Drug Screening by Enzymatic Immunoassay with the Centrifugal Analyzer

Alan Broughton and Doris L. Ross

We describe a method for determination of barbiturates, amphetamines, opiates, and methadone by the "enzyme multiplied immunoassay technique" (EMIT) applied to the centrifugal analyzer. This method gives results that agree well (92% for barbiturates, 97% for opiates, 94% for methadone, and 96% for amphetamines) with those obtained by the same technique with the Syva-Gilford EMIT instrumentation. Its advantages include use of half as much of the expensive reagents; capability of batch operation, which removes the need for continual attention; and more nearly complete automation. For individual samples it is as fast as the manual method; for large numbers of specimens it is faster.

The "enzyme multiplied immunoassay technique" (EMIT) (1, 2) for drug screening affords a rapid, sensitive (3), and relatively specific method and for these reasons it has gained acceptance in many drug-screening clinics. The simplicity of the technique makes it preferable to those assays developed for a continuous-flow analyzer (4, 5) and its sensitivity gives it an advantage over thin-layer chromatographic methods (6, 7). The cost of reagents is a disadvantage (6).

Enzyme activity is used for detection in the EMIT system; therefore, this appeared to be an appropriate technique for the centrifugal analyzer. The methods for barbiturates, opiates, methadone, and amphetamine were chosen for adaptation to this type of instrumentation. Our approach was to investigate the effect of centrifugal movement upon the bacterial suspension, to study the kinetics of the reaction under various conditions, and then to compare the results obtained by the Drug Urine Analysis Laboratory at the Texas Research Institute of Mental Sciences (TRIMS) in the Texas Medical Center, Houston, with those obtained by this method in our laboratory. The TRIMS laboratory uses the Syva-Gilford instrumentation described elsewhere (2).

Materials and Methods

Specimens

Human urines that had been screened for one or more of the drugs to be assayed were obtained from the Drug Urine Analysis Laboratory of TRIMS. These specimens were identified by number and the results were not known to the operator. On receipt in our laboratory, the specimens were centrifuged and the pH was adjusted, if necessary, to 5.5 to 8.0 just before assay. Those specimens that could not be assayed on the same day as they were received were refrigerated at 5-8 °C and were assayed within six days. The inherent enzyme activity of each urine was determined and the assay values were corrected on all specimens that gave positive results.

Reagents

The EMIT reagents (Syva Corp., Palo Alto, Calif. 94304) were reconstituted and stored according to the manufacturer's directions. These reagents are: the antibodies to the drug in solution, a solution of lysozyme (EC 3.2.1.17) conjugated with the drug, lyophilized bacteria (M. luteus), concentrated buffer, and four calibrators, each containing all four drugs.

The University of Texas Medical School, Hermann Hospital, 12003 Ross Sterling Ave., Houston, Tex. 77025.
Presented in part at the 26th National Meeting of the AACC, August 20, 1974, Las Vegas, Nev.
Received Aug. 24, 1974; accepted Nov. 5, 1974.
The calibrators are reconstituted (distilled water) to give negative (0 µg/ml), low (0.5 or 1.0 µg/ml, depending on the drug), medium (5.0 µg/ml), and high (50.0 µg/ml) concentrations. The principle of the EMIT technique and one set of these reagents has been described previously (2).

Apparatus

GEMSAEC Analyzer System, Serial No. 2114 (Electro-Nucleonics, Inc. 368 Passaic Ave., Caldwell, N. J. 07006). The principle and design of the centrifugal analyzer have been described previously (8).

Rotoloader III, Serial No. 2157 (Electro-Nucleonics, Inc.), and automatic loader that includes a pick-up and dispenser unit (Micromedic Systems, 1312 Meridian St., Huntsville, Ala. 35810), which is used manually for this procedure with the foot switch for loading the Teflon rotor.

Constant-temperature circulating water bath (at 37 ± 0.02 °C) Lauda Serial K2/R, Type 11739 (Lauda Instruments Division, Brinkmann Instruments, Inc., Westbury, N. Y. 11590) connected to the GEMSAEC analyzer.

Thermometers: For temperatures 42 °C and below we used a “TRI-R” Thermistor, Model TML (TRI-R Instruments, Rockville Centre, N. Y., and Electro-Nucleonics, Inc.), which has a range of +20 to +45 °C with a precision of ±0.3 °C. For temperatures above 42 °C we used a “Y.S.I. Telethermometer,” Model 43TC, with a range of +15 to 100 °C and a precision of ±0.8 °C. The probes for these thermometers were inserted into the water jacket surrounding the cuvettes in the GEMSAEC analyzer module.

Procedure

The procedure we adopted after studying the effect of varying the volume of reagents and time increment is as follows:

Using the Micromedic unit containing a 50-µl sample pump and 200-µl flush pump, deposit the following volumes of reagents in the specified wells of the Teflon rotor of the GEMSAEC analyzer:

25 µl of EMIT Reagent A (antibody) flushed by 116 µl of buffer into well C of the rotor, 25 µl of urine sample (or calibrator) flushed by 116 µl of buffer into well C of the rotor, and 25 µl of EMIT Reagent B (enzyme–drug conjugate) flushed by 116 µl of buffer into well B of the rotor.

Deliver 100 µl of bacterial suspension with an Eppendorf pipet into well C of the rotor.

Before reagents are added, keep the rotor at 37 °C for at least 10 min; after the additions and before starting the reaction, incubate it at 37 °C for 5 min. Determine the enzyme activity on the analyzer by using the parameters indicated in Table 1.

Record a positive result when the difference in absorbance between the 10-s and 50-s readings is equal to or greater than that of the calibrator chosen as the “cut-off” shown in Table 2.

Results

Analytical Variables

Stability of bacterial suspension. We showed that the bacterial suspension was stable during the centrifugal movement of the analyzer by performing an analysis without the presence of enzyme. The maximum change in absorbance during the 40-s period was a decrease of 0.003 units, with a mean of 0.002 units. This was so for bacterial suspensions that had initial absorbances of 0.714, 0.592, and 0.423 units in the 1-cm cuvet at 436 nm. Analyzer rotation (2900 rpm for 8 s and 480 rpm for the duration of the run) was insufficient to cause sedimentation of the bacteria within the time of the assay.

Effect of substrate concentration. The minimal concentration of bacteria in suspension allowing maximal activity had an absorbance of 0.75 at 436 nm. Figure 1 shows the activities of the opiate-conju-
gated enzyme and the barbiturate-conjugated enzyme in the presence of their respective antibodies and the 50 μg/ml calibrators.

**Effect of temperature and antibody.** The reaction between the lysozyme and *M. luteus* suspension was recorded for 105 s. The change in absorbance was found to be linear up to 50 s at 37 °C. Table 3 shows the initial rate of the reaction in 5 °C increments from 32 to 52 °C in the absence of antibody, in the presence of antibody, and with both antibody and free hapten (opiate) present. The rates increased with increasing temperature, but at 52 °C declined sharply. Inhibition of activity by anti-opiate antibody was maximal at 48 °C and restoration of activity in the presence of free hapten is maximal at that temperature if one discounts the 102% restoration at 52 °C. We elected to perform our assays at 37 °C, in accord with the cooperating laboratory, although 48 °C appears to be an attractive alternative for this procedure.

**Effect of urea concentration.** Urea is known to cause conformational changes of some proteins and has been reported to interfere in antigen-antibody precipitin reactions in as little as 0.2 mol/liter (9). There are two proteins that have functions important to this reaction; therefore we studied the effect of a high concentration of urea on the results of an opiate assay. A urine specimen that was negative for opiates and another that was positive were re-assayed after making the urea concentration of each urine 3 mol/liter. The absorbance was measured over a 105-min period by the method described. The change in absorbance over the first 40 s was 0.073 for the positive specimen, before and after the addition of urea. The negative specimen had a change of absorbance of 0.018 before urea was added and 0.019 after. Because there was no real difference, we conclude that the presence of 3 mol/liter urea does not interfere in the opiate EMIT test.

**Comparison of Results**

The specimens that had been analyzed for one or more of the drugs at TRIMS were analyzed in our laboratory on the GEMSAEC by the method described. Table 4 shows the results of this comparison study. Whenever specimen volume permitted, thin-layer chromatography (10) and a fluorescent method for morphine (11) were used to check differing results between the laboratories. If the referee method agreed with our results, the TRIMS result was entered as being that of the referee method. If the referee result agreed with TRIMS it was entered as TRIMS reported it. For the purpose of this study, only positive and negative results were compared, because no nearby facility could be located that quantitated the results by the EMIT system. The concentrations used by investigators for classifying results as positive or negative appear to vary. Table 2 shows the values above which samples have been classified as positive by the TRIMS laboratory, another local laboratory, and from the literature (6, 12). During this study the “cut-off” value for opiates was changed by the TRIMS laboratory from the 1 μg/ml listed in Table 2 to 0.5 μg/ml and our “cut-off” value was adjusted accordingly.

**Discussion**

The EMIT method can be used on the GEMSAEC for drug screening. The bacterial suspension is stable and the reaction can be monitored in the method we have adapted.

It became obvious to us during this study that the level of a cut-off value is critical. Drug-screening laboratories in this area determine the cut-off level by feedback from the counselors in the drug-screening clinics. In at least one hospital in this area, one cut-off value is used for the specimens from the drug-screening clinic and another for specimens from the emergency room and hospital patients. The EMIT system for drug screening, both manually and by the method described here, is in our opinion best used to detect the presence of drugs rather than their quantitation.

Mulé et al. (6) compared EMIT results with those of thin-layer chromatography. Using the thin-layer chromatography as the true result, they found 5.1%, 5.6%, 2.6%, and 12.5% “false” positives for barbiturates, opiates, methadone, and amphetamines, respectively, for the EMIT method. The “false” negatives they reported were 0.2%, 0.1%, 2.3%, and 0.7%, respectively. We found the EMIT system on the GEMSAEC to give, when compared with thin-layer chromatography, 2%, 0.9%, 0%, and 0.5% “false” positives, and 3.7%, 0%, 3.6%, and 0.2% “false” negatives.

---

**Table 3. Effect of Temperature on Initial Reaction Rates**

<table>
<thead>
<tr>
<th>Temp., °C</th>
<th>ΔA/40 s</th>
</tr>
</thead>
<tbody>
<tr>
<td>32</td>
<td>0.072</td>
</tr>
<tr>
<td>37</td>
<td>0.082</td>
</tr>
<tr>
<td>42</td>
<td>0.094</td>
</tr>
<tr>
<td>48</td>
<td>0.115</td>
</tr>
<tr>
<td>52</td>
<td>0.083</td>
</tr>
</tbody>
</table>

A. Enzyme and bacteria | 0.072 | 0.082 | 0.094 | 0.115 | 0.083 |
B. Enzyme, bacteria and antibody ( % activity) | 0.025 | 0.015 | 0.020 | 0.019 | 0.019 |
C. Enzyme, bacteria, antibody and free hapten ( % activity) | 0.063 | 0.077 | 0.084 | 0.112 | 0.085 |

---

**Table 4. Comparison of EMIT Results by Two Different Systems**

<table>
<thead>
<tr>
<th>Drug</th>
<th>T+ H+</th>
<th>T+ H-</th>
<th>T+ H-</th>
<th>T- H+</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Barbiturates</td>
<td>41</td>
<td>226</td>
<td>16</td>
<td>9</td>
<td>292</td>
</tr>
<tr>
<td>Opiates</td>
<td>198</td>
<td>98</td>
<td>6</td>
<td>3</td>
<td>305</td>
</tr>
<tr>
<td>Methadone</td>
<td>77</td>
<td>81</td>
<td>3</td>
<td>169</td>
<td></td>
</tr>
<tr>
<td>Amphetamine</td>
<td>5</td>
<td>186</td>
<td>4</td>
<td>198</td>
<td></td>
</tr>
</tbody>
</table>

*T, results by Drug Urine Analysis Laboratory or reference method; H, our GEMSAEC results.*
for barbiturates, opiates, methadone, and amphetamines, respectively. Mulé et al. indicate that the increased sensitivity of detection by EMIT contributed to their number of false positives when compared to the less-sensitive thin-layer chromatographic method. The difference between our percentage of “false” negative and positive values and theirs may reflect use of different cut-off values for the EMIT, the use of a different thin-layer chromatographic method, and confirmation of opiates by a fluorescence procedure. There is very good agreement between the Syva–Gilford system at TRIMS and the GEMSAEC: 92% for barbiturates, 97% for opiates, 94% for methadone, and 96% for amphetamines.

One of the disadvantages of the EMIT method is the high cost of reagents. The cost of each test, calculated from prices for the EMIT assay kits for 100 assays, calibrators, concentrated buffer, bacteria and chart paper but excluding instrument cost and technologist’s time, is approximately $1.00 per test when run according to the manufacturer’s instructions. In the method described here, one-half the volume of the expensive reagents is required, and so each test costs about $0.50.

The amount of technical involvement is also decreased with the use of automation and in large drug-screening clinics results can be produced at the rate of 1.75 min per test. The operator time can be reduced even more by the incorporation of the Rotor-loader IV (Electro-Nucleonics, Inc.) automated sample- and reagent-dispenser.

The combination of an enzyme immunoassay system with a centrifugal analyzer presents an attractive alternative to the somewhat laborious and potentially hazardous immunoassay procedures in which radioisotopes are used.

We thank Dr. David C. Fennimore and Ms. Bobette Zettel of the Drug Urine Analysis Laboratory, TRIMS, for supplying the urine specimens and their data; to Dr. F. M. Urry, The University of Texas Medical Branch, Galveston, for performing several of the thin-layer chromatographic assays; and to Mr. Paul Nelson, M.T. (ASCP), for his technical assistance.

References