<td>100.3 ± 0.8</td>
</tr>
<tr>
<td>Capsule</td>
<td></td>
<td>103.5 ± 1.9</td>
<td>102.2 ± 1.8</td>
<td>99.9 ± 0.9</td>
<td>100.9 ± 1.0</td>
<td>102.3 ± 1.6</td>
<td>101.2 ± 0.7</td>
<td>99.3 ± 1.0</td>
</tr>
<tr>
<td>Teabag</td>
<td></td>
<td>104.8 ± 2.1</td>
<td>103.6 ± 1.1</td>
<td>100.4 ± 1.5</td>
<td>102.3 ± 1.4</td>
<td>101.0 ± 2.0</td>
<td>98.5 ± 0.6</td>
<td>101.7 ± 1.1</td>
</tr>
</tbody>
</table>

Each value represents recovery (%; mean ± S.D.).

Sample Analysis

Fifteen samples were assayed using the procedure developed in this study. Seven BZDs were not detected in 14 samples using HPLC with the photodiode array. However, a peak in the capsule-type sample containing a yellow-white powder was identified as DIA in UV spectral analysis. The retention time of DIA in this HPLC analysis was 16.0 min, and the peak in the capsule-type sample was identified. Figure 4 shows the UV spectra of the DIA standard and the capsule-type sample containing DIA monitored over the range of 220 to 340 nm. The maximum wavelengths for DIA were 237 and 285 nm. Quantitative analysis was performed at 225 nm, and no interference was observed in the chromatograms. Figure 4 shows the HPLC chromatogram.

Identification System for DIA in a Dietary Supplement

TLC is widely used in pharmaceutical analytical laboratories for identity testing because it is
simple, rapid, robust, and inexpensive. The sample test solution was prepared by dilution with methanol and filtered to remove endogenous insoluble components. After development, a dark blue spot of DIA was observed under UV light at 254 nm, and it was clearly resolved and well separated from the other components. The Rf value of DIA was 0.3, and UV measurement allows reliable identification of DIA up to 0.1 µg.

LC/MS is a powerful qualitative technique for the accurate determination of molecular masses of analytes, because analyte identification on the basis of molecular mass is extremely selective. LC/MS using the ESI interface in positive-ion mode was used to determine the molecular marker ions of DIA. To improve sensitivity, the LC mobile phase was water/acetonitrile (1 : 1, v/v). The retention time of DIA in this LC/MS analysis was 8.3 min, and a peak in the supplement was identified. The protonated molecule [M+H]+ for DIA was found at m/z 285. From a peak in the supplement, the protonated molecule [M+H]+ at m/z 285 was also observed. Figure 5 shows the mass spectra of the DIA standard and a supplement containing DIA generated in positive-ion mode. The peak in the supplement was identified as DIA based on both mass and LC/MS spectra.

The sample was found to contain DIA 1.9 mg per capsule using HPLC. A single oral dose of DIA is 2 mg in Japan.18 Such supplements are especially troublesome and frequently lead to overuse and have undesirable effects on the health of consumers.

In conclusion, an HPLC method for the simultaneous analysis of seven BZDs is presented for its application in the screening examination of dietary supplements as adulterants. After analysis of the 15 samples, DIA in the capsule-type supplement was detected. Finally, DIA was identified by a combination of three different analytical methods: HPLC/photodiode array, TLC, and LC/MS. The identification of adulterants in dietary supplements poses an even greater challenge to laboratories required to conduct routine surveillance programs. Because of the great variation in the chemical properties, it is not possible to devise a single screening method to cover all medicinal ingredients. The procedure described here can provide an adequately broad analysis in a single run within 17 min and is available for the screening of seven BZDs in adulterated supplements with minimal sample preparation.

Acknowledgements This study was supported by a Health Sciences Research Grant from the Ministry of Health, Labor and Welfare, Japan.

REFERENCES
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