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Evaluation of the Abuscreen® for Methadone

In recent years radioimmunoassay has provided the toxicologist with a rapid, simple way to identify and quantitate drugs of abuse. This paper deals with an evaluation of a radioimmunoassay[2] for methadone.

Experimental

A typical reagent kit contains the following:

20 ml methadone antibody,
50 ml saturated ammonium sulfate,
4 ml negative standard,
4 ml positive standard with methadone at 100 ng/ml, and
20 ml ^{125}I-labeled methadone antigen.

Ten milligrams of each compound listed in Table 1 were separately dissolved in ethanol. Aliquots were taken and diluted in "negative" urine to a concentration of 10 μg/ml and 2.5 μg/ml.

Methods

Urine Screening

Into separate culture tubes, 0.1 ml negative urine control, 0.1 ml positive urine control, and 0.1 ml unknown specimen to be tested were pipeted. To each tube was added 0.2 ml methadone antibody, followed by 0.2 ml methadone ^{125}I-labeled antigen. Samples were then mixed and allowed to equilibrate at room temperature for 1 h. To each sample, 0.5 ml saturated ammonium sulfate was added. The samples were mixed again and allowed to stand for at least ten minutes (extending this time to at least ½ h will insure a better protein precipitation).

The samples were then centrifuged at 4000 *g* for 10 min. An aliquot of the supernatant was then counted in a gamma counter (0.5 ml is suggested in the kit, but we used 0.8 ml), and the counts were compared to the negative and positive (100 ng/ml) methadone controls.

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[2] Information received from Roche Diagnostics indicates Abuscreen® for the detection of methadone will be marketed in the very near future.

Positive and negative urines were confirmed with gas chromatographic (GC) and thin-layer chromatographic (TLC) analysis by the following method: 30 ml of urine was extracted at pH 8.5 with 60 ml of ether. The ether was filtered, a drop of 6N HCl in ethanol was added, and the ether was allowed to evaporate. The residues were resuspended in a small amount of ethanol, half of which was spotted on a silica gel TLC plate and then developed in the Davidow et al system [2].

The plates were dried and sprayed with a 5% sulfuric acid, followed by iodoplatinate spray to locate the methadone spot. The remaining residue was resuspended in 50 μl of chloroform, 1 μl of which was injected on a Varian 1700 gas chromatograph.

Pure methadone standards were analyzed simultaneously with unknown samples by both GC and TLC. These samples were gas chromatographed on two glass columns, a 3% SE30 and a 3% OV17. Flame ionization was used for detection with column temperature set at 240°C. Under these conditions the retention time for methadone is about 2.5 min.

Instrumentation

A Packard Tricarb® scintillation spectrometer (Model 3002) was used for counting radioimmunoassay (RIA) samples. Gas chromatography was performed on a Varian 1700 dual column gas chromatograph. Thin-layer chromatographic plates were precoated silica gel G, 250 μm thick, a product of Analtech Inc., Newark, Del.

Quantitative Aspects

Liver, kidney, blood, brain, urine, and bile were each diluted or homogenized in a Waring® blender with water in the following proportions: liver and kidney, 1:10; blood and brain, 1.5; urine and bile, 1:25. The homogenates were spun at 4000 *g* for 20 min. The fat layer was then aspirated and the supernatant assayed as follows: To 100 μl aliquots of the supernatants were added 500 μl of antiserums and 500 μl of antigen. The samples were then mixed and allowed to equilibrate at room temperature for 3 h, after which 1 ml of saturate ammonium sulfate was added to each sample. These were mixed thoroughly and allowed to stand for an additional hour. Following centrifugation at 4000 *g* for 10 min, the supernatant (1.8 ml) was counted for 1 min.

This technique was duplicated, using drug-free tissue homogenates to which had been added known amounts of methadone, ranging from 50 to 500 ng/ml of homogenate. By extrapolating from the standard curve for that particular tissue, we were able to determine the tissue methadone levels. The results of this technique (on liver) were compared with Robinson's and William's gas chromatographic method [3], which we modified by using meperidine as an internal standard.

Discussion

The Abuscreen® for methadone appears to provide a relatively simple method of screening urines. To check the specificity of this kit, we tested for cross-reactivity against the compounds listed in Table 1. No cross-reaction was found with these drugs at a level of 2.5 μg/ml when compared to a 100 ng/ml methadone standard. To be considered positive the counts must equal the 100 ng/ml methadone control. To be negative the count must be equal to or below the negative control.

Phenobarbital did cross-react at a concentration of 10 μg/ml, but the counts were between a negative and the 100 ng/ml methadone standard and therefore was called a questionable positive. The other drugs listed in Table 1 did not cross-react at a concentration of 10 μg/ml.

TABLE 1—*Pharmaceuticals tested in pure form.*

Generic Name	Commercial Name	Manufacturer
Acetaminophen	Tylenol®	McNeil
Amitriptyline	Elavil®	Merck Sharpe & Dome
d-Amphetamine	Benzedrine®	Smith Kline & French
Caffeine	...	Merck Sharpe & Dome
Chlordiazepoxide	Librium®	Roche
Cocaine	...	Mallinckrodt
Dextromethorphan	Romilar®	Abbott
Diazepam	Valium®	Roche
Ephedrine	...	Winthrop
Glutethimide	Doriden®	USVitamin
Imipramine	Tofranil®	Geigy
Meperidine	Demerol®	Winthrop
Meprobamate	Miltown®	Wallace
Methamphetamine	...	Winthrop
Methaqualone	Quāālude®	Rorer
Morphine	...	Mallinkrodt
Oxazepam	Serax®	Wyeth
Oxycodone	Percodan®	Endo
Pentazocine	Talwin®	Winthrop
Pheniramine	Phenaphen®	Robins
Phenobarbital	Luminal®	Mallinkrodt
Phenylpropanolamine	...	Smith Kline & French
Propoxyphene	Darvon®	Eli Lilly
Quinine	...	Matheson, Coleman, Bell
Secobarbital	Seconal®	Eli Lilly
Sodium salicylate	...	Mallinckrodt
Thioridazine	Mellaril®	Sandoz
Chlorpromazine	Thorazine®	Smith Kline & French
Metabolites		
Norpropoxyphene	...	Eli Lilly
2-ethylidene, 5-dimethyl-3,3 diphenyl 1-pyrrolidium perchlorate	methadone metabolite I [*1*]	Eli Lilly
2 ethyl 5 methyl 3,3 diphenyl 1 pyrroline HCl	methadone metabolite II [*1*]	Eli Lilly

The two major metabolites of methadone (see Table 1) were screened with the kit. Only at a concentration of 10 μg/ml was there any cross-reaction and this, as in the case of phenobarbital, was questionable. At 2.5 μg/ml neither metabolite cross-reacted.

Because of the structural similarity to methadone, a metabolite of propoxyphene (norpropoxyphene) was also tested. At a concentration of 10 μg/ml no cross-reaction could be detected. Thus the methadone antibody appears to be extremely specific and will eliminate the need for more than one or two other methods of confirmation.

In a blind study, 292 samples were checked by RIA: 204 samples from the Narcotics Probation Department and 88 samples submitted to us for proficiency testing by the New York State Department of Health and the United States Communicable Disease Center. The entire 292 samples were then subjected to TLC and GC for confirmation. The results of these studies are shown in Table 2.

It should be noted that of the 10 nonconfirmed RIA positives, four were from patients on the methadone maintenance program. With *one* exception, all TLC and GC positives (listed in Table 2) were duplicated with RIA positives. The one exception gave a negative RIA but a positive TLC and GC (Line 1, Table 2). The RIA method was

TABLE 2—*Comparison of radioimmunoassay (RIA) to the standard gas chromatographic (GC) and thin layer chromatographic (TLC) methods.*

Specimens	RIA Positive	TLC Positive	GC Positive
100 positive urines	99	100	100
100 negative urines	0	0	0
292 unknowns	68	56	58

repeated on this sample, but the results were still negative. Due to insufficient quantity of the specimen, TLC and GC could not be similarly repeated. Of the 88 proficiency samples tested, all methadone positives were detected, and no false positive or false negative was observed.

The present methods for quantitating methadone in tissue from medical examiner's cases are poor. The problem appears to be in the extraction of the drug from the tissues [*4*]. Since radioimmunoassay detects the drug without extracting it, this technique lends itself nicely to quantitation in tissues [*5*].

The kit (as supplied) is linear only between 50 and 150 ng with a range of 8000 to 12 000 counts per minute (cpm) for those respective amounts. This narrow range lends itself to considerable error when trying to quantitate tissue methadone levels. For this reason, we increased the amounts of reagents used by 2½ times. This provides us with a linear range of between 50 and 600 ng/ml (Figure 1). Although the cost is increased by 2½ times, the number of dilutions that must be analyzed (to fall in the linear range) is greatly reduced. Thus the overall cost of analysis per case is essentially the same.

When standard curves were set up in tissue supernatants, the curves were essentially the same as in water, with the exception of liver and kidney tissue, which appear to give a slightly higher blank and stand curve (Figs. 2 and 3). Since there did not appear to be any nonspecific binding in blood, brain, urine, or bile, one can use a standard curve in water for concentration estimates in these fluids and tissues. When attempting

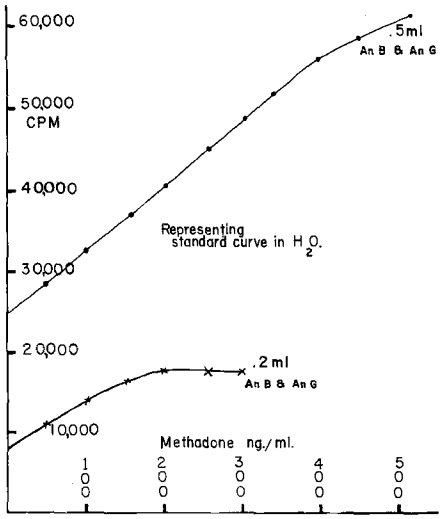

FIG. 1—*Comparison of methadone standard curves in water, with 0.2 ml of antibody and antigen and 0.5 ml of antibody and antigen.*

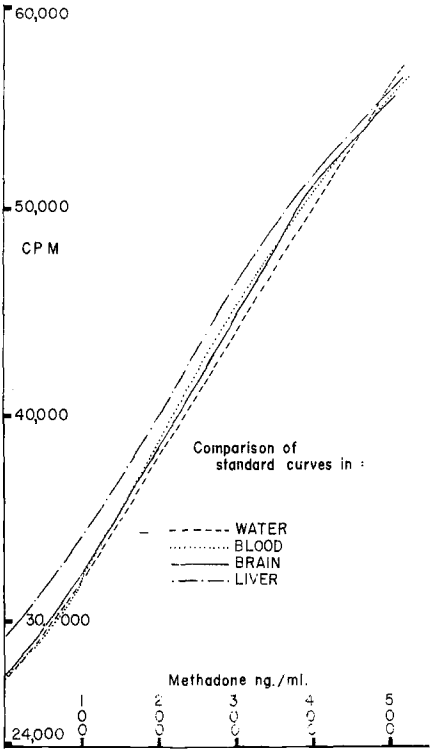

FIG. 2—*Comparison of standard curve in water, blood, liver, and brain homogenates; 0.5 ml of antibody and 0.5 ml of antigen were used.*

to determine the methadone levels in kidney and liver, concentration curves should be run with negative liver and kidney supernatants. A comparison of tissue levels assayed by this method is seen in Table 3.

Case Histories

Values reported in these case histories were obtained using our modification of the Robinson method [*3*]. Statements within quotation marks are witnesses' remarks noted in the medical examiner's reports.

Case 1

A 22-year-old male, who had been discharged from a methadone maintenance program a few months earlier, was found dead in bed at approximately 5:00 p.m. on 23 June 1974. He had last been seen alive at 9:00 a.m. on 21 June 1974.

Autopsy revealed no needle marks, but there were mild decomposition and visceral congestion. Toxicological analyses showed methadone and metabolites present in the brain, bile, stomach, and liver (liver quantitation: methadone, 0.5 mg/100 g; metabolite I, 0.4 mg/100 g; and metabolite II, 0.1 mg/100 g). Cause of death was certified as methadone intoxication.

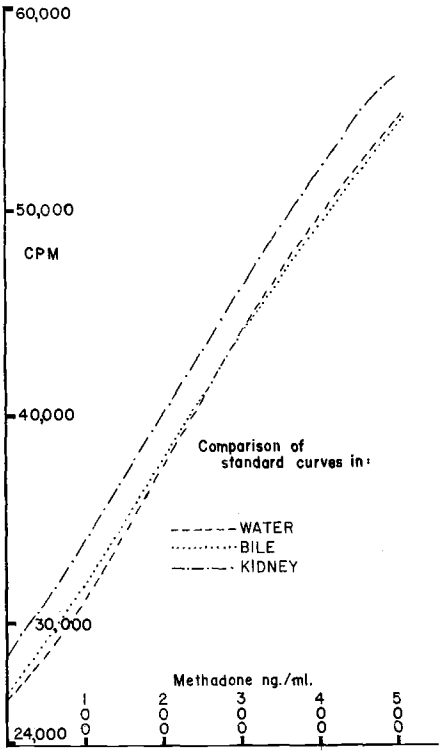

FIG. 3—*Comparison of standard curves in water, bile, and kidney homogenates; 0.5 ml of antibody and 0.5 ml of antigen were used.*

Case 2

A 19-year-old female had a known three-year history of drug abuse (including methaqualone and methadone), although she was not in a maintenance program. Two weeks prior to her death, she had overdosed and survived. On the day of her demise, she was seen to ingest methadone at approximately 10:30 a.m. and lapse into unconsciousness; she was pronounced dead on arrival at the hospital at 11:00 a.m.

Autopsy revealed visceral congestion. Toxicological analyses showed methadone present in the brain, stomach, and liver (liver quantitation: 0.35 mg/100 g); metabolites were not detected. Cause of death was certified as methadone intoxication.

Case 3

A 24-year-old male, who was not on a maintenance program, was seen to ingest three wheat germ capsules and some wine while visiting a friend. At approximately 7:30 p.m. he fell asleep. At 8:40 p.m. he was found to be unresponsive and brought to the hospital where he was pronounced dead on arrival at 9:40 p.m.

Autopsy revealed needle puncture marks of the antecubital fossae, bilaterally. Toxicological analyses showed an ethanol level of less than 0.01% in the brain. Methadone and metabolites were present in the brain, stomach, urine, and liver (liver quantitation: methadone, 0.25 mg/100 g; metabolite I, negative; metabolite II, 0.26 mg/100 g). Cause of death was certified as methadone intoxication.

TABLE 3—*Comparison of tissue methadone levels with radioimmunoassay.*

Case	Blood, mg/100 ml	Brain, mg/100 g	Kidney, mg/100 g	Bile, mg/100 ml	Liver, mg/100 g	Liver VPC, mg/100 g
1	0.11	0.12	0.28	1.00	0.32	0.48
2	0.08	0.13	0.60	0.70	0.45	0.35
3	0.12	0.08	0.16	0.60	0.34	0.25
4	0.06	0.11	0.13	0.38	0.31	0.27
5	0.09	0.07	0.35	0.29	0.35	0.50
6	...	0.09	0.28	1.30	0.75	0.90
7	0.18	0.14	0.51	1.80	0.43	0.78
8	0.17	0.08	0.30	0.45	0.42	0.55
9	0.04	0.08	0.19	0.50	0.25	0.20
10	0.09	0.05	0.11	0.50	0.18	0.22

Case 4

A 20-year-old male was known to be a drug addict, but he was not in a maintenance program. The medical examiner's report states that at 5:30 p.m. he arrived at a friend's home in a "drowsy and somnabulant state." He slept intermittently until 2:00 a.m., when he awoke, "gurgled," vomited, and lapsed into unconsciousness. He was pronounced dead on arrival at the hospital at 2:26 a.m.

Autopsy revealed three recent needle puncture marks of the antecubital fossae and another recent needle mark over the dorsal aspects of both hands, which were swollen and showed blood extravasation. Toxicological analyses revealed methadone present in the brain, bile, and liver (liver quantitation: 0.27 mg/100 g); but methadone was not detected in the stomach. These findings suggest the methadone had been injected. Cause of death was certified as methadone intoxication.

Case 5

A 24-year-old male with a history of arrests for narcotic and other drug (pills) abuse was currently on probation for car theft. At approximately 9:00 p.m. on 13 Dec. 1974 he arrived at a friend's home, appearing "high and hyperactive." At 11:00 p.m. he became sleepy and displayed respiratory difficulty. He fell asleep and was placed in a cold shower for approximately 45 min, after which his friend brought him outside to his parked truck and stayed with him until 2:00 a.m. At that time he appeared to be sleeping. At 10:30 a.m., when the friend returned to check, he was in the same prone, face-down position in which he'd been left. Death was pronounced at the scene at 12:01 p.m. on 14 Dec. 1974.

Autopsy revealed visceral congestion. Toxicological analyses showed a trace of amobarbital-secobarbital in the stomach, brain, and blood (less than 0.1 mg/100 ml); a trace of meprobamate in the stomach, but negative in the brain; methadone and metabolites present in the brain, stomach, and liver (liver quantitation: methadone, 0.5 mg/100 g; metabolite I, 0.1 mg/100 g; metabolite II, 0.5 mg/100 g). Cause of death was certified as methadone intoxication.

Case 6

A 26-year-old male, who was not in a maintenance program, had a 10-year history of arrests for "possession of dangerous drugs, possession of a dangerous instrument, car theft, burglary, reckless and drunken driving." He was found dead on the couch at home

at 1:00 a.m. with an open bottle of beer at his side. He had last been seen alive five hours earlier.

Autopsy revealed visceral congestion and orange material in the stomach contents. Toxicological analyses showed an ethanol level of 0.11% in the brain; methadone and metabolites were present in the brain, stomach, and liver (liver quantitation: methadone, 0.9 mg/100 g; metabolite I, not detected; metabolite II, 0.1 mg/100 g). Cause of death was certified as methadone intoxication.

Case 7

An extremely obese (347 lb or 157 kg) 28-year-old male was on a maintenance program. After dinner the deceased felt weak and faint, and he went to sleep in a reclining chair, where he was found unresponsive at 5:20 a.m. He was pronounced dead on arrival at the hospital at 5:55 a.m.

Autopsy revealed needle punctures and extensive recent interstitial hemorrhage beneath the skin of the antecubital fossa and advanced fatty metamorphosis and portal cirrhosis of the liver. Toxicological analyses revealed the presence of ethchlorvynol in the brain (2.0 mg/100 g), liver (3.3 mg/100 g), kidney (3.2 mg/100 g), blood (1.5 mg/100 ml), and stomach. Methadone and metabolites were present in the liver (liver quantitation: methadone, 0.78 mg/100 g; metabolite I, not detected; metabolite II, 1.3 mg/100 g). Because of the high methadone level, death was certified as methadone intoxication.

Case 8

A 21-year-old male had a history of drug abuse and was on a maintenance program. While operating a vehicle on a parkway, he struck a pole and was pronounced dead on arrival at the hospital.

Autopsy revealed multiple fractures and fatal injuries. Toxicological analyses showed an ethanol level of 0.03% in the brain and methadone and metabolites in the liver (liver quantitation: methadone, 0.55 mg/100 g; metabolite I, not detected; metabolite II, 0.38 mg/100 g). Cause of death was certified as massive hemothorax-accidental.

Case 9

A 21-year-old male, who was not on a maintenance program, arrived at his home at 2:30 a.m. in what appeared to be an "intoxicated state." At 7:40 a.m. he developed breathing difficulty and was brought to the hospital where he was pronounced dead at 8:50 a.m.

Autopsy revealed both old and fresh needle puncture marks of the left antecubital fossa. Toxicological analyses showed quinine present in the brain, stomach, bile, urine, and liver. Morphine was present (3 μg/100 ml blood; 3 μg/100 g brain; 4 μg/100 g liver; 24 μg/100 g kidney; 200 μg/100 ml bile; 110 μg/100 ml urine). Methadone liver quantitations were 0.2 mg/100 g; metabolites I and II were not detected. Cause of death was certified as methadone and opiate derivative intoxication.

Case 10

A 26-year-old male had a prolonged history of drug addiction and four arrests for "possession of drugs, public intoxication, drunken driving, and shoplifting," all in a period of two months, from 20 June 1974 to 24 Aug. 1974. He was currently on a maintenance program and had codeine with Phenaphen® and Valium® prescribed (in the past few days) by a dentist who had been doing root canal dentistry on the deceased. While dining out, the deceased is said to have ingested three Valium® tablets, becoming incoherent shortly thereafter. When he returned home, he went to bed. At

7:00 a.m. the next morning he was found unresponsive and pronounced dead at the scene.

Autopsy revealed needle puncture scars of the left antecubital fossa and visceral congestion. Toxicological analyses showed the presence of phenobarbital in the stomach and less than 0.5 mg/100 g in the liver. Diazepam was present in the stomach and liver (0.39 mg/100 g) but was not detected in the brain, urine, or bile. Codeine was present as follows: 0.05 mg/100 ml in blood; 0.04 mg/100 g in brain; 0.08 mg/100 g in liver; 0.06 mg/100 g in kidney; 0.3 mg/100 ml in bile; and it was also present in the stomach. Methadone was present in the stomach and liver (liver quantitation: methadone, 0.22 mg/100 g; metabolite I, not detected; metabolite II, 0.5 mg/100 g. Cause of death was certified as multiple drug ingestion.

The tissue levels reported here correspond well with overdose levels of methadone previously reported [*3,6*]. It is noteworthy that the brain levels appear to be the determining criteria of actual toxicity in overdose situations.

Summary

The increased use and abuse of methadone in recent years has posed a problem of both its identification and quantitation in body tissues. Recent development of a radioimmunoassay for methadone appears to have solved the problem.

In our hands the assay was extremely sensitive and specific. It also appears to be an excellent tool when quantitative estimates are to be obtained. Although it initially appears to be relatively expensive, the time saved in doing a complete tissue distribution equalizes the cost. It is hoped that other radioimmunoassays currently under development will prove as satisfactory.

Acknowledgment

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