Diagnostic Performance of the EMIT-tox®
Benzodiazepine Immunoassay, FPIA Serum
Benzodiazepine Immunoassay, and Radioreceptor
Assay in Suspected Acute Poisoning*

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Abstract
We evaluated the diagnostic performance of the EMIT-tox serum benzodiazepine assay adapted to a Hitachi 717 analyzer (EMIT),
the Abbott ADx™ serum benzodiazepine fluorescence polarization immunoassay (FPIA), and a radioreceptor assay (RRA) in 113
patients with suspected acute poisoning. The reference method
was high-performance liquid chromatography with ultraviolet
detection after solid-phase extraction. For the discrimination
between negative and positive samples, the areas under the
receiver-operating characteristic (ROC) curves were 0.976, 0.991,
and 0.991 for EMIT (cutoff, 50-ng/mL diazepam), FPIA (cutoff,
12-ng/mL nordiazepam), and RRA (cutoff, 50-ng/mL diazepam),
respectively. For the discrimination between non-toxic and toxic
concentrations, the areas under the ROC curves were 0.896,
0.893, and 0.933, respectively. EMIT (with the cutoff lowered to
50 ng/mL), FPIA, and RRA can be reliably used to screen for the
presence of benzodiazepines in serum, but in many cases they
cannot discriminate between toxic and nontoxic concentrations.

Introduction
In many countries, benzodiazepines are involved in approx-
imately 50% of the acute suicidal poisonings (1–7). Laborato-
ries thus frequently receive requests for the determination of
benzodiazepines in serum, urine, or gastric lavage fluid. Toxic-
ological analysis of benzodiazepines in body fluids is compi-
lated by the diversity of benzodiazepines available on the
market and the fact that each product has a particular therape-
utic and toxic range. Numerous detection methods exist,
but not one of them is ideal. Chromatographic procedures
such as gas chromatography (GC) (8,9) and high-performance
liquid chromatography (HPLC) (10,11) allow the identifica-
tion and quantitation of the individual benzodiazepines, but
they require specialized personnel and instrumentation that is
rarely accessible on a 24-h basis. Radioreceptor assays (RRA)
(12) will give a result that is an integration of all the benzodi-
azepines and active metabolites present in the sample, but
they do not provide identification and are not suitable for use
in an emergency setting. Immunoassays such as EMIT (13,14)
and fluorescence polarization immunoassay (FPIA) (15,16) are
automated and provide fast results. The number of studies
that have compared immunoassays and chromatographic
methods for the determination of benzodiazepines in serum
of patients with suspected poisoning is very limited: we could
find only two articles for EMIT (13,17), one for the Du Pont
aca® (Du Pont, Wilmington, DE) (18), and one abstract for
FPIA (16). In these comparisons, the immunoassays seem to
have inferior diagnostic sensitivity (13,18), particularly the
EMIT assay when a cutoff at 300 ng/mL was used. The results
of such evaluations are dependent on the population examined.
Immunoassays will score better in a population that uses only
diazepam or nordiazepam (good cross-reactivity, concentra-
tions in the µg/mL range) than in a population that uses ria-
zolam or lorazepam (poor cross-reactivity, concentrations
in the ng/mL range). We compared the diagnostic performance
of two immunoassays, EMIT and FPIA, and RRA to detect
benzodiazepines in the serum of patients in whom an acute
poisoning was suspected. HPLC served as the gold standard.

Materials and Methods
Patients
Samples from 113 patients in whom overdose was suspected
were analyzed. These samples were sent to our laboratory for
toxicological analysis in cases of suspected accidental or

* Part of this study was presented at the 33rd International Congress on Forensic Toxicology (TIAFT), August 27–31, 1995, in Thessaloniki, Greece.
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intentional overdose. Samples for which only ethanol was requested were excluded from the study, as were samples with a volume less than 1 mL.

EMIT
An adaptation of the Behring EMIT-tox serum benzodiazepine assay (Behring Diagnostics, San Jose, CA) on a Hitachi 717 (Boehringer Mannheim, Germany) analyzer was used. The assay was calibrated semiquantitatively using Du Pont aca Barbiturates screen/benzodiazepine screen calibrator. Samples yielding results higher than 2000 ng/mL nordiazepam were analyzed again after dilution with benzodiazepine-free serum. The detection limit (zero calibrator plus three standard deviations) was determined to be 50 ng/mL of diazepam (19). Day-to-day coefficients of variation were lower than 10.2% (19).

FPIA
The Abbott ADx benzodiazepines serum reagent pack (Abbott Diagnostics Division, North Chicago, IL) was used on an ADx analyzer according to the manufacturer's instructions. Samples yielding results higher than 1000 ng/mL nordiazepam were analyzed again after dilution with benzodiazepine-free serum. The detection limit was 12 ng/mL according to the manufacturer.

RRA
The radioreceptor assay was a modification of the method used by Lund (20) and used 3H-flunitrazepam as a tracer. The modifications consisted of a decrease in the volume of receptor suspension so that the assay could be carried out in microtiter plates with incubation at 4°C over 90 min and filtration with a Skatron 96-well cell harvester (Skatron Instrument AS, Lier, Norway). The benzodiazepine receptor suspension was obtained from Research Biochemicals International (Natick, MA). The detection limit of the assay was 50-ng/mL diazepam (19).

HPLC
The benzodiazepines were determined by HPLC (21) after solid-phase extraction on Analytichem Bond Elut C2 columns (Varian, Harbor City, CA) (10). The columns were washed with 4 x 1 mL of methanol, 4 x 1 mL of water, 1 mL of acetone, and 1 mL of a solution consisting of 10% acetonitrile and 90% 0.1M K2HPO4 (pH 9.1). Five hundred microliters of plasma was added to 100 µL of internal standard (camazepam, 30 µg/mL) and 1 mL of 0.1M K2HPO4 (pH 9.1) and slowly (approximately 1 mL/min) passed through the extraction column. The column was washed with 1 mL of a solution consisting of 10% acetonitrile and 90% 0.1M K2HPO4 (pH 9.1). The benzodiazepines were eluted with 2 x 250 µL of methanol. The extracts were evaporated to dryness and reconstituted in 100 µL of mobile phase. Twenty microliters of the sample was injected into the HPLC system.

The separation was performed using a Chrompack Microsphere C18 column (10 x 0.46 cm, 3-µm particle size, Chrompack BV, Middelburg, The Netherlands) on an HPLC system consisting of a Waters 700 Satellite WISP autoinjector, a Waters 600E pump, and a Waters 490E programmable multiwavelength UV detector (Millipore Waters, Milford, MA). Integration was performed using Baseline 815 software. The mobile phase consisted of acetonitrile, methanol, 0.1M phosphate buffer (pH 2.7), and triethylamine (60:3:37:0.01). The flow rate was 1 mL/min. The detection limit was approximately 5 to 10 ng/mL for most benzodiazepines: 6 ng/mL for alprazolam, 4 ng/mL for flunitrazepam, and 8 ng/mL for triazolam (19).

Data analysis
The samples were classified in four groups according to the HPLC results: negative, low (i.e., benzodiazepines present at concentrations lower than the lowest therapeutic value), therapeutic (i.e., at least one benzodiazepine present at a therapeutic concentration), and toxic (i.e., at least one benzodiazepine present at a concentration greater than the highest therapeutic concentration). The therapeutic concentrations published by Schütz (22) were used (Table I).

The analysis of the receiver-operating (ROC) curves was performed according to Zweig and Campbell (23) and Henderson (24), using MedCalc software (Mariakerke, Belgium) (25).

<table>
<thead>
<tr>
<th>Benzodiazepine</th>
<th>Lowest therapeutic concentration (ng/mL)</th>
<th>Highest therapeutic concentration (ng/mL)</th>
<th>Number of positives in the samples</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Lower than therapeutic</td>
<td>Therapeutic</td>
<td>Toxic</td>
</tr>
<tr>
<td>Alprazolam</td>
<td>10</td>
<td>80</td>
<td>-</td>
</tr>
<tr>
<td>Bromazepam</td>
<td>80</td>
<td>170</td>
<td>1</td>
</tr>
<tr>
<td>Clobazam</td>
<td>100</td>
<td>400</td>
<td>1</td>
</tr>
<tr>
<td>Clonazepam</td>
<td>30</td>
<td>60</td>
<td>2</td>
</tr>
<tr>
<td>Clotiazepam</td>
<td>100</td>
<td>400</td>
<td>-</td>
</tr>
<tr>
<td>Delorazepam</td>
<td>10</td>
<td>60</td>
<td>-</td>
</tr>
<tr>
<td>Desalkylflurazepam</td>
<td>40</td>
<td>150</td>
<td>-</td>
</tr>
<tr>
<td>Desmethylclobazam</td>
<td>2000</td>
<td>3000</td>
<td>-</td>
</tr>
<tr>
<td>Desmethyldiazepam</td>
<td>200</td>
<td>800</td>
<td>4</td>
</tr>
<tr>
<td>Diazepam</td>
<td>500</td>
<td>750</td>
<td>10</td>
</tr>
<tr>
<td>Flunitrazepam</td>
<td>5</td>
<td>15</td>
<td>-</td>
</tr>
<tr>
<td>Flurazepam</td>
<td>1</td>
<td>10</td>
<td>-</td>
</tr>
<tr>
<td>Lorazepam</td>
<td>20</td>
<td>200</td>
<td>-</td>
</tr>
<tr>
<td>Lorometazepam</td>
<td>2</td>
<td>10</td>
<td>-</td>
</tr>
<tr>
<td>Oxazepam</td>
<td>1000</td>
<td>2000</td>
<td>14</td>
</tr>
<tr>
<td>Temazepam</td>
<td>350</td>
<td>850</td>
<td>2</td>
</tr>
<tr>
<td>Triazolam</td>
<td>2</td>
<td>10</td>
<td>-</td>
</tr>
</tbody>
</table>

Total 34 32 51 117

* Twenty-seven samples contained more than one benzodiazepine; therefore, the total incidence is higher than 53. The highest and lowest therapeutic concentrations used to classify the samples are also shown.
Results

HPLC analysis provided the following results: 50 sera (44%) contained no benzodiazepines, 7 sera (6%) had lower than therapeutic levels, 17 (15%) were within the therapeutic range, and 39 (35%) contained toxic levels. Table I shows the frequency with which each benzodiazepine was detected and in what concentration it was present.

Discrimination between negative and positive samples

The ROC curves for the discrimination between negative and positive samples are shown in Figure 1A. The areas under the curve are 0.976 (confidence interval, 0.927–0.995), 0.991 (0.951–0.999), and 0.991 (0.961–0.999) for EMIT, FPIA, and RRA, respectively. The differences between the areas under the ROC-curves obtained with the different techniques were not statistically significant (EMIT versus FPIA: P = 0.093; EMIT versus RRA: P = 0.198; FPIA versus RRA: P = 0.892). The optimal cutoff values were calculated for the hypothesis that the cost of a false-positive result is equal to the cost of a false-negative result. The cutoff values were 73-, 22-, and 50-ng/mL (nor)diazepam equivalents for EMIT, FPIA, and RRA, respectively (Table II). If the cutoff of 300 ng/mL is used for EMIT, as suggested by the manufacturer, only 65% of the samples are correctly classified (sensitivity 38%, specificity 100%). In the false-negative samples, there was a sample that contained 8 ng/mL triazolam (EMIT and FPIA negative) and two samples that contained lorazepam (8 and 21 ng/mL) that were negative by EMIT. The false-positive samples included one extremely lipemic sample (positive by FPIA) and two samples that contained zolpidem, an imidazopyridine that binds to the benzodiazepine receptor (only positive by RRA). The other false positives could not be explained.

Discrimination between toxic and nontoxic concentrations

Figure 1B shows ROC curves for the discrimination between toxic and nontoxic concentrations of benzodiazepines. The areas under the curves are 0.896 (0.824–0.945), 0.893 (0.821–0.943), and 0.933 (0.870–0.971) for EMIT, FPIA, and RRA, respectively. In Table III, the “optimal” cutoffs are compared for the three techniques. At these cutoffs, 19, 18, and 16 samples are misclassified by EMIT, FPIA, and RRA, respectively. The differences between the areas under the ROC curves

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**Table II. Comparison of EMIT, FPIA, and RRA Versus the Reference Method (HPLC) for the Discrimination between Negative and Positive Samples (n = 113)**

<table>
<thead>
<tr>
<th>Analytical method</th>
<th>EMIT</th>
<th>FPIA</th>
<th>RRA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cutoff (ng/mL (nor)diazepam equivalents)</td>
<td>73</td>
<td>22</td>
<td>50</td>
</tr>
<tr>
<td>Number of false positives</td>
<td>1</td>
<td>1</td>
<td>5*</td>
</tr>
<tr>
<td>Number of false negatives</td>
<td>3</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Sensitivity (%)</td>
<td>95</td>
<td>98</td>
<td>95</td>
</tr>
<tr>
<td>Specificity (%)</td>
<td>98</td>
<td>98</td>
<td>90*</td>
</tr>
<tr>
<td>Predictive value of a positive result (%)</td>
<td>98</td>
<td>98</td>
<td>91</td>
</tr>
<tr>
<td>Predictive value of a negative result (%)</td>
<td>94</td>
<td>98</td>
<td>95*</td>
</tr>
</tbody>
</table>

* Two samples contained zolpidem, an imidazopyridine that binds on the benzodiazepine receptor (34).

**Table III. Comparison of EMIT, FPIA, and RRA Versus the Reference Method (HPLC) for the Discrimination Between Toxic and Nontoxic Concentrations (n = 113)**

<table>
<thead>
<tr>
<th>Analytical method</th>
<th>EMIT</th>
<th>FPIA</th>
<th>RRA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cutoff (ng/mL (nor)diazepam equivalents)</td>
<td>122</td>
<td>47</td>
<td>740</td>
</tr>
<tr>
<td>Number of false positives</td>
<td>13</td>
<td>16</td>
<td>3</td>
</tr>
<tr>
<td>Number of false negatives</td>
<td>6</td>
<td>2</td>
<td>13</td>
</tr>
<tr>
<td>Sensitivity (%)</td>
<td>85</td>
<td>95</td>
<td>67</td>
</tr>
<tr>
<td>Specificity (%)</td>
<td>82</td>
<td>78</td>
<td>95</td>
</tr>
<tr>
<td>Predictive value of a positive result (%)</td>
<td>72</td>
<td>70</td>
<td>90</td>
</tr>
<tr>
<td>Predictive value of a negative result (%)</td>
<td>90</td>
<td>96</td>
<td>84</td>
</tr>
<tr>
<td>Rule-out cutoff (number of false positives)</td>
<td>70</td>
<td>30</td>
<td>110</td>
</tr>
<tr>
<td>(number of false positives)</td>
<td>(25)</td>
<td>(19)</td>
<td>(22)</td>
</tr>
<tr>
<td>Rule-in cutoff (number of false negatives)</td>
<td>400</td>
<td>650</td>
<td>740</td>
</tr>
<tr>
<td>(number of false negatives)</td>
<td>(21)</td>
<td>(21)</td>
<td>(15)</td>
</tr>
</tbody>
</table>
obtained with the different techniques are not statistically significant (EMIT versus FPIA: \( P = 0.851 \); EMIT versus RRA: \( P = 0.140 \); FPIA versus RRA: \( P = 0.062 \)). We therefore determined rule-out (the value at which the sensitivity is 95%) and rule-in cutoffs (specificity is 95%). Results lower than the rule-out cutoff are unlikely to correspond to toxic concentrations. Results higher than the rule-in cutoff are very likely to correspond to toxic concentrations. These cutoffs are shown in Table III.

**Discussion**

In our survey of serum samples from 113 subjects with suspected acute intoxication, we found benzodiazepines in 56% of the samples. This percentage is in agreement with a previous survey of acute poisonings in our emergency department (26) and in many other centers (1,4,5,7). We found toxic concentrations of benzodiazepines in 35% of the samples (62% of the positive samples).

The benzodiazepines that were found are, in decreasing order of frequency, nordiazepam, diazepam, oxazepam, bromazepam, desalkylflurazepam, and lormetazepam. Other authors have also observed that nordiazepam and diazepam are the most frequently found (11,27).

Our results show that non-chromatographic techniques can make a good discrimination between negative and positive samples. FPIA (cutoff of 22-ng/mL nordiazepam equivalents) gave the best results with one false negative and one false positive. With EMIT (cutoff of 73-ng/mL diazepam equivalents), there were three false negatives and one false positive. For EMIT, these results are much better than most other published studies, which used a cutoff of 300 ng/mL as suggested by the manufacturer. The number of false negatives varied between 13% (16) and 100% (therapeutic concentrations of alprazolam) (28). By lowering the cutoff from 300 to 73 ng/mL, we reduced the number of false negatives from 39 to 3 without increasing the number of false positives. Hallbach and Guder (17) observed a better sensitivity of the EMIT serum benzodiazepine assay without decreasing the specificity by using a cutoff of 50 ng/mL. RRA (cutoff of 50-ng/mL diazepam equivalents) yielded one false-negative sample and five false positives, two of which were caused by zolpidem. The high number of false positives with RRA is somewhat surprising. The false positives could be explained by the presence of low concentrations of potent benzodiazepines or metabolites that were not detected by the other techniques.

The areas under the ROC curves were higher than 0.976, which confirmed the good discriminating power of the tests according to the criteria of Swets (29) (area > 0.9: good discriminating power). This good performance can partly be explained by the high prevalence of diazepam and nordiazepam in our samples. These benzodiazepines are present in higher concentrations in the serum, and the immunoassays have a higher cross-reactivity for them. If a majority of our patients had taken triazolam or lormetazepam, more false negatives would have occurred. One could ask if determination of benzodiazepine concentrations in blood from acutely poisoned patients is really needed. No correlation could yet be found between the benzodiazepine concentrations in serum or plasma and the clinical status of the patient (30,31). However, benzodiazepine use is so prevalent in the population (32) that finding benzodiazepines does not imply that they were ingested in the acute poisoning episode, but that they represent the chronic therapy of the patient. In our samples, 21% contained therapeutic or less than therapeutic concentrations of benzodiazepines. In many clinical cases it will be sufficient to do a qualitative analysis to confirm or exclude the presence of benzodiazepines and to give sufficient information for the acute management of the patient, but in a number of cases more information will be needed and a frequent question is whether the concentrations are therapeutic or toxic. In these individual cases (when the patient characteristics and history can be taken into account for the interpretation), semi-quantitative analysis of benzodiazepines in serum and the determination of the absence or presence of toxic concentrations can be relevant to the management of the patient. We therefore tried to determine if a semiquantitative result of an immunoassay or RRA could be used to determine if toxic concentrations of benzodiazepines are present.

EMIT, FPIA, and even RRA do not discriminate well between nontoxic and toxic concentrations of benzodiazepines. The areas under the ROC curve are lower than 0.9, except for RRA where it is 0.933. At the “optimal” cutoff, 17, 16, and 14% of the samples are misclassified with the respective techniques. This is not unexpected for immunoassays because the affinity of the antibodies for the different benzodiazepines is not proportional to the potency of the molecule. In general, immunoassays have a lower cross-reactivity for the more potent benzodiazepines (e.g., triazolam, lormetazepam). We were surprised to see that RRA discriminates only marginally better than the immunoassays. A possible explanation lies in the great diversity of the therapeutic values for benzodiazepines that does not disappear when the correction is made for the receptor affinity by comparing the RRA results (in nanogram-per-milliliter diazepam equivalents) that correspond to the therapeutic values. Therapeutic values for bromazepam (1–10 ng/mL) would yield 60–88-ng/mL diazepam equivalents in the RRA, whereas therapeutic values for lorazepam would yield 145–673-ng/mL diazepam equivalents. Other explanations are the different indications of the benzodiazepines (anxiolytic, hypnotic, and anti-epileptic), the differences in protein binding, and the presence of metabolites. Moreover, there is no consensus on the therapeutic concentrations of benzodiazepines. For diazepam, according to Schütz (22), the therapeutic values are 500–750 ng/mL, and toxic concentrations start at 1000 ng/mL. Tietz (33) considers 100–1000 ng/mL therapeutic; toxic concentrations start at 5000 ng/mL.

Trying to determine if a sample contains toxic or nontoxic concentrations of benzodiazepines with immunoassay or RRA alone does not seem feasible. Use of the rule-in and rule-out cutoffs allows the classification of a result as very likely or unlikely to be toxic. With this approach, 36, 31, and 26% of the results determined by EMIT, FPIA, and RRA, respectively, remain in the gray zone (between the rule-out and the rule-in cutoffs).
For these samples, consultation between the toxicologist and the clinician would be needed to interpret the results and determine if chromatographic analysis is needed. Another approach is to screen all samples with immunoassay and to perform chromatographic analysis on all samples that are above the detection limit. In this way, only a few false-negative samples would be missed.

Conclusion

Our study shows that, in a population of patients with suspected poisoning, EMIT (cutoff, 73-ng/mL diazepam), FPIA (cutoff, 22-ng/mL nordiazepam), or RRA (cutoff, 50-ng/mL diazepam) can be reliably used to screen for the presence of benzodiazepines in serum. Only a few false negatives and false positives were observed with each method. However, these methods cannot be used to discriminate between toxic and nontoxic concentrations. This requires the use of a chromatographic method.

Acknowledgments

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References


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