Quantitative Determination of Medazepam, Diazepam, and Nitrazepam in Whole Blood by Flame-Ionization Gas–Liquid Chromatography

Mavis S. Greaves

Accurate methods are described for the qualitative and quantitative determination of medazepam, diazepam, and nitrazepam in 5 ml of whole blood. Medazepam and diazepam are analyzed intact and nitrazepam is chromatographed as its trimethylsilyl derivative by flame-ionization gas-chromatography on "1% OV-17." A supplementary column of "2% OV-1" is used to separate nitrazepam TMS from diazepam when both are present in the same extract. Essential data given include the percentage recovery of medazepam, and the flame-ionization detector responses of diazepam and silylated nitrazepam relative to medazepam, calculated after extraction from blood and gas-chromatographic analysis. Chromatograms are illustrated of extracts of blood taken from patients on medazepam and diazepam therapy and from a patient who had taken an overdose of nitrazepam.

Additional Keyphrases: toxicology • tranquilizers • monitoring therapy

Various methods have been described in the literature for determining the benzodiazepines medazepam ("Nobrium"), diazepam ("Valium") and nitrazepam ("Mogodon") in whole blood. Diazepam has been determined by ultra-violet spectrophotometry (1) and GLC1 after formation of a benzophenone derivative (2). Medazepam and diazepam have been measured by using electron-capture GLC (3). Nitrazepam has been determined by a colorimetric method (4) and by electron-capture GLC after formation of 2-amino-5-nitro-benzophenone (5). GLC analysis of intact benzodiazepines was shown to be possible by Marcucci et al. (6), who used a flame-ionization detector (6).

Because most of the methods described in the literature are too time consuming for emergency toxicological analysis in a hospital laboratory, I decided to develop methods to estimate benzodiazepines that would be an acceptable compromise between accuracy, sensitivity, and speed of the determinations.

In the methods described here, gas chromatograms are used that are equipped with flame-ionization detectors. They are accurate and reproducible, and sensitive enough to determine medazepam and diazepam concentrations that are greater than 80 \( \mu \text{g}/\text{liter} \) of whole blood, and nitrazepam concentrations greater than 200 \( \mu \text{g}/\text{liter} \) of whole blood. This sensitivity enables therapeutic concentrations of medazepam and diazepam in whole blood to be measured.

Because of the lack of sensitivity of the drug and its asymmetrical peak shape on GLC analysis, nitrazepam is chromatographed as its TMS derivative whilst the two tranquilizers are chromatographed intact.

The procedure takes 1.5 to 2 h, depending on which drug is to be estimated.

Materials and Methods

Gas Chromatography

Two Pye 104 gas chromatographs equipped with dual-flame ionization detectors were used in conjunction with Honeywell 1 mV recorders.

The silanized glass columns, each 213 cm long and 0.4 cm i.d., were packed with either "1% OV-17" (selective phase) or "2% OV-1" (nonselective phase) (Phase Separations Ltd., England). The phases were supported on acid washed and silanized "Diatomite C" (100–120 mesh) and the coating was carried out...
by the filtration technique. Each column was pre-
conditioned at a temperature of 290 °C (7) before its
initial use by passing a trickle of argon through it
overnight.

Conditions for the gas chromatographic analysis of
the benzodiazepines are summarized in Table 1.

Reagents

_Diethyl ether_, AR grade (BDH Chemicals Ltd.).
Pass this through a 1.5-cm (i.d.) column containing
25 g of active aluminum oxide (Woelm, basic, ac-
activity 1) to remove oxidation products such as perox-
ides.

_Borate buffer, pH 9._ Solution A. Dissolve 61.8 g of
boric acid and 74.6 g of potassium chloride per liter
of distilled water.

Solution B. Dissolve 106 g of sodium carbonate per
liter of distilled water.

Mix 630 ml of Solution A with 370 ml of Solution
B to obtain a buffer of pH 9 that is 1 mol/liter with
respect to the salts. Store at 37 °C to prevent crys-
tallization. If the buffer is kept at a room tempera-
ture of 20 °C, the salts crystallize out after a few
days.

_Ethanol, absolute_, AR grade. (Burroughs Ltd.)

_Standard solutions_, 1 g/liter. Dissolve 25-mg
quantities of medazepam, and nitrazepam, respec-
tively, in ethanol in 25-ml volumetric flasks.

_Internal standard solution_, 200 mg/liter. Pipette 5
ml of the standard solution of medazepam (1 g/liter)
into a 25-ml volumetric flask and dilute with ethanol
to give a concentration of 200 μg of medazepam per
milliliter of ethanol.

_Mixed drug standard solution_, 200 mg/liter. Add 5
mg of each of the following drugs to a 25-ml vol-
umetric flask and dissolve in ethanol: amitriptyline,
imipramine, n-desmethyl diazepam, desipramine,
protriptyline, medazepam, clomipramine, n-des-
methyl medazepam, “Ro5-3350,” nitrazepam, n-des-
methyl Ro5-3350, diazepam, n-desmethyl diazepam,
and chloridiazepoxide.

_Bis(trimethylsilyl) trifluoroacetamide_ (Pierce
Chemical Co., Rockford, Ill. 61105).

Procedure

The basic extraction procedure was the same for
each benzodiazepine and was a modified version of
that used by De Silva and Puglisi (3). No internal
standard was added to the blood when medazepam
was analyzed. Medazepam, 5 μg, was added as inter-
nal standard when diazepam or nitrazepam was being
extracted. The extracts were analyzed on a 1%
OV-17 column. If diazepam and nitrazepam were
present in the same specimen of blood, the two were
separated by using a 2% OV-1 column, because ni-
trazepam TMS, and diazepam are not separated on
1% OV-17. The method follows:

1. Pipette 5 ml of blood into a stoppered tube and
add 5 μg of medazepam as internal standard (for es-
timation of diazepam and nitrazepam only).

<table>
<thead>
<tr>
<th>Table 1. GLC Conditions for the Analysis of Some Basic Drugs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Support</td>
</tr>
<tr>
<td>(100-120 mesh)</td>
</tr>
<tr>
<td>Column length, cm</td>
</tr>
<tr>
<td>Column diameter, cm</td>
</tr>
<tr>
<td>Carrier gas</td>
</tr>
<tr>
<td>Inlet pressure, kg/cm²</td>
</tr>
<tr>
<td>Flow rate, ml/min</td>
</tr>
<tr>
<td>Column temp., °C</td>
</tr>
<tr>
<td>Detector temp., °C</td>
</tr>
<tr>
<td>Attenuation, A</td>
</tr>
<tr>
<td>Chart speed, cm/h</td>
</tr>
</tbody>
</table>

For concentrations of the order of 80 μg benzodiazepine/liter
of blood the attenuation must be increased to 2 × 10⁻¹⁰ A.

2. Add 5 ml of borate buffer (pH 9) and mix thor-
oughly.

3. Extract twice with 30-ml aliquots of ether by
mixing for 3-min intervals on a vortex-type mixer.

4. Separate the phases after each extraction by
centrifuging.

5. Combine the ether phases and discard the
aqueous phases.

6. Extract the benzodiazepines from the ether by
shaking for 2 min with 5 ml of 2 mol/liter hydrochlo-
ric acid.

7. Remove the lipid material from the acid phase
by shaking for 15 s with 10 ml of ether. This “clean-
ing” step was repeated twice, and the ether phases
were discarded.

8. Add 3 drops of bromothymol blue indicator to
the acid phase and then sodium hydroxide (2 mol/
liter) until the indicator turns blue, indicating that
the pH of the solution is about 8.

9. Extract the alkaline phase for 2-min and 1-min
intervals with 15-ml aliquots of ether. Combine the
two ether extracts.

10. Dry the pooled extract by passing the ether
through anhydrous sodium sulfate that had pre-
viously been washed with ether.

11. Concentrate the dried extract with a rotary
evaporator and then transfer to a stoppered centri-
fuge tube. Take to dryness under a stream of filtered
nitrogen in a water bath at 40 °C.

If either medazepam or diazepam is being estimat-
ed the extract is dissolved in 50 μl of ethanol and ali-
quotes are analyzed by GLC on a 1% OV-17 column.

If nitrazepam is being quantitated, form the TMS
derivative by adding 50 μl of BSTFA to the dried ex-
tract, stoppering the tube, and heating at 60 °C
for 15 min. Cool and analyze aliquots by GLC on a
column of 1% OV-17. (An aliquot is chromato-
graphed on 2% OV-1 to see if diazepam is present in
the extract). The 2% OV-1 column is used for the separation of diazepam and nitrazepam TMS.

Results

Identification of the benzodiazepines was based on the relative retention times of peaks obtained by GLC analysis on a 1% OV-17 column. Two percent OV-1 was used as a supplementary column to determine the concentration of nitrazepam in the presence of diazepam. Retention data for a variety of basic drugs are summarized in Table 2.

The retention times of some of the benzodiazepine metabolites likely to be encountered with the parent compound in the blood, and also of a number of commonly prescribed antidepressant drugs, was established. Aliquots (25 μl) of the mixed drug standard solution (1 mg of each drug per liter of blood) were added to four tubes, each containing 5 ml of blood, and analyzed as described in the procedure. In each analysis the retention times of the drugs extracted from the blood were the same as those of authentic standards when chromatographed on 1% OV-17 and 2% OV-1. Figure 1 shows that these substances are well separated on 1% OV-17, except for diazepam and nitrazepam TMS.

Patients on therapeutic doses of aspirin, amobarbital, secobarbital, phenobarbital, acetaminophen, or phenacetin had 1 μg of either medazepam, diazepam, or nitrazepam added to 5-ml aliquots of their blood, which were then analyzed as described in the procedure. These commonly prescribed drugs did not interfere with the quantitation of the benzodiazepines.

Before quantitation of medazepam, diazepam, and nitrazepam could be carried out, certain preliminary data were obtained.

First, the flame-ionization detector responses of these three benzodiazepines and nitrazepam TMS were determined over the range of 0–10 μg. The peak areas (peak height × width at half height) were plotted vs. concentration. Each drug showed a linear response, but the slopes varied, showing that the flame-ionization detector responses per unit of drug were each different. The TMS derivative of nitrazepam gave a greater detector response than did the parent compound; thus a lower concentration of nitrazepam in blood could be determined.

By measuring the concentration in blood and correcting for procedural losses, the concentration of medazepam was determined indirectly. Therefore it was necessary to know the percentage recovery of medazepam from blood when the described procedure was used.

Medazepam (range: 80–1000 μg/liter of blood) was added to 5 ml aliquots of blood, extracted and chromatographed on 1% OV-17. The peak areas were measured and the recovery was calculated as follows:

\[
\text{% recovery} = \frac{\text{concentration measured in blood}}{\text{concentration added to blood}} \times 100
\]

From the data given in Table 3 the mean recovery was 75.49%, the coefficient of variation 7.94%

In addition, medazepam was added to a control sample of blood and divided into 10 equal aliquots consisting of 5 ml of blood and 2.5 μg of medazepam (500 μg of medazepam per liter of blood). Six of the

![Table 2. Retention Data for Benzodiazepines and Some Other Basic Drugs]

<table>
<thead>
<tr>
<th>Drug</th>
<th>1% OV-17</th>
<th>Relative retention time</th>
<th>2% OV-1</th>
<th>Relative retention time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amitriptyline</td>
<td>2.6</td>
<td>0.68</td>
<td>2.4</td>
<td>0.92</td>
</tr>
<tr>
<td>Imipramine</td>
<td>2.7</td>
<td>0.71</td>
<td>2.6</td>
<td>1.00</td>
</tr>
<tr>
<td>N-Desmethyl diazepam TMS</td>
<td>3.1</td>
<td>0.82</td>
<td>2.7</td>
<td>1.04</td>
</tr>
<tr>
<td>Desipramine</td>
<td>3.5</td>
<td>0.92</td>
<td>2.6</td>
<td>1.00</td>
</tr>
<tr>
<td>Proptyltyline</td>
<td>3.6</td>
<td>0.95</td>
<td>2.6</td>
<td>1.00</td>
</tr>
<tr>
<td>Medazepam</td>
<td>3.8</td>
<td>1.00</td>
<td>2.6</td>
<td>1.00</td>
</tr>
<tr>
<td>Clomipramide</td>
<td>4.9</td>
<td>1.29</td>
<td>4.1</td>
<td>1.58</td>
</tr>
<tr>
<td>N-Desmethyl medazepam</td>
<td>5.4</td>
<td>1.42</td>
<td>3.0</td>
<td>1.15</td>
</tr>
<tr>
<td>R05-3350 TMS</td>
<td>6.2</td>
<td>1.63</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Nitrazepam TMS</td>
<td>7.4</td>
<td>1.95</td>
<td>5.3</td>
<td>2.04</td>
</tr>
<tr>
<td>Diazepam</td>
<td>7.7</td>
<td>2.03</td>
<td>4.2</td>
<td>1.62</td>
</tr>
<tr>
<td>N-Desmethyl medazepam</td>
<td>9.2</td>
<td>2.42</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>R05-3350</td>
<td>10.6</td>
<td>2.79</td>
<td>4.7</td>
<td>1.81</td>
</tr>
<tr>
<td>N-Desmethyl Diazepam</td>
<td>10.6</td>
<td>2.79</td>
<td>4.7</td>
<td>1.81</td>
</tr>
<tr>
<td>Chloridiazepoxide</td>
<td>11.3</td>
<td>2.98</td>
<td>4.9</td>
<td>1.89</td>
</tr>
<tr>
<td>R05-3350</td>
<td>18.5</td>
<td>4.86</td>
<td>6.3</td>
<td>2.42</td>
</tr>
<tr>
<td>Nitrazepam</td>
<td>27.8</td>
<td>7.31</td>
<td>8.7</td>
<td>3.35</td>
</tr>
</tbody>
</table>

a GLC conditions as described in Table 1.
b Relative to medazepam.
samples were extracted and chromatographed on the same day and the other four samples were analyzed at intervals of two or three days. This day-to-day and within-run precision of the method (Table 5), taken in conjunction with the recovery data given in Table 3, shows the accuracy and reproducibility of the method.

The ratio of the flame-ionization detector responses to known concentrations of medazepam and diazepam was determined. Diazepam (range 80–5000 μg/liter of blood) was added to 5-ml aliquots of blood which contained 5 μg of medazepam (1000 μg medazepam per liter of blood). The blood was extracted and chromatographed on 1% OV-17. The peak areas were measured and, because of the linearity of the detector response over a wide concentration of medazepam and diazepam, the ratio of equivalent quantities (1 μg) of these drugs was determined. This ratio takes into account procedural losses and the relative flame-ionization detector responses of the two drugs, which are slightly different. The use of an internal standard medazepam is a more desirable method of quantitation than the indirect procedure of making an allowance for the overall percentage recovery from the blood.

Medazepam was used as the internal standard for the quantitation of nitrazepam in blood. Nitrazepam (range: 200–2000 μg/liter of whole blood) was added to 5-ml aliquots of blood containing 5 μg of medazepam (1000 μg/liter blood), extracted and the TMS derivative of nitrazepam was formed. The ratio medazepam:nitrazepam TMS after chromatography on 1% OV-17 and 2% OV-1 was shown to be the same on both columns. This ratio can be applied to all future quantitations of nitrazepam in blood. Data pertaining to the quantitation of diazepam and nitrazepam are given in Table 4.

Day-to-day and within-run precision were measured by adding either diazepam or nitrazepam to two large control samples of blood, both of which contained medazepam as internal standard. These two samples were each divided into 10 5-ml aliquots containing 1 μg of diazepam (200 μg/liter of blood) and 1 μg of medazepam (200 μg/liter of blood) and 10 5-ml aliquots of blood containing 2 μg of nitrazepam (400 μg/liter of blood) and 1 μg of medazepam (200 μg/liter of blood). The bloods were analyzed six times the first day and on four other occasions at two- or three-day intervals. The results are given in Table 5.

During a six-month period, and using the methods described in the procedure, I performed the preliminary experiments to determine the percentage recovery of medazepam from whole blood and the relative flame ionization responses of diazepam and nitrazepam TMS relative to medazepam. It can be seen from Tables 3 and 4 that the results obtained during this time are reproducible, and taken in conjunction with the day-to-day and within-run precision data given in Table 5 show that the accuracy and reproducibili-

<p>| Table 3. Recovery of Medazepam from Whole Blood |</p>
<table>
<thead>
<tr>
<th>Added, μg/liter</th>
<th>Extracted</th>
<th>Recovery, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>80</td>
<td>61</td>
<td>76.2</td>
</tr>
<tr>
<td>80</td>
<td>51</td>
<td>63.7</td>
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<tr>
<td>200</td>
<td>152</td>
<td>76.0</td>
</tr>
<tr>
<td>200</td>
<td>153</td>
<td>76.5</td>
</tr>
<tr>
<td>200</td>
<td>158</td>
<td>79.0</td>
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<td>200</td>
<td>169</td>
<td>84.5</td>
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<tr>
<td>400</td>
<td>311</td>
<td>77.7</td>
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<td>400</td>
<td>316</td>
<td>79.0</td>
</tr>
<tr>
<td>500</td>
<td>398</td>
<td>79.6</td>
</tr>
<tr>
<td>500</td>
<td>365</td>
<td>73.0</td>
</tr>
<tr>
<td>1000</td>
<td>741</td>
<td>74.1</td>
</tr>
<tr>
<td>1000</td>
<td>869</td>
<td>86.9</td>
</tr>
<tr>
<td>1000</td>
<td>780</td>
<td>78.0</td>
</tr>
<tr>
<td>1000</td>
<td>690</td>
<td>69.0</td>
</tr>
<tr>
<td>1000</td>
<td>695</td>
<td>69.5</td>
</tr>
<tr>
<td>1000</td>
<td>660</td>
<td>66.0</td>
</tr>
<tr>
<td>1000</td>
<td>814</td>
<td>81.4</td>
</tr>
<tr>
<td>1000</td>
<td>748</td>
<td>74.8</td>
</tr>
<tr>
<td>1000</td>
<td>695</td>
<td>69.5</td>
</tr>
</tbody>
</table>

Mean = 75.49%
SD = 6.02%
CV = 7.94%

<p>| Table 4. Ratio of the FID Response of Diazepam and Nitrazepam TMS Relative to Medazepam after Extraction from Whole Blood and GLC on 1% OV 17 |</p>
<table>
<thead>
<tr>
<th>Diazezpam added, μg/liter</th>
<th>Ratio: diazepam/medazepam</th>
<th>Nitrazepam added, μg/liter</th>
<th>Ratio: nitrazepam TMS/medazepam</th>
</tr>
</thead>
<tbody>
<tr>
<td>80</td>
<td>1.070</td>
<td>200</td>
<td>0.431</td>
</tr>
<tr>
<td>200</td>
<td>0.820</td>
<td>400</td>
<td>0.600</td>
</tr>
<tr>
<td>200</td>
<td>0.906</td>
<td>1000</td>
<td>1.062</td>
</tr>
<tr>
<td>400</td>
<td>0.820</td>
<td>1000</td>
<td>0.527</td>
</tr>
<tr>
<td>400</td>
<td>0.841</td>
<td>2000</td>
<td>0.434</td>
</tr>
<tr>
<td>500</td>
<td>0.943</td>
<td>2000</td>
<td>0.623</td>
</tr>
<tr>
<td>1000</td>
<td>0.947</td>
<td>2000</td>
<td>0.589</td>
</tr>
<tr>
<td>1000</td>
<td>0.939</td>
<td>2000</td>
<td>0.682</td>
</tr>
</tbody>
</table>

Mean = 0.570
SD = 0.089
CV = 15.61%

* Flame ionization detector.
ty of the method is adequate for emergency analysis in a routine chemical pathology laboratory.

Figure 2a shows the chromatogram of the blood of a patient on medazepam therapy. The metabolites diazepam, n-desmethyl diazepam and n-desmethyl medazepam are also seen to be present. The medazepam measured represented an overall recovery of 75.49% after procedural losses. The concentration present in the blood was calculated to be 158 μg/liter of blood.

Figure 2b shows the chromatogram of the blood of a patient receiving a therapeutic dose of 5 mg of diazepam thrice daily. The metabolite n-desmethyl diazepam was present. The diazepam concentration was calculated by reference to the internal standard medazepam (1 mg/liter of blood).

The relative areas of the peaks in 0.5 ml blood are:

Medazepam  142 units = 0.5 μg (internal standard)
Diazepam   87 units

Equivalent concentrations of medazepam:diazepam give a ratio of 1.000:0.932 (Table 4)

\[
1 \text{ μg diazepam} = \frac{0.932 \times 284}{1000} = 264 \text{ units}
\]

264 units of diazepam = 1 μg
87 units of diazepam = 0.330 μg
0.33 μg diazepam is present in 0.5 ml of blood.

\[
\text{\textcircled{3}} \text{ the concentration is } 660 \text{ μg diazepam/liter of blood.}
\]

Figure 2c is the chromatogram of a specimen from a patient who had taken an overdose of nitrazepam the previous day. Four minutes after injection of the sample, the attenuation used was 2 × 10^{-19} A to 2 × 10^{-10} A, thus increasing the sensitivity × 5. The calculation of the concentration of nitrazepam was analogous to that of diazepam described previously and was based on the ratio of medazepam

<table>
<thead>
<tr>
<th>Day</th>
<th>Medazepam added, μg/liter</th>
<th>Recovery, %</th>
<th>Diazepam added, μg/liter</th>
<th>Diazepam*/Medazepam</th>
<th>Nitrazepam added, μg/liter</th>
<th>Nitrazepam TMS*/Medazepam</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 1</td>
<td>500</td>
<td>76.2</td>
<td>200</td>
<td>0.833</td>
<td>400</td>
<td>0.604</td>
</tr>
<tr>
<td>Day 2</td>
<td>500</td>
<td>72.0</td>
<td>200</td>
<td>0.987</td>
<td>400</td>
<td>0.521</td>
</tr>
<tr>
<td>Day 3</td>
<td>500</td>
<td>78.4</td>
<td>200</td>
<td>0.992</td>
<td>400</td>
<td>0.537</td>
</tr>
<tr>
<td>Day 4</td>
<td>500</td>
<td>72.8</td>
<td>200</td>
<td>1.035</td>
<td>400</td>
<td>0.575</td>
</tr>
<tr>
<td>Day 5</td>
<td>500</td>
<td>80.6</td>
<td>200</td>
<td>0.954</td>
<td>400</td>
<td>0.582</td>
</tr>
<tr>
<td>Mean</td>
<td>77.44</td>
<td>9.33</td>
<td>9.567</td>
<td>0.053</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SD</td>
<td>3.91</td>
<td>5.05</td>
<td>4.3</td>
<td>9.35</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CV, %</td>
<td>5.05</td>
<td>6.43</td>
<td>9.35</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Ratio of the flame-ionization detector responses of equivalent concentrations of benzodiazepines.

Fig. 2. (a) Basic extract of whole blood taken from a patient on a therapeutic dose of medazepam and chromatographed on 1% OV-17. The attenuation used was 2 × 10^{-19} A. 1. medazepam; 2, n-desmethyl medazepam; 3, diazepam; 4, n-desmethyl diazepam; and 5, lipid material. (b) Basic extract of whole blood taken from a patient on diazepam therapy. The separation was on 1% OV-17 and the attenuation used was 1 × 10^{-10} A. 1, medazepam (internal standard); 2, diazepam; and 3, n-desmethyl diazepam. (c) Basic extract of whole blood from a patient who had taken an overdose of nitrazepam. Nitrazepam TMS was formed and chromatographed on 1% OV-17. 1, medazepam (internal standard) and 2, nitrazepam TMS (internal standard) to nitrazepam TMS (1.000:0.570). The concentration in the blood was calculated to be 250 μg/liter of blood.

Discussion

In the methods described, a flame-ionization detector is used in the GLC analysis of benzodiazepines extracted from blood. Although not as sensitive as an electron-capture detector, it is less easily contaminated and the linearity of the flame-ionization detector responses of the drugs analyzed extend over a much wider range of concentration, a fact which makes it more useful than the electron-capture det-
tector when overdose concentrations of drugs in the blood are being quantitated. It is relatively trouble-free, making its use in a routine chemical pathology laboratory very acceptable.

Although patients on long-term diazepam therapy may have drug concentrations in their blood of the order of 1000 μg/liter, it is necessary to be able to detect at least 250 μg of diazepam per liter of blood. Toxic doses of medazepam and diazepam give low concentrations in blood; e.g., a single dose of 100 mg of diazepam will give a concentration on blood of the order of 250 μg/liter (8). Nitrazepam concentrations in toxic cases are relatively low, and <1000 μg/liter of blood is not conclusive evidence of overdose. Because of the possibility of low benzodiazepine concentrations found after some overdoses, the blood extract should be free from contaminants that could mask the drug peaks on GLC analysis. The described extraction procedure is time consuming, but essential for meaningful chromatograms that are relatively free from extraneous materials and will allow low blood concentrations of medazepam, diazepam, and nitrazepam after toxic overdoses to be measured by flame-ionization GLC.

The methods described will measure concentrations >80 μg medazepam or diazepam per liter of blood. This enables therapeutic concentrations to be measured. The lower limit of detection of nitrazepam is 200 μg/liter of blood.

Medazepam and diazepam are chromatographed intact. Acid hydrolysis of each will result in the formation of MACB. The presence of several of their metabolites in the blood would yield either MACB or ACB on hydrolysis, making the published procedures of De Silva et al. (1, 2) unsuitable because of the lack of specificity. Nitrazepam is chromatographed as nitrazepam TMS, a derivatization that is completed within 15 min at 60 °C. This is much quicker than the formation of 2-amino-5-nitrobenzophenone, which takes 1 h at 105 °C to complete (5).

It is necessary to derivatize nitrazepam before GLC, otherwise a chromatogram is obtained that shows a peak of poor shape, unsuitable for quantitation. Furthermore, the chromatogram is not reproducible, indicating some absorption of the nitrazepam on the column. The peak shape of nitrazepam TMS is symmetrical and the sensitivity of the determination is increased. This enables toxic drug concentrations to be measured by flame-ionization gas chromatography.

On reacting the dried extract with 50 μl of BSTFA at 60 °C for 15 min, more than 95% of the nitrazepam was converted to the TMS derivative. On-column derivatization of nitrazepam was tried, in order to speed up the quantitation. The extract (dissolved in acetone) and BSTFA were taken into the same syringe and injected onto the 1% OV-17 column. Only about 30% of the nitrazepam was converted to its TMS derivative, and even this was not consistent.

Figure 3 illustrates the structural formulas of several benzodiazepines and some of their metabolites found in the blood. When the methyl group is present at the N-1 position, as in medazepam and diazepam, no reaction occurs with BSTFA. Nitrazepam, Ro5-3350, n-desmethyl medazepam and n-desmethyl diazepam each have a hydrogen atom at the N-position, and this is replaced by the TMS group when reacted with BSTFA under suitable conditions.

No suitable internal standard was found that could be used in the determination of medazepam. Diazepam could not be used because it is a metabolite of medazepam that is found in the blood (3). “Ro5-3350” (7-bromo-1,3-dihydro-5-(2 pyridyl)-2H-1,4-benzodiazepin-2-one) was tried as an internal standard, but because it was not quantitatively extracted by the described procedure in the text, it was considered to be unsuitable. Although De Silva and Kaplan (9), after extraction and formation of 2-amino-5-bromo-benzoylpyridine, obtained an overall recovery of 61% when determined by electron-capture GLC, I was unable quantitatively to extract the Ro5-3350 from the blood. Nitrazepam was rejected as an internal standard for the determination of medazepam in blood for the following reasons: It would be necessary to form nitrazepam TMS for ac-
accurate quantitation and analysis would have to be carried out by using the 2% OV-1 column because of the presence of the medazepam metabolite, diazepam, in the blood. The possibility that other drugs with the same retention time on 2% OV-1 as medazepam could be present (Table 2) makes the use of nitrazepam as an internal standard undesirable.

Conversely, however, medazepam is used as the internal standard for determination of nitrazepam in the blood. Nitrazepam TMS is formed and GLC analysis is carried out on 1% OV-17. If diazepam is also present, it is separated from nitrazepam TMS on 2% OV-1 and the relative areas of the peaks give a ratio that can be applied to the combined peak on 1% OV-17, allowing the quantitation of each substance to be carried out.

The column of choice for the analysis of the benzodiazepines is 1% OV-17 because it has good separation properties for a large number of basic drugs, some of which have been added to blood and taken through the described analytical procedure. None of these were seen to interfere with the quantitation of medazepam, diazepam, and nitrazepam on 1% OV-17 (Table 2). 2% OV-1 will separate the benzodiazepines, but a number of anti-depressant drugs have the same retention time and are not separated from medazepam. As a supplementary column used to separate diazepam and nitrazepam TMS, 2% OV-1 serves a useful purpose.

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References