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A Comparison of Enzyme Immunoassay and Gas Chromatography/Mass Spectrometry in Forensic Toxicology

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ABSTRACT: A series of 137 urine samples were analyzed for drugs of abuse by enzyme immunoassay (EMIT®) and by gas chromatography/mass spectrometry (GC/MS). Agreement between these methods was excellent and ranged from 93.4% for benzodiazepines to 98.5% for propoxyphene. EMIT false negatives were traced to the presence of elevated endogenous lysozyme or other interfering materials. In the case of moderate amounts of lysozyme the use of a blank would lead to correct results. Disagreement in the identification of nine benzodiazepine samples was found to be due to a low recovery of benzodiazepine metabolites from urine. Recovery could be improved by incubation of the urine sample with the enzyme β-glucuronidase.

KEY WORDS: toxicology, chemical analysis, immunoassay

In 1972, Rubenstein and co-workers [*1,2*] described the enzyme immunoassay (EMIT®) for morphine. Commercially available reagents for this assay and those of several other drugs subject to abuse soon appeared and are now in widespread use in clinical and forensic toxicology laboratories.

Mulé and others [*3*] evaluated the EMIT kits for methadone, amphetamine, cocaine, opiates, and barbiturates using thin-layer chromatography as the reference method. They noted a low percentage of false negatives for all assays and false positives ranging from a low of 2.6% for methadone to a high of 12.5% for amphetamine. It was thought by these workers that the high percentages of false positives for amphetamine and cocaine were exaggerated results that reflected, in part, the lesser sensitivity of thin-layer chromatography in comparison with EMIT.

Spiehler et al [*4*] compared morphine determinations by using radioimmunoassay, EMIT, and spectrofluorometry. They observed a good correlation between the two immunoassays but frequent disagreement with the fluorometric method. This discrepancy was ascribed to cross-reactivity between either codeine or naloxone and the morphine antibody used in the immunoassays.

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Van der Slooten and Van der Helm [5], using gas chromatography/mass spectrometry (GC/MS) as the reference method in an evaluation of the EMIT opiate assay, found poor quantitative correlations between these two techniques, although at the cutoff level of 0.5 μg/ml for both methods the enzyme immunoassay gave only 4% false positives and 5.6% false negatives in comparison with the reference method.

The present study was undertaken to evaluate all of the EMIT reagent sets for drugs of abuse and used computerized GC/MS as the reference method. Specifically, this study aimed at evaluating the accuracy of the EMIT method when it is applied to postmortem samples.

Materials and Methods

Reagents for the EMIT method were obtained from the Syva Corp. (Palo Alto, Calif.) and were employed according to the manufacturer's directions. Absorbance changes were determined at 436 nm on a Beckman Model 25 double-beam spectrophotometer with a heated sipper cell. A Beckman timer-printer was used with a delay time of 10 s and a measurement time of 40 s.

For the GC/MS analysis, a urine sample was divided into two aliquots, one of which was subjected to the extraction procedure of Christopoulos and Kerch [6] for morphine assay. The second aliquot, intended for a general GC/MS search, was extracted twice with 50 ml of chloroform, after which the aqueous phase was rendered alkaline and again twice extracted with chloroform. Extracts were pooled and filtered. Five drops of concentrated hydrochloric acid were added before evaporation on a steam bath. The residue was reconstituted in 0.5*M* hydrochloric acid and extracted with chloroform. The aqueous phase was again made alkaline and twice extracted with chloroform. Finally, chloroform extracts were pooled, evaporated over steam, and reconstituted in a small volume of methanol before the injection.

A Finnegan Model 3200 GC/MS was used. Samples were injected onto a 2-m column of 3% OV-1 and the chromatograph was temperature-programmed from 190 to 250°C at a rate of 12°C/min. Upon exiting the column, the sample was struck by electrons of 70 eV energy. Mass spectra were recorded and evaluated by a Finnegan Model 6100 data system.

Lysozyme assay was conducted by measuring the degree of clearing of a suspension of *Micrococcus luteus* cells at 436 nm during a 40-s interval. Standards of lysozyme were obtained from the Worthington Biochemical Corp. (Freehold, N.J.). The assay was observed to be linear over the range of 0 to 10 μg/ml.

Urinary protein determination was carried out by a turbidimetric assay involving protein precipitation with trichloroacetic acid and measurement of absorbance at 470 nm [7].

Results and Discussion

Table 1 shows the results for 137 urine samples analyzed by GC/MS and by enzyme immunoassay. The generally good agreement is evident and ranges from 93.4% for benzodiazepines to 98.5% for propoxyphene. A significant pattern was the small percentage of false positives except for the benzodiazepine assay. Cocaine and amphetamine also showed a small percentage of false positives but were ultimately excluded from the study because of the small number of true positives as well.

Fifteen false negatives were observed. Five of these errors were traced to unknown materials that interfered with the assay while the presence of large amounts of lysozyme was responsible for six false negatives.

Table 2 shows the effect of interfering substances. The low calibrators for each drug contain that drug at the detection limit of the assay while negative calibrators consist of drug-free urine. A sample is positive if the adjusted absorbance change (total absorbance change minus blank) at 436 nm exceeds that of the low calibrator. It was note-

TABLE 1—*Comparison of EMIT with GC/MS in urine.*

	EMIT Assays									
	Methadone		Opiates		Barbiturates		Benzodiazepines		Propoxyphene	
Results	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%
True positive	14	10.2	25	18.2	17	12.4	20	14.6	21	15.3
True negative	120	87.6	104	75.9	115	83.9	108	78.8	114	83.2
Total	134	97.8	129	94.2	132	96.4	128	93.4	135	98.5
False positive	0	0	1	0.7	0	0	9	6.6	2	1.5
False negative	3	2.2	7	5.1	5	3.6	0	0	0	0
Total	3	2.2	8	5.8	5	3.6	9	6.6	2	1.5

TABLE 2—*False negatives resulting from interfering substances.*

	ΔOD_{436} Blank	ΔOD_{436} Adjusted				
		Barbiturate	Propoxyphene	Benzodiazepine	Opiate	Methadone
Negative calibrator	2	63	42	54	50	30
Low calibrator	2	112	59	71	78	46
Case 15029	7	26	21	25	32	23
Case 17649	8	98	31	45	41	26
Case 18894	11	23	7	20	62	21
Case 18947	10	53	19	28	69	23

worthy that, in the four cases shown, the absorbance changes are not merely less than the low calibrators but generally less than those of the negative (drug-free) calibrators as well. This failure to exhibit background activity suggests that some interfering material is nullifying the assay. False negatives are known to occur in older specimens that become alkaline during storage or in the presence of alkalizing drugs. Such pH elevations lead to false negatives by enzyme inactivation [*8*]. These samples, however, were not alkaline. The interference in this case may be due to a specific, unidentified inhibitor of lysozyme. Samples 15029, 18894, and 18947 were positive for opiates by GC/MS. Samples 17649 and 18947 were positive by GC/MS for barbiturates. Although these samples were not correctly analyzed by EMIT the presence of drugs was suggested by absorbance changes that were much greater for the drugs present (opiates or barbiturates) than for drugs absent from the urine sample.

Occasionally, urine samples had levels of lysozyme so elevated that the enzyme immunoassay was ineffective. Table 3 illustrates six cases in which a drug was shown to be present by GC/MS but the EMIT result was negative. In all cases, the absorbance changes were greater than those of the low calibrators. However, the absorbance changes of the blanks, owing to endogenous lysozyme, were also high so that the corrected changes were too low for positive identification.

Since a large number of samples contained a significant amount of lysozyme, an experiment was conducted to determine at what level urinary lysozyme causes false negatives. Figure 1 shows results when a urine sample containing 1 μg/ml of morphine is spiked with lysozyme. The absorbance changes resulting from endogenous lysozyme and from drug-labeled lysozyme were additive over a very narrow range. In the sample illustrated, endogenous enzyme levels greater than 8 μg/ml resulted in false negatives. It was further observed in similar experiments that the magnitude of interference resulting from urinary lysozyme is inversely proportional to the concentration of drug in the sample and depends on the absorbance change associated with the low calibrator for the drug in question.

TABLE 3—*False negatives resulting from lysozymuria.*

	Sample ΔOD_{436}	Blank ΔOD_{436}	Adjusted ΔOD_{436}
		Barbiturates	
Negative calibrator	63	2	61
Low calibrator	114	3	111
Case 17658	149	56	93
Case 18010	135	51	84
Case 18693	194	178	16
		Opiates	
Negative calibrator	51	2	49
Low calibrator	79	2	77
Case 17615	98	22	76
Case 18645	226	231	(−5)
		Methadone	
Negative calibrator	32	2	30
Low calibrator	48	3	45
Case 18706	161	141	20

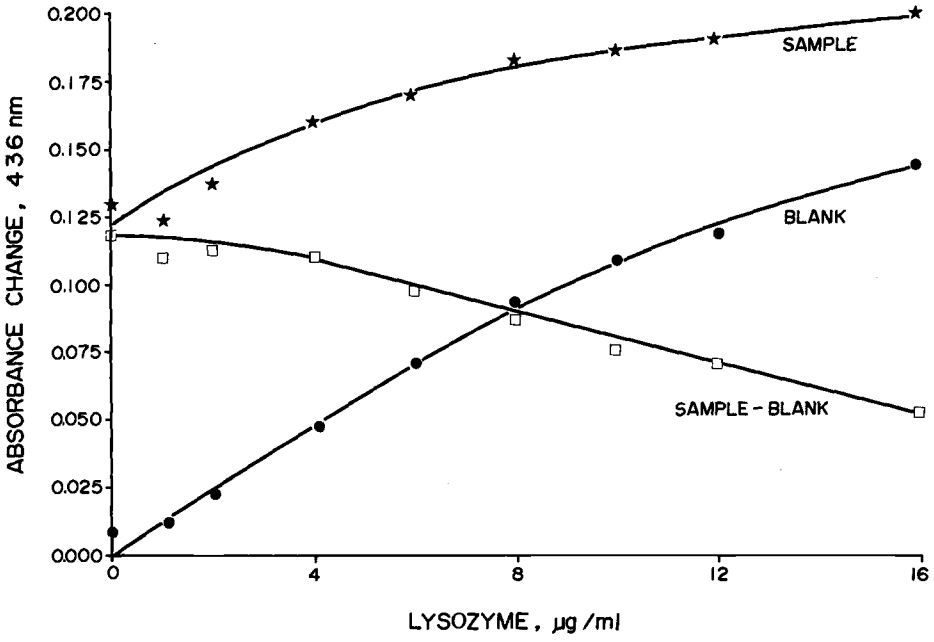

FIG. 1—*The effect of endogenous urinary lysozyme on EMIT opiate assay. The addition of increasing amounts of lysozyme to the sample causes the blank absorbance change to increase at a rate faster than the sample absorbance change. The net absorbance change decreases and false negatives result if endogenous lysozyme exceeds approximately 8 µg/ml.*

Lysozyme levels were measured in a large series of normal and postmortem urines. Results showed mean levels of 0.9 ± 1.2 and 5.3 ± 9.4 µg/ml, respectively. These results suggest that the problem of false negatives in immunoassay caused by endogenous lysozyme, while uncommon for normal urines, is a potentially serious problem for postmortem urines, which contain, on the average, six-fold as much lysozyme.

The source of lysozyme in postmortem urine is not known. We have, however, observed a

correlation ($r = 0.60, P < 0.01$) between lysozyme and protein in postmortem urine (Fig. 2). Anan'eva [9] reported increases in serum lysozyme in dogs experiencing blood loss leading to hypovolemic hypotension. The serum lysozyme level was therefore suggested as a possible indicator of hypoxic damage to the internal organs in terminal states. The elevated urinary lysozyme observed in this study may therefore originate as a consequence of terminal hypoxia. In the normal kidney, lysozyme is filtered and reabsorbed from the tubules into the blood. Immediately before death, renal function is presumably impaired and elevated lysozyme levels together with serum proteins would be found in the urine.

Whereas false negatives were the more common type of error for most drugs with EMIT, false positives appeared to be more frequent in the benzodiazepine assay (Table 1). Examination of these nine EMIT positives suggested that some or all of them are in fact true positives and that the enzyme immunoassay is more sensitive than GC/MS under the conditions employed. This conclusion was supported by the fact that four urine samples that were positive by EMIT and negative by GC/MS came from decedents whose blood was positive by GC/MS for benzodiazepines. This observation suggested that our recognition of benzodiazepines, especially diazepam, was better for the parent drug in blood than the metabolites in urine. It was especially likely that the major diazepam metabolite, oxazepam glucuronide, was too polar to be recovered after the extraction used in the GC/MS procedure. In an attempt to improve the recovery of benzodiazepine urinary metabolites, we tried a pre-incubation of urine with β-glucuronidase. This improved our recognition by GC/MS of oxazepam, again suggesting that the discrepancy between EMIT and GC/MS for benzodiazepines was due to extraction difficulties before the GC/MS analysis. On the basis of these findings, we now regard the presence of the diazepam base peak at *m/e* 256 at the appropriate retention time of 7 min as presumptive evidence for the presence of diazepam.

Summary

The EMIT system for assay of drugs of abuse correlates well with GC/MS on urine samples collected at autopsy. Generally good agreement is found between the two methods although the enzyme immunoassay, relying on lysozyme activity and an appropriate pH and ionic strength for antigen-antibody interaction, is affected when these parameters are disturbed. In particular, elevated levels of urinary lysozyme are problematic because it is not possible to correct for them by running a simple blank. An advantage of the EMIT method over GC/MS and many other procedures is its applicability to untreated samples and

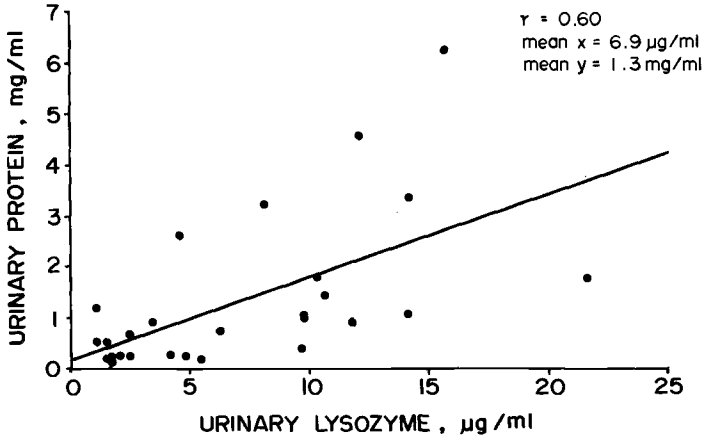

FIG. 2—*Correlation between urinary lysozyme and protein in postmortem urine.*

therefore drug loss during extraction is eliminated. EMIT assays require only 50 μl of sample and are thus useful in situations where limited specimen renders most other methods unsuitable. We conclude that EMIT has no inherent limitations that severely restrict its accuracy in the forensic toxicology laboratory.

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