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E. L. Slightom, [1] *M.S.*

The Analysis of Drugs in Blood, Bile, and Tissue with an Indirect Homogeneous Enzyme Immunoassay

For the past six or seven years analytical methods using antibodies have become an area of serious interest in clinical laboratories, and, as evidenced by the content of recent meetings and publications [*1–4*], such methods have promise in forensic science.

Presented here is work we have done for the past two years concerning the adaptation of the enzyme multiplied immunoassay technique (EMIT®) drug abuse urine assay to the analysis of drugs in blood, bile, and tissues. EMIT is a homogeneous immunoassay designed to provide a direct and rapid method for the detection of drugs, primarily in urine and serum. The assay principles and techniques have been described in detail by the manufacturer (Syva Corporation, Palo Alto, Calif.) [*5–7*]. The method of detection prevents the direct analysis of blood, bile, and tissues. As a result the assay has been primarily the tool of the clinical and drug abuse screening laboratory and has been limited in forensic toxicology to urine screening. The EMIT serum assay is primarily limited to antiepileptic drugs.

The assay has been extended in our laboratory to the analysis of whole blood, bile, and tissue through the sampling of reconstituted organic solvent extracts. This modification has the immediate advantage of controlling the concentration of the drug or drugs and would provide a cleaner and more controlled matrix for analysis. This has proved very helpful in some day-to-day problems encountered in our laboratory.

Experimental Procedure

Reagents and Solutions

Lysozyme with attached drug was supplied for all drugs except tetrahydrocannabinol (THC) with no alterations by the Syva Corp. (Palo Alto, Calif.). For THC, maleate dehydrogenase was used as supplied by the Syva Corp. and the National Institute of Drug Abuse (NIDA) (Rockville, Md.). Antibodies for all drugs except THC were supplied by Syva Corp. and were used as supplied. The THC antibody was supplied by the Syva Corp. and NIDA. In all cases the buffers used were supplied by the Syva Corp.

The standard lysozyme solution was composed of 3.24 mg Muramidase® [the mucopeptide *N*-acetylmuramyl hydrolase, supplied by Sigma Chemical Co. (No. L-6876) as 3 × crystallized, dialyzed, and lyophilized powder] per 100 ml of EMIT buffer. The extraction reagents were as follows:

(1) chloroform, used as supplied by Matheson-Coleman-Bell;
(2) anhydrous ether, used as supplied by Fisher (No. E-138);

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[1] Forensic toxicologist, Toxicology Section, Georgia Crime Laboratory, Atlanta, Ga. 30301.

(3) 0.13*N* sodium hydroxide, 5.12 g/litre;
(4) 0.067*M* disodium hydrogen phosphate, 9.5 g/litre;
(5) 4% sodium bicarbonate, 4 g/litre;
(6) 100% reagent-quality ethanol, used as supplied by U.S. Industrial Chemical Co.;
(7) hexane, used as supplied by Fisher (No. H-292);
(8) methanolic potassium hydroxide, prepared by dissolving 37 g potassium hydroxide in 20 ml of water and 100 ml of methanol; and
(9) 0.5*N* sulfuric acid, 14.4 ml/litre.

Standard Drug Solutions

Drugs were prepared in distilled water over the following range of concentrations (the EMIT sensitivity was used as a guide):

(1) phenobarbital, 1 to 10 μg/ml;
(2) pentobarbital, 1 to 10 μg/ml;
(3) morphine sulfate, 0.25 to 12 μg/ml, calculated as free base;
(4) codeine sulfate, 0.05 to 2 μg/ml, calculated as free base;
(5) hydromorphone hydrochloride, 0.25 to 10 μg/ml, calculated as free base;
(6) meperidine hydrochloride, 25 to 100 μg/ml, calculated as free base;
(7) cocaine hydrochloride, 1 to 10 μg/ml, calculated as free base;
(8) nordiazepam, 0.25 to 10 μg/ml;
(9) diazepam, 0.5 to 10 μg/ml; and
(10) propoxyphene hydrochloride, 1 to 10 μg/ml, calculated as free base.

For the THC assay (maleate dehydrogenase), pure Δ^9-THC in ethanol (10 mg/ml) was diluted with pure ethanol to a concentration of 1 μg/ml. Dilution of this solution was made with a 5% ethanol-water mixture to the concentration range needed (10 to 200 ng/ml).

Equipment

The spectrophotometer was a Gilford Stasar III equipped with a temperature control flow cell; for lysozyme, the wavelength was set at 436 nm, and for maleate dehydrogenase the wavelength was set at 340 nm. A Monroe 1305 timer-printer was used.

Extraction

To extract barbiturates, blood (5 ml) and homogenized tissue (5 g), adjusted to pH 7 or less, were extracted with chloroform (50 ml), washed with 4% sodium bicarbonate (10 ml) and then back extracted with 0.13*N* sodium hydroxide (5 ml). The aqueous phase was separated and the pH was adjusted to 7 or less before the extraction with chloroform. The final chloroform extract was evaporated to dryness and distilled water was added prior to EMIT analysis. For ultraviolet analysis the sodium hydroxide phase was adjusted to pH 9.4 with an equal volume of 0.2*M* boric acid solution. The absorption was recorded at 240 nm.

To extract strong and amphoteric bases, blood (10 ml) and homogenized tissue (10 g) were adjusted with potassium carbonate to pH 8 to 9 and extracted with either chloroform (codeine and propoxyphene), chloroform/ethanol (9:1) (amphoteric bases), or diethyl ether (cocaine and meperidine). The organic solvent was filtered, washed with 0.067*M* disodium hydrogen phosphate, and extracted with 0.5*N* sulfuric acid. The sulfuric acid phase was adjusted to pH 8 to 9 and extracted with organic solvent. The organic solvent was evaporated to dryness and reconstituted with distilled water before analysis. Reconstituted

cocaine extracts were heated until hydrolysis to benzoylecgonine was complete or until maximum activity was attained.

To extract nordiazepam and diazepam, blood (10 ml) and homogenized tissue (10 g) were extracted at physiological pH into chloroform (50 ml). The chloroform phase was separated and washed with 0.1*N* hydrochloric acid (10 ml) and 0.13*N* sodium hydroxide (10 ml). The chloroform phase was evaporated to dryness and reconstituted with distilled water.

To extract THC, blood samples (10 ml) were extracted twice with hexane (30 ml each); then the extracts were pooled and extracted with methanolic potassium hydroxide (10 ml). The methanolic potassium hydroxide phase was separated, made acidic, and back-extracted with two 20-ml portions of hexane. The hexane was evaporated to dryness and the residue reconstituted with a 5% ethanol-water mixture.

Results

To extend the EMIT drug abuse urine assay to the analysis of biological fluids (other than urine) and tissues, through the sampling of extracts, several criteria must be met.

1. The drug must be extracted.
2. The partitioning of native lysozyme must not take place, or if it does it must be irreversibly denatured by the organic solvent.
3. The partitioning of the products of the lysozyme reaction or any other inhibitors must not take place.

The extraction of the drugs of interest was not to be a part of this study, and therefore we used our standard laboratory methods. Other methods may be equally adaptable as long as the other criteria for the EMIT analysis are met.

The interference of native lysozyme in biological samples is a serious problem in the EMIT assay, even for urine. Table 1 summarizes the results of the extraction of a standard lysozyme solution with two solvents, chloroform and diethyl ether. At a concentration of 3.24 mg/100 ml, the solution registered an EMIT reading of 200. This number represents the observed absorbance change $\times 10^3$. This was very similar to the reading obtained when the EMIT assay was run in the absence of antibody. After extraction of this solution, filtering, evaporation, and reconstitution, EMIT analysis gave results similar to that recorded for EMIT buffer, showing the elimination of lysozyme after a single partitioning. Chloroform/alcohol mixtures were not studied, but the results of extracting amphoteric bases in actual cases showed that lysozyme elimination was possible with these solvent mixtures. The elimination of lysozyme with organic solvents is not unexpected considering the solubility and stability of globular proteins.

The problem of inhibitors, however, presented a more varied and less resolved problem. Many inhibitors of lysozyme, both natural and man-made, have been reported in the literature [*8-13*]. They include pneumococcus polysaccharides; RNA; DNA; heparin; copolymers of glutamic acid with tyrosine, phenylalanine, or leucine; and cationic detergents.

TABLE 1—*EMIT values expressed as change in optical density for the extraction procedure to eliminate lysozyme. Ten millilitres of lysozyme solution in EMIT buffer was extracted with 50 ml of organic solvent, filtered, evaporated to dryness, and reconstituted with 10 ml of EMIT buffer.*

Solution	EMIT Value, ΔOD
Lysozyme solution (EMIT buffer), 3.24 mg/100 ml	200
Reconstituted chloroform extract	<10
Reconstituted diethyl ether extract	<10

No attempt was made to study these inhibitors because of their number, but several observations were made concerning the inhibition problem. Urine specimens inhibited by direct analysis were analyzed after extraction for the common drugs of abuse. The reconstituted (distilled water) extracts were shown to have no inhibition and gave results similar to uninhibited specimens. A close look at the list of the inhibitors reveals that they are polar, water-soluble molecules, which would not be expected to extract into organic solvents. The extraction of specimens with organic solvent solves many of the problems of lysozyme inhibition.

One of the advantages of the indirect EMIT assay is the control one has over the concentration of the drugs in the sample being analyzed. Assuming a direct assay sensitivity of 1 μg/ml, one actually analyzes an absolute amount of drug equal to 50 ng because 50 μl is sampled. If a 10-ml sample is extracted and the residue reconstituted with 0.25 ml of water, a drug concentration of 25 ng/ml in the original specimen can be detected if there is 100% extraction efficiency. This is a concentration factor of 40. The minimal reconstitution volume is about 0.25 ml. Several assays should be run on each sample and the values averaged. An analysis should also be run with EMIT buffer in place of EMIT enzyme and antibody to check for any high background that could give a false positive, irrespective of any cross-reactivity.

Table 2 lists the direct and indirect sensitivities for various drugs of abuse analyzed by EMIT. This immunoassay can be extended to a larger group of drugs because of its inherent cross-reactivity. However, this can be a problem unless other biological specimens such as gastric contents, bile, or urine are available to help detect the presence of other drugs by more specific methods. The direct sensitivities (Table 2) are those reported by the Syva Corp. as the low calibrator values for urine. The detection limit in the reconstituted samples can be placed at even lower values than those reported in urine because of the greater uniformity in the matrix.

For pentobarbital the reported direct sensitivity minimum in urine is 2.6 μg/ml, and therefore 0.13 μg of pentobarbital is actually sampled. If 10 ml of sample is extracted and reconstituted with 0.25 ml of water, the indirect sensitivity in the original sample would be 0.065 μg/ml. For the other barbiturates a similar situation is applicable.

The EMIT opiate assay is a very sensitive and useful analytical tool. Morphine is the drug most often analyzed by the indirect EMIT. The reported direct sensitivity is 0.5 μg/ml, and therefore 0.025 μg of morphine is sampled during each analysis and the indirect sensitivity is 0.0125 μg/ml in the original sample. Hydromorphone has the same detection limits as morphine, and codeine is even more sensitive to the EMIT assay. Meperidine, a

TABLE 2—*Comparison of specific direct and indirect EMIT sensitivity.*

Drug	Amount of Drug Analyzed, μg	Direct Sensitivity, μg/ml	Indirect Sensitivity,[a] μg/ml
Pentobarbital	0.13	2.6	0.065
Phenobarbital	0.095	1.9	0.0475
Morphine	0.025	0.5	0.0125
Codeine	0.01	0.2	0.005
Cocaine	0.08	1.6	0.04
Hydromorphone	0.025	0.5	0.0125
Nordiazepam	0.04	0.8	0.02
Diazepam	0.04	0.8	0.02
Meperidine	1.5	30	0.75
Propoxyphene	0.1	2.0	0.05

[a] Ten millilitres of sample was extracted, and the residue was reconstituted with 0.25 ml of aqueous solvent.

drug that cross-reacts very poorly on the opiate assay, can be detected at a concentration of 100 μg/100 ml in 10 ml of blood or 100 μg/100 mg in 10 g of tissue. This sensitivity allows not only the detection of low levels but also detection of higher levels on a much smaller volume of sample. For example, morphine and hydromorphone can be detected in a 1-ml sample of blood at levels as low as 5 μg/100 ml.

As previously discussed, the extraction procedures are those already in use in our laboratory. The procedure for extraction and analysis of morphine is described in Fig. 1. The extraction procedure is for total bases, but if there is a need to separate strongly basic drugs from amphoteric drugs an extra step can be added. The final organic extract is split, one portion for EMIT and the other for fluorometry. Table 3 lists the results comparing EMIT and fluoresence values. The higher EMIT value in the liver of Case 1 prob-

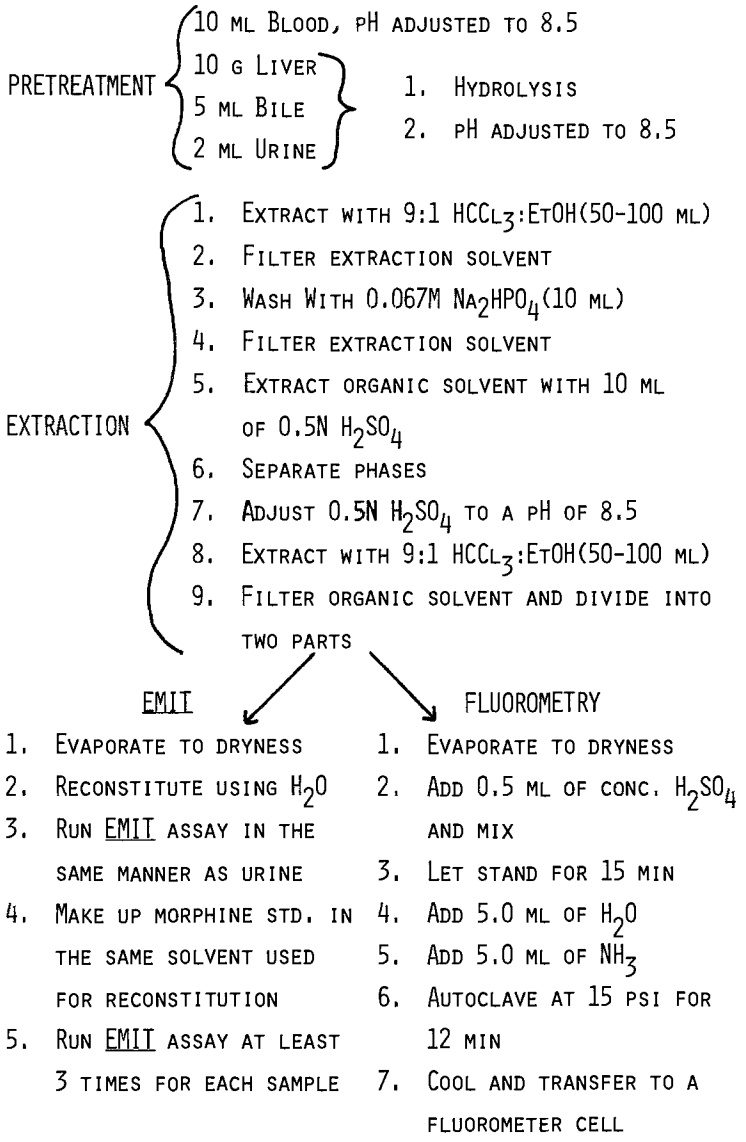

FIG. 1—*Extraction and analysis of biological specimens for morphine (15 psi = 103 kPa).*

ably was a result of cross-reacting metabolites that could not form fluorescent derivatives. This high value could also result from other cross-reacting drugs [*3*]; however, since the blood fluorometric and indirect EMIT levels correlate so well, this does not seem probable. This reasoning fails only in two instances: (1) an acute overdose from a cross-reacting drug in which an elevated nonequilibrium liver/blood ratio occurs or (2) a drug having an inherently high equilibrium liver/blood ratio. In all instances there was a good correlation between EMIT and fluorometric blood morphine levels. Figure 2 represents a typical standard curve generated for morphine by EMIT. The solvent used for the standard curves and the reconstituted extracts was distilled water. On the *y*-axis is plotted the optical density change minus the blank. The blank here refers to the optical density change recorded for the solvent, which in this case was distilled water. The points are an average of the difference in optical density minus blank values obtained, and the horizontal lines represent the high and low values. All subsequent curves were plotted in a similar manner.

Since the EMIT analysis depends on an equilibrium shift, the morphine concentration is plotted as the log value. This should theoretically give a sigmoidal curve if a wide range of drug concentrations is plotted. In many cases the entire concentration range is not reproduced, so most plots vary as to their curvature.

Two barbiturate blood standards were prepared and analyzed with the indirect EMIT assay. The results are shown in Table 4. In the pentobarbital blood standard the sample was extracted for ultraviolet analysis by using a modified extraction scheme [*14*] (see Experimental Procedures), and the aqueous solution was back-extracted with organic solvent, evaporated to dryness, and reconstituted with distilled water. The blood sample used was putrefied and therefore difficult to clean for ultraviolet analysis. The phenobarbital standard was prepared in fresh blood, and a simple one-step extraction with

TABLE 3—*Morphine results: EMIT versus fluorescence.*

Sample	EMIT Value, μg/100 ml or 100 mg	Fluorescence Value, μg/100 ml or 100 mg
Case 1, blood	27	28
Case 1, liver	59	34
Case 2, blood	17	20
Case 3, blood	6.8	6.6

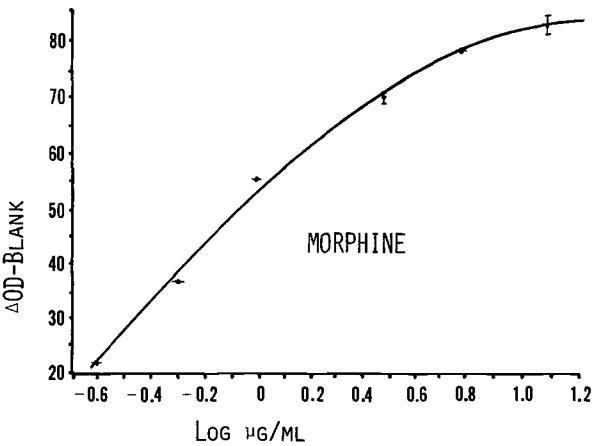

FIG. 2—*Standard curves for morphine with distilled water; horizontal bars represent high and low values obtained at each point.*

chloroform was done. The chloroform phase was filtered, evaporated to dryness, reconstituted with distilled water, and analyzed by EMIT. The 86% recovery may very well represent the loss resulting from the partitioning of phenobarbital between blood and chloroform after one extraction. Figures 3 and 4 show the standard curves generated for pentobarbital and phenobarbital with the EMIT assay. The solvent used was distilled water.

Tables 5 and 6 show the results of the EMIT opiate assay for a quantitation of codeine and hydromorphone in blood and tissues in actual toxicology cases. In both cases analysis of bile and urine showed the absence of other drugs. The use of EMIT provided an addi-

TABLE 4—*EMIT barbiturate results.*

Sample	EMIT Value, mg/100 ml	Ultraviolet Value, mg/100 ml	Actual Value, mg/100 ml
Pentobarbital standard	1.47	1.5	1.8
Phenobarbital standard	8.6	...	10

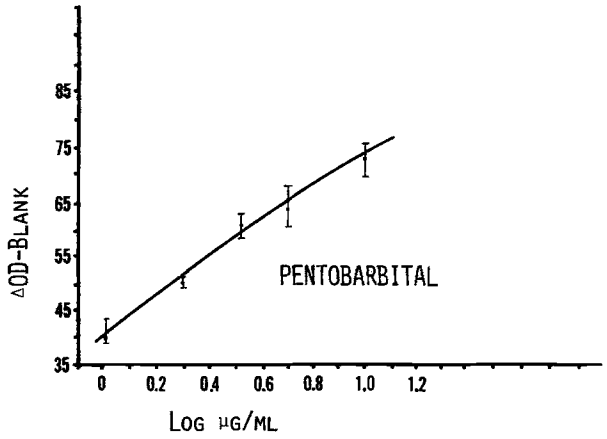

FIG. 3—*Standard curve for pentobarbital with distilled water; horizontal bars represent high and low values obtained at each point.*

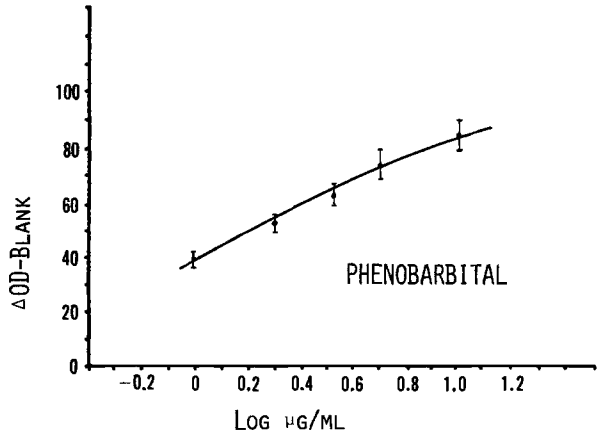

FIG. 4—*Standard curve for phenobarbital with distilled water; horizontal curves represent high and low values obtained at each point.*

tional method, both qualitative and quantitative, for analysis of these two drugs. Figures 5 and 6 represent typical standard curves generated for codeine and hydromorphone with distilled water as the solvent.

The analysis of cocaine with the indirect EMIT procedure represents a problem not encountered with the other assays. The antibody is produced in response to the protein-bound hapten benzoylecgonine. As a result the antibody binds cocaine very poorly. The hydrolysis of cocaine to benzoylecgonine can be brought about very easily in distilled water by gently warming the solution until maximum activity is attained. By using a selective extraction procedure for the separation of cocaine and benzoylecgonine these two compounds can be determined separately.

Meperidine, propoxyphene, nordiazepam, and diazepam have also been analyzed in actual toxicology cases with the indirect EMIT procedure, and the results were confirmed by using other methods of analysis (thin-layer and gas-liquid chromatography).

Figure 7 shows the standard curve generated for Δ^9-THC with a 5% ethanol-water

TABLE 5—*Indirect EMIT codeine analysis.*

Sample	EMIT Value, mg/100 ml or 100 mg
Blood	0.117
Liver	0.44
Brain	0.16

[a] Analysis of bile and urine showed the absence of other drugs.

TABLE 6—*Indirect EMIT hydromorphone analysis.* [a]

Sample	EMIT Value, μg/100 ml or 100 mg
Blood	17
Liver	6.7
Kidney	12.7

[a] Analysis of bile and urine showed the absence of other drugs.

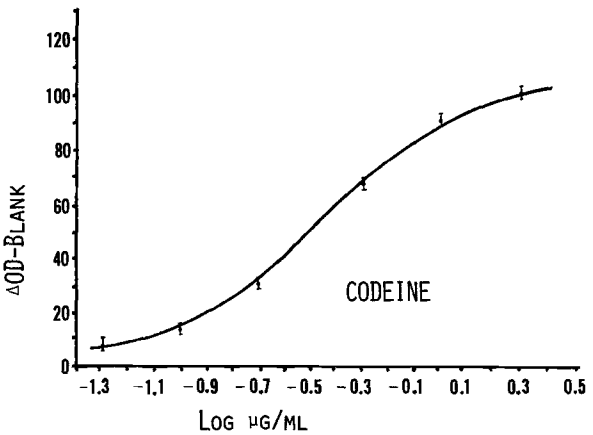

FIG. 5—*Standard curve for codeine with distilled water; horizontal bars represent high and low values obtained at each point.*

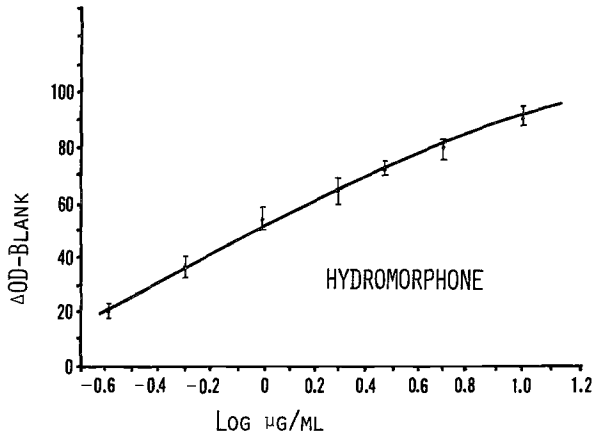

FIG. 6—*Standard curve for hydromorphone with distilled water; horizontal bars represent high and low values obtained at each point.*

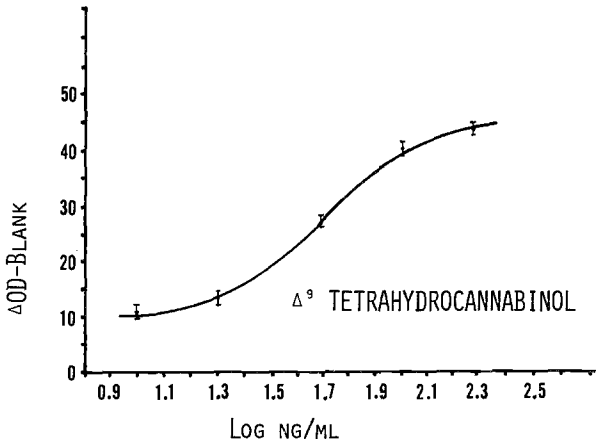

FIG. 7—*Standard curve for Δ^9-THC with a 5% ethanol/water mixture; horizontal bars represent high and low values obtained at each point.*

mixture as the solvent. This was the only EMIT assay included in this study that used nicotinamide adenine dinucleotide (NAD-NADH) for detection. This system was not evaluated to the same extent as the lysozyme system; however, it was thought that it may present fewer problems regarding native enzyme levels and inhibition (Syva uses a NAD-NADH detection method for direct serum assay for antiepileptic drugs). By using the same concentration factors as previously discussed for the EMIT drug abuse urine assay and by assuming a direct sensitivity of 10 ng/ml, an indirect sensitivity in blood of 0.25 ng/ml Δ^9-THC could theoretically be obtained. A positive response for Δ^9-THC in spiked blood at levels as low as 1 ng/ml has been attained. The blood was extracted in a manner similar to that developed by Vinson [*14*]. Table 7 summarizes the experimental data of several EMIT assays. Figure 8 shows the quantitation curves for all of the EMIT assays discussed and illustrates their relationship to each other.

TABLE 7—*Summary of EMIT experimental data.*

Drug	Specimen	Amount of Specimen, ml or g	Solvent	Solvent Recovery, %	Percentage of Extract for EMIT	Water Added, ml	ΔOD – Blank	Concentration of Drug, μg/ml	Concentration of Drug in Specimen, μg/100 ml or 100 mg
Morphine	blood	10	4:1 chloroform/ isopropanol	82	80	0.3	108	6.13	27.4
Morphine	liver	5	9:1 chloroform/ ethanol	61	50	0.3	116	6.0	59
Morphine	blood	10	9:1 chloroform/ ethanol	94	100	0.2	113	5.63	17
Morphine	blood	10	9:1 chloroform/ ethanol	86	50	0.25	...	1.18	6.8
Codeine	blood	5	9:1 chloroform/ ethanol	81,5	100	5	77	0.96	117
Codeine	liver	5	9:1 chloroform/ ethanol	72	100	5	94	3.16	440
Codeine	brain	5	9:1 chloroform/ ethanol	81	100	5	83	1.3	160
Phenobarbital	blood	5	chloroform	73.4	100	50	66	6.3	8600
Pentobarbital	blood	5	chloroform	72	100	20	55.6	2.7	1470
Cocaine	blood	10	chloroform	84	100	5	58[a]	2.1	125
Cocaine	blood	10	chloroform	...	100	1	113[a]	12.2	122
Hydromorphone	blood	10	9:1 chloroform/ ethanol	55	100	1	50	0.93	17
Hydromorphone	liver	10	9:1 chloroform/ ethanol	70	100	0.5	50.5	0.94	6.7
Hydromorphone	kidney	10	9:1 chloroform/ ethanol	82	100	0.5	82.5	2.09	12.7
Nordiazepam	blood	5	chloroform	91	100	10	28.8	1.74	390[b]

[a] After heating.
[b] Found to be a mixture of related benzodiazepins.

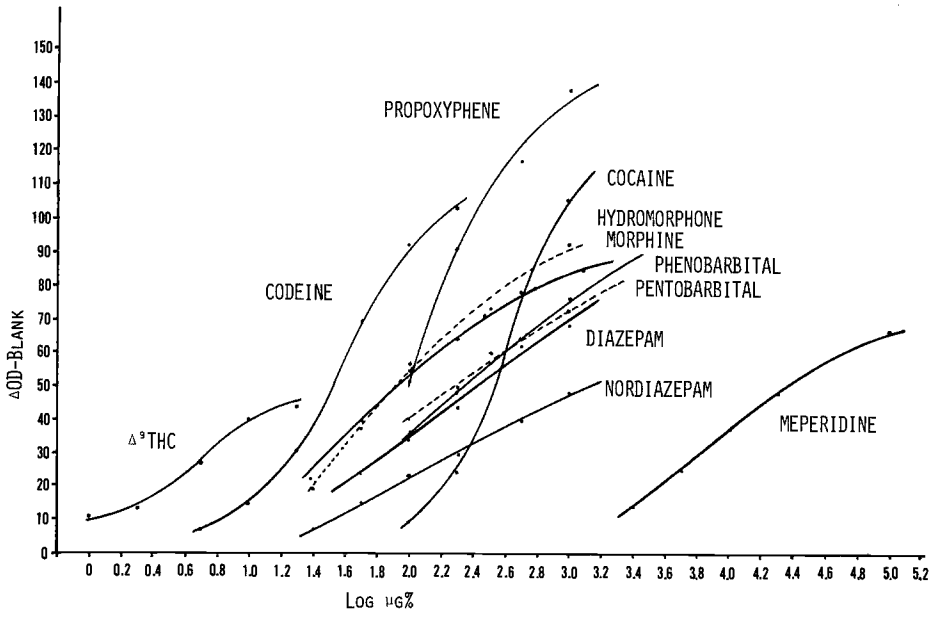

FIG. 8—*A summary of EMIT quantitation curves showing their relationship to each other.*

Conclusions

The data demonstrate the applicability of extending the EMIT assay to specimens other than urine through the analysis of reconstituted extracts. This may be useful for screening, confirming, and quantitation of drugs in biological specimens where other methods may be time-consuming, insensitive, or unavailable. As with most analytical methods the EMIT assay has advantages and disadvantages. The advantages of the indirect method are these:

1. It extends the EMIT assay to more toxicologically significant specimens.
2. It eliminates the interference of native lysozyme in postmortem samples.
3. It eliminates interferences from inhibitors that may be present in toxicological specimens.
4. It controls the pH and ionic strength under which the sample is analyzed.
5. It concentrates the drug being analyzed, thereby increasing the sensitivity of the analysis.
6. It detects the presence of drugs such as hydromorphone that may not be detected by more classical methods.
7. It separates certain cross-reacting drugs (codeine and morphine).
8. It quantitates drugs, providing the possibility of cross-reactivity can be overcome.
9. It requires minimal technical skill.
10. It gives rapid results compared to certain other methods (radioimmunoassay).

The disadvantages of the indirect method are these:

1. It is not structurally specific and suffers from various degrees of cross-reactivity for certain structurally related molecules.
2. It increases the time of the EMIT assay.
3. It is limited in the number of drugs that can be analyzed at the present time.
4. The cost of instrumentation and reagents is relatively high, although the instrumen-

tation is not absolutely necessary (a good timepiece, a small-volume cuvette, and standard ultraviolet equipment can be used).

Those toxicology laboratories already using EMIT for urine screening may find the indirect method described here a valuable extension to their drug-testing capability. The indirect analysis of biological extracts could theoretically be extended to other immunoassay techniques.

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Address requests for reprints or additional information to
E. L. Slightom
Toxicology Section
Georgia Crime Laboratory
P.O. Box 1456
Atlanta, Ga. 30301