Rapid Monitoring of Benzodiazepines in Clinical Samples by Using On-Line Column Switching HPLC
C. M. Moore, K. Sato, and Y. Katsumata

We have determined routinely administered benzodiazepines and some of their metabolites in clinical samples by using on-line column switching for sample clean-up and reversed-phase high-pressure liquid chromatography (HPLC). The plasma sample is directly injected onto a cyanopropyl (CN) pre-column, washed with buffer, and eluted onto an analytical column (C18, octadecysilane). Analytical recovery from drug-supplemented plasma samples was >85% for all the benzodiazepines tested. The response of the analytical column varied linearly with drug concentrations over the range 0.01–1 mg/L in plasma, with CVs ranging from 0.3% to 1.8% within-day and 5.5% to 15.7% between days. The detection limit (signal-to-noise ratio >3) was 0.01 mg/L (1 ng on column) for all of the benzodiazepines and their metabolites. The advantages of the technique include the extreme cleanliness of the traces, no requirement for off-line sample preparation, and ease of automation.

Additional Keyphrases: monitoring drug therapy · chromatography, reversed-phase · psychotropic drugs

Benzodiazepines are often used as anti-anxiety agents in the treatment of psychiatric disorders (1). Various researchers have reported total plasma concentrations of benzodiazepines in relation to both clinical effect and toxicity, indicating that the therapeutic monitoring of these drugs is important.

Analytical methods have been described for the determination of benzodiazepines in biological fluids. Common methods include thin-layer chromatography, which tends to lack specificity (2), and gas chromatography (3), which often requires formation of derivatives for the determination of thermally labile benzodiazepines, e.g., oxazepam and chlordiazepoxide. Hence, high-pressure liquid chromatography (HPLC) has become the most widely used analytical technique for the determination of benzodiazepines (4, 5).

The HPLC methods reported are often applicable to only one drug and its metabolites (6–8). However, those papers that do report screening procedures (9) do not specifically address the problems of clinical screening, where very often two or more unrelated drugs are co-administered with the benzodiazepine.

The chromatographic theory and method development behind on-line pre-concentration or column switching techniques have been well reviewed and described (10, 11). Again, however, the applications of such techniques rarely address the problems of clinical samples and, when applied to benzodiazepines, are often limited to one drug and its metabolites (12). Indeed, Koenigbauer et al. (12) observed a great deal of interference from endogenous plasma components in their method.

Pre-column drug screening that does not cover benzodiazepines has been described (13–15), as has a double pre-column procedure for determinations of drugs in urine samples (16).

In our paper, we describe a column-switching technique in which the plasma samples are directly injected onto a cyanopropyl pre-column and washed with buffer. This ensures an extremely clean chromatographic trace. The trapped drugs are then eluted onto a reversed-phase analytical column for determination.

Our method is rapid, simple, and easily automated and allows the determination of benzodiazepines and their metabolites, which we regularly administer to psychiatric patients, in clinical samples.

Materials and Methods

Materials

Chemicals and reagents. Purified free base benzodiazepines for research purposes only were obtained from various pharmaceutical companies in Japan. Chlordiazepoxide, desmethyldiazepam, diazepam, and estazolam were obtained from Takeda Co., Osaka; chloziazepam and etizolam from Yosohiti Co., Osaka; triazolam from the Upjohn Co., Tokyo; nitrazepam and medazepam from Shionogi Pharmaceutical Co., Osaka; tofisopam from Mochida Co., Tokyo; oxazepam and flutoprazepam from Banyu Co., Tokyo; flunitrazepam from Ezi Co., Tokyo; flurazepam from Roche Pharmaceuticals, Tokyo; haloxazolam from the Sankyo Chemical Co., Tokyo; fludizepam, nimetazepam, and clonazepam from the Sumitomo Chemical Co., Osaka.

All solvents used were HPLC grade, and all chemicals were analytical grade or better.

Standards. Standard aqueous solutions of the benzodiazepines under investigation were prepared at concentrations of 0.01, 0.1, 0.5, and 1 mg/L. When the free base benzodiazepines would not dissolve directly in water, methanolic solutions at high concentrations were diluted with de-ionized water to the required concentration. Also, plasma samples were supplemented with the drugs at these same concentrations. All samples were stored at 4 °C until analysis.

Clinical samples. Blood samples were taken in the early morning from 22 psychiatric patients, 12 h after
the routine administration of a single oral dose of benzodiazepines. All the patients were also receiving other drugs—chlorpromazine, levomepromazine, phentoin, valproate sodium, bromocriptine mesylate, meprone, pimozide, clozapamine, bromeridol, haloperidol, timiperone, perizacazine, phenazine, carbamazepine, promethazine, or profenamine—at various therapeutic concentrations.

The samples were taken with the full permission of the patients.

Apparatus

The chromatographic system (Figure 1) essentially consisted of a 4.0 × 30 mm cyano propyl pre-column packed with 10-μm CN particles, a 4.6 × 250 mm reversed-phase analytical column packed with octadecylsilane (ODS)-C18 5-μm particles (Nomura Chemical Co., Seto, Japan), and a Rheodyne injection valve incorporating a 100-μL fixed-volume loop. The use of a fixed loop eliminates the need for an internal standard because injection volumes are reproducible.

The system contained two continuous-flow pumps, A and B, both Model LC-6As (Shimadzu, Kyoto, Japan). Pump B was connected to a six-port switching valve, which allowed one of six possible solvents to be pumped over the pre-column at a given time. Pump A was used only for the mobile phase required for the main analytical column.

Two rotary valves, A and C (Shimadzu), were required to fully automate the analytical procedure. Rotary valve A (RVA) connected the flow over the main column to the flow over the pre-column, and rotary valve C (RVC) allowed the specific wash solvents from the six solvent reservoirs to be selected.

In position 0, RVA allowed the analytical mobile phase to pass over the analytical column only. In position 1, the mobile phase was pumped over both the pre-column and the analytical column. In position 1, RVA pumped water over the pre-column; in position 2, it pumped 0.1 mol/L (17.42 g/L) dipotassium hydrogen phosphate buffer, pH 9.0; and in position 3, methanol.

The analytical mobile phase, methanol/de-ionized water/acetic acid (60/35/5 by vol), was pumped at a rate of 0.6 mL/min.

The detector (Model SPD-6A) was a variable-wave- length ultraviolet detector set at 241 nm. The system controller was a Model SCL-6B and the recorder was a Model C-R6A Chromatopac (both from Shimadzu).

Chromatographic Assay

The untreated plasma sample (100 μL) was directly injected into the sample loop, and the valve was turned from the load to the inject position. This procedure started the computer program shown in Table 1. For 1 min, the sample was taken from the loop and pumped onto the cyano pre-column in water (RVC position 1; pump B), at a flow rate of 1.5 mL/min. Then the column was washed with 0.1 mol/L dipotassium hydrogen phosphate buffer (pH 9.0) for 3 min (RVC position 2) to remove any endogenous plasma components. After 4 min, the mobile phase for the analytical column (pump A) was automatically pumped over the pre-column and onto the main column (RVA position 1), eluting the retained drugs from the CN column. After 15 min, water was pumped over the pre-column (RVC position 1) for 3 min to prevent phosphate crystallization, followed by a third solvent, methanol (RVC position 3), for 4 min to wash away any remaining endogenous material and to re-condition the CN column before injection of the next sample. Finally, water was again passed over the pre-column to prepare it for the next injection (RVC position 1). The total cycle time was 25 min.

Other Procedures

Analytical recovery. The recovery of the parent drugs added to plasma was determined by comparing the peak heights obtained from an injection of a drug standard with that obtained from the injection of drug-supplemented plasma.

Precision and linearity. The response of the detector to each benzodiazepine was determined at four concentra-

Table 1. Program for Column Switching Procedure for Determining Benzodiazepines in Clinical Samples

<table>
<thead>
<tr>
<th>Time, min</th>
<th>System component</th>
<th>Position*</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.01</td>
<td>RVA</td>
<td>1</td>
</tr>
<tr>
<td>1</td>
<td>RVA</td>
<td>2</td>
</tr>
<tr>
<td>4</td>
<td>RVA</td>
<td>1</td>
</tr>
<tr>
<td>4</td>
<td>Start</td>
<td>1</td>
</tr>
<tr>
<td>14</td>
<td>B. flowb</td>
<td>1.5</td>
</tr>
<tr>
<td>15</td>
<td>B. flow</td>
<td>2.0</td>
</tr>
<tr>
<td>15</td>
<td>RVA</td>
<td>1</td>
</tr>
<tr>
<td>16</td>
<td>RVA</td>
<td>0</td>
</tr>
<tr>
<td>18</td>
<td>RVA</td>
<td>3</td>
</tr>
<tr>
<td>20</td>
<td>B. flow</td>
<td>2.0</td>
</tr>
<tr>
<td>22</td>
<td>RVA</td>
<td>1</td>
</tr>
<tr>
<td>25</td>
<td>B. flow</td>
<td>1.5</td>
</tr>
<tr>
<td>25</td>
<td>Stop</td>
<td>End</td>
</tr>
</tbody>
</table>

RV = rotary valve.

* RVA: position 1, water; 2, 0.1 mol/L dipotassium hydrogen phosphate, pH 9.0; 3, methanol. RVA: position 0, mobile phase passed through analytical column only; 1, mobile phase passed through pre-column before analytical column.

b Pump B flow, 1.5 mL/min.
tions. Each sample was injected at least twice on each of 20 days within a period of four months.

Interferences. We checked for possible interferences not only from various drugs that were co-administered with the benzodiazepines, but also from other common drugs: caffeine, theophylline, theobromine, quinine, atropine, lidocaine, sulpiride, diphenhydramine, bupivacaine, papaverine, chlorzoxazone, meprobamate, ami-tryptiline, imipramine, trimipramine, and amoxapine were all tested at greater than therapeutic concentrations (17, 18).

Other benzodiazepines. Aqueous standards of etizolam, haloxazolam, fludiazepam, flurazepam, nimetazepam, clonazepam, and nitroprazepam were also determined with this procedure, although they were not adminis-tered to our patients.

Results and Discussion

Analytical Recovery

The system was highly reproducible and efficient. Table 2 lists the retention times of benzodiazepines and recoveries from drug-supplemented plasma samples.

Precision and Linearity

Results by this analytical system varied linearly with drug concentrations for all the benzodiazepines administered to patients. The respective within-day and between-day CVs for these drug determinations are shown in parentheses: oxazepam (0.3%, 8.3%), diazepam (0.4%, 5.5%), triazolam (1.0%, 11.1%), chlordiazepoxide (1.1%, 6.9%), chlordiazepoxide (1.1%, 15.7%), tofisopam (1.3%, 11.4%), nitrazepam (1.4%, 10.2%), medazepam (1.4%, 7.8%), estazolam (1.8%, 11.6%), and flunitrazepam (1.2%, 7.6%). The slopes, intercepts, correlation coefficients, and standard errors of the estimate are listed in Table 3.

Patients' Samples

Blood samples treated with heparin to prevent coagulation were centrifuged, and the plasma supernates (100 μL) were injected into the sample loop. Because the pre-column was washed with 100% methanol and pre-conditioned on-line before the injection of each sample, we could inject 200 samples (20 mL of plasma) before having to change the pre-column. The chromatographic peaks were extremely clean because endogenous substances had been washed away by the buffer on the pre-column before the drugs were eluted onto the analytical column (Figure 2a).

Benzodiazepines tested were well separated on the chromatographic system (Figure 2b), and the total cycle

<table>
<thead>
<tr>
<th>Drug</th>
<th>Retention time, min</th>
<th>Recovery, %</th>
<th>No of samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medazepam</td>
<td>9.9</td>
<td>93.4 (7.2)</td>
<td>9</td>
</tr>
<tr>
<td>Chlordiazepoxide</td>
<td>10.4</td>
<td>85.5 (11.6)</td>
<td>15</td>
</tr>
<tr>
<td>Tofisopam</td>
<td>13.3</td>
<td>85.4 (12.4)</td>
<td>11</td>
</tr>
<tr>
<td>Nitrazepam</td>
<td>14.5</td>
<td>96.9 (4.6)</td>
<td>10</td>
</tr>
<tr>
<td>Flunitrazepam</td>
<td>14.8</td>
<td>94.9 (5.5)</td>
<td>10</td>
</tr>
<tr>
<td>Estazolam</td>
<td>15.8</td>
<td>101.2 (14.0)</td>
<td>10</td>
</tr>
<tr>
<td>Triazolam</td>
<td>16.3</td>
<td>99.5 (14.5)</td>
<td>10</td>
</tr>
<tr>
<td>Oxazepam</td>
<td>17.4</td>
<td>88.9 (9.5)</td>
<td>11</td>
</tr>
<tr>
<td>Chlortizolam</td>
<td>19.2</td>
<td>103.7 (11.2)</td>
<td>9</td>
</tr>
<tr>
<td>Diazepam</td>
<td>22.8</td>
<td>97.9 (8.8)</td>
<td>11</td>
</tr>
</tbody>
</table>

* Mean (SD) recovery of drugs added to plasma at concentrations of 1 mg/L.

Table 3. Benzodiazepine Concentration vs Detector Response

<table>
<thead>
<tr>
<th>Drug</th>
<th>Slope</th>
<th>y-Intercept</th>
<th>r</th>
<th>S slopes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chlordiazepoxide</td>
<td>81250.0</td>
<td>+3000</td>
<td>0.998</td>
<td>331.7</td>
</tr>
<tr>
<td>Medazepam</td>
<td>28571.4</td>
<td>0.001</td>
<td>0.997</td>
<td>127.1</td>
</tr>
<tr>
<td>Nitrazepam</td>
<td>25219.3</td>
<td>-0.100</td>
<td>0.999</td>
<td>93.1</td>
</tr>
<tr>
<td>Oxazepam</td>
<td>25000.0</td>
<td>0.000</td>
<td>0.987</td>
<td>102.2</td>
</tr>
<tr>
<td>Diazepam</td>
<td>19195.1</td>
<td>0.005</td>
<td>0.998</td>
<td>137.1</td>
</tr>
<tr>
<td>Estazolam</td>
<td>17647.0</td>
<td>-0.001</td>
<td>0.997</td>
<td>168.4</td>
</tr>
<tr>
<td>Flunitrazepam</td>
<td>12350.3</td>
<td>0.000</td>
<td>0.972</td>
<td>279.8</td>
</tr>
<tr>
<td>Chlortizolam</td>
<td>9381.3</td>
<td>-0.030</td>
<td>0.988</td>
<td>75.3</td>
</tr>
<tr>
<td>Triazolam</td>
<td>9198.2</td>
<td>0.200</td>
<td>0.999</td>
<td>96.4</td>
</tr>
<tr>
<td>Tofisopam</td>
<td>5000.0</td>
<td>0.000</td>
<td>0.997</td>
<td>52.2</td>
</tr>
</tbody>
</table>

Based on the response of the liquid chromatograph to aqueous drug standards at four concentrations (0.01, 0.1, 0.5, and 1 mg/L). n = 12.
time was 25 min. All the drugs were detected (i.e., signal-to-noise ratio >3) at 0.01 mg/L (1 ng on column). Flunitrazepam co-eluted with nitrazepam and so is not shown; however, the two drugs would never be administered simultaneously, so they would never appear in the same chromatogram.

Clinical Samples

Chlordiazepoxide and its metabolites desmethylchlorodiazepoxide, desmethyldiazepam, and oxazepam were detected in all the samples taken after the administration of chlordiazepoxide. The parent drugs estazolam, nitrazepam, tofisopam, medazepam, and chlordiazepoxide were detected in all samples after their respective administrations (Figure 2c). Desmethyldiazepam was also detected in the plasma sample from the patient receiving medazepam.

Parent diazepam and its metabolites desmethyldiazepam and oxazepam were identified in all patients’ samples after diazepam administration (Figure 2d). Parent flunitrazepam and triazolam were not detected because the sampling was 12 h after administration, a time at which these highly potent benzdiazepines would be present at very low concentrations, if at all.

The peak shape of flutazolam in this system was not good, owing to its low absorbance at 241 nm. We did not detect it in patients’ samples as parent flutazolam (retention time 11.0 min); however, samples from those patients contained a peak at 17.3 min, which was later identified by super microbore liquid chromatography–mass spectrometry as a major metabolite of flutazolam with a molecular mass of 288 Da (manuscript submitted for publication).

The following benzdiazepines were administered once a month for three months, with samples taken 12 h after each dose. We quantified these parent drugs and some of their metabolites with the following results:

Diazepam: Two patients received 10 mg. The mean (SD) concentration of diazepam detected was 0.081 (0.097) mg/L; oxazepam, 0.025 (0.027) mg/L; and desmethyldiazepam, 0.81 (0.51) mg/L (n = 6).

Four patients received 20 mg. The mean (SD) concentrations measured were: diazepam, 0.19 (0.07) mg/L; oxazepam, 0.03 (0.04) mg/L; and desmethyldiazepam, 1.13 (0.33) mg/L (n = 12).

Chlordiazepoxide: One patient received 10 mg. Mean (SD) concentrations were as follows: chlordiazepoxide, 0.04 (0.05) mg/L; desmethyldiazepam, 0.14 (0.06) mg/L (n = 2); and oxazepam, 0.006 mg/L (one sample only).

One patient received 15 mg. Mean (SD) concentrations were as follows: chlordiazepoxide, 0.12 (0.056) mg/L; desmethyldiazepam, 0.34 (0.09) mg/L; and oxazepam, 0.018 (0.022) mg/L (n = 3).

One patient received 20 mg. Mean concentrations were

\[\text{as follows: chlordiazepoxide}, 0.27 \text{ mg/L; desmethyldiazepam}, 0.55 \text{ mg/L; and oxazepam}, 0.03 \text{ mg/L (n = 3).}\]

Four patients received 30 mg. Mean concentrations were as follows: chlordiazepoxide, 0.15 (0.097) mg/L; desmethyldiazepam, 0.62 (0.31) mg/L; and oxazepam, 0.013 (0.01) mg/L (n = 12).

One patient received 40 mg. Mean concentrations were as follows: chlordiazepoxide, 0.33 (0.061) mg/L; desmethyldiazepam, 1.27 (0.44) mg/L; and oxazepam, 0.035 (0.018) mg/L (n = 3).

Estazolam: Three patients received 2 mg. The mean concentration detected was 0.103 (SD 0.015) mg/L (n = 9).

Tofisopam: One patient received 150 mg. The mean (SD) concentration detected was 0.091 (0.057) mg/L (n = 3).

Nitrazepam: Two patients received 5 mg. The mean (SD) concentration detected was 0.062 (0.015) mg/L (n = 8).

Chloretiazepam: One patient received 15 mg. The plasma concentration was determined to be 0.136 mg/L (n = 1).

Medazepam: One patient received 10 mg. The plasma concentrations of medazepam and desmethyldiazepam were 0.13 and 0.38 mg/L (n = 1), respectively.

Interferences

The results reported here are based on clinical samples taken from authentic psychiatric patients. This assay was developed for use in our clinical laboratory. Several drugs were co-administered to the patients during the course of their normal treatment; therefore, the specificity of this assay was largely based on the feasibility of interference from these drugs, rather than from all drugs that could feasibly be co-administered to psychiatric patients. We recommend that each laboratory determine their own likely “in-house” interfering drugs before this procedure is used routinely.

The following co-administered drugs did not interfere in any way with the assay: phenytoin, valproate sodium, bromocriptine mesylate, moperone, pimozone, clozapamine, bromoperidol, haloperidol, timiperone, propriociazine, and phenazine. However, at doses ≥100 mg, chlorpromazine, with a retention time of 13.3 min, was detected. Although it did not interfere with the assay, it possibly could interfere with the determination of tofisopam. In this study, however, tofisopam and chlorpromazine were not co-administered, so we did not actually observe such interference. When given at doses <100 mg, chlorpromazine was not detected.

At doses of 5, 10, 15, and 25 mg, levomepromazine (retention time 11.6 min) was not detected. When 50 mg was administered, a small peak was observed.

Promethazine (retention time 10.5 min) was detected in all samples from patients who had received it. This could cause a problem with the determination of chlordiazepoxide, because their retention times are very close. Only one patient was given both these drugs, but we were able to identify, quantify, and confirm the presence of chlordiazepoxide by mass spectrometry.

\[\text{Desmethylchlordiazepoxide was unavailable as a standard in Japan during this study; therefore, even though it was detected in some samples after chlordiazepoxide administration, we could not quantify it.}\]
Profenamine, administered at 40 mg, was not detected. However, its retention time of 11.0 min and its characteristic peak shape made it easily identifiable in patients’ samples after doses of 150 or 200 mg.

The retention times of carbamazepine and its metabolite were 15.0 and 13.4 min, respectively. Hence, parent carbamazepine interfered with the determination of nitrazepam and flunitrazepam (if present). Nitrazepam was detected when carbamazepine was not co-administered. Flunitrazepam was not detected in any samples.

Possible alternative interferences. Papaverine, bupivacaine, lidocaine, quinine, caffeine, theophylline, theobromine, atropine, diphenhydramine, sulpiride, chlorpromazine, and meprobamate were not detected by the system at greater than therapeutic concentrations. The antidepressants amitriptyline, imipramine, trimipramine, and amoxapine at a concentration of 1 mg/L gave peaks eluting earlier than 15 min. The presence or absence of these drugs in clinical samples would be known, and analysts should be aware that their presence may cause interference, depending on the amount administered.

Other benzodiazepines. Standard aqueous solutions of the other benzodiazepines tested gave the following retention times (minutes): etizolam 17.58, haloxazolam 10.92, fludiazepam 20.08, flurazepam 9.79, nimetazepam 15.85, clonazepam 15.03, and flutoprazepam >35. The detection limit for all these drugs was 0.01 mg/L. The linearity of detector response to these benzodiazepines was also good, with a maximum within-day CV of 2.1% and between-day CV of 9.5%.

The purpose of this testing was to assess applicability of the procedure to other available benzodiazepines.

In conclusion, the system described is rapid, efficient, reproducible, and easily automated. It is sufficiently sensitive for the determination of benzodiazepines and their metabolites 12 h after therapeutic administration to patients. Few interferences exist, because of the selectivity of the cyanopropyl pre-column for benzodiazepines. Only carbamazepine interferes significantly with the determination of nitrazepam and flunitrazepam in plasma samples. The elimination of off-line sample extraction procedures enhances the speed of determination and cuts the costs of extraction columns and (or) extraction solvents. Overall, the system is highly suitable for benzodiazepine determinations in clinical samples and is a substantial improvement on existing procedures.

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References
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