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*T. S. Sulkowski,*[1] *Ph.D.; G. D. Lathrop,*[1] *M.D., M.P.H., Ph.D.; J. H. Merritt,*[1] *B.S.; J. H. Landez,*[1] *M.S.; and E. R. Noe,*[1] *M.S.*

# A Semiautomated Radioimmunoassay for Mass Screening of Drugs of Abuse

The initiation of a mass drug abuse screening program of military personnel as part of the nation's effort to stem the use of illegal or otherwise abused drugs placed an extreme demand on normal laboratory tests and personnel. Although several methods existed for the detection of opiates, barbiturates, and amphetamines in urine [*1–4*], none had been employed for a testing program in which 3000 to 4000 urine specimens were to be analyzed daily for each of the three classes of drugs.

The screening procedures initially used (1970) within the Drug Abuse Testing Facility of the U.S. Air Force (USAF) School of Aerospace Medicine consisted of thin-layer chromatographic (TLC) and gas-liquid chromatographic (GLC) methods. Along with their advantages, these methods posed certain disadvantages that were magnified in mass screening, such as the use of large volumes of noxious solvents, multiple manual steps, and procedures requiring subjective judgment by thoroughly trained technical personnel. Also, both TLC and GLC methods required an acid hydrolysis at elevated temperatures to liberate morphine from its urine conjugate.

To eliminate undesirable aspects and to reduce error-prone steps and technician requirements in the screening program, other procedures were considered [*5–7*]. Preliminary considerations indicated that a radioimmunoassay (RIA) procedure would be most advantageous. Beyond considerations of sensitivity and specificity, the RIA methodology lends itself well to automation and does not require large volumes of reagents or urine samples. Subjective judgment and hydrolysis steps are not necessary. The RIA technique involves a reaction in which an antibody equilibrates between "free" and "bound" states. If the antigen in the initial mix is radioactively labeled, the addition of unlabeled antigen to the reaction mix will displace the labeled antigen from the bound state in proportion to the labeled/unlabeled ratio. Separation of free from bound antigen with ammonium sulfate or other means provides a method by which unlabeled antigen (morphine, barbiturate, etc. in liquids such as urine or serum) can be quantitated.

The present report deals with the development of a semiautomated RIA procedure for the detection of morphine, barbiturates, and amphetamines. The initial studies were conducted with commercially available RIA reagents for morphine. The later studies for barbiturates and amphetamines were undertaken with reagents prior to their commercial

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[1]Chief of Biochemistry Function, Division Chief, Chemist, Chemist, and Chief of Drug Abuse Detection Branch, respectively, Epidemiology Division, U.S. Air Force School of Aerospace Medicine, Aerospace Medical Division, AFSC, U.S. Air Force, Brooks Air Force Base, Texas.

introduction. All reagents for evaluation studies were supplied by Hoffman-La Roche, Inc., Nutley, N.J.

Since the essential thrust of this report is the automation of existing tests to large-scale screening procedures, only typical and necessary data pertinent to this end are reported. For additional basic investigations and consequent discussions, the literature and procedures supplied by the manufacturer should be consulted [*8*].

## Experimental

### *Apparatus*

The heart of the semiautomated procedure is the automatic pipetting station (Model No. 24004) manufactured by Micromedic Systems, Inc., Philadelphia, which is designed to dispense repetitively or dilute microvolumes into test tubes, as specially designed racks of tubes are fed automatically across the station. The racks are mated together so that a double row of tubes is presented, 14 per row. In these RIA procedures the instrument is set up in the sample dilution mode, that is, a sequential aspiration of a urine sample from a tube in front, and a transfer of that sample with a volume of diluent into a companion tube in the rear. The tubes are 7 by 12-mm disposable polystyrene test tubes made by Falcon Plastics (Oxnard, Calif.). Some other brands of disposable 7 by 12-mm tubes would not fit the racks.

Centrifuges and a gamma spectrometer are also required equipment. Centrifuges at the Drug Abuse Testing Facility at the USAF School of Aerospace Medicine are adapted to spin the tubes in the racks. The centrifuge head can accommodate 8 racks of 14 tubes each.

### *Reagents*

Morphine, barbiturate, and amphetamine antibodies and $^{125}$I-labeled morphine, barbiturate, and amphetamine antigens were obtained from Hoffman-La Roche, Inc., Nutley, N.J. Morphine and barbiturate antigens and antibodies were received separately and mixed before use. The amphetamine antigen and antibody, as well as the combined morphine-barbiturate divalent reagents, were mixed by the manufacturer and shipped as complete reagents. The reagents were dated to indicate the useful shelf life.

Other materials used included distilled water and a saturated solution of ammonium sulfate, American Chemical Society (ACS) reagent. A few crystals of the salt were allowed to settle to the bottom of the bottle to insure saturation, an essential feature to insure proper precipitation.

### *Radiological Aspects*

Designated Atomic Energy Commission (AEC) procedures required are essentially minimal since only small volumes of radioactive reagents are used and the activity level is low (0.015 microcuries per sample). Disposable test tubes containing the liquid wastes were stored in plastic bags for several half-lives before disposal. Throughout the one-year period of this report, no excessive contamination was encountered within the working area.

### *Procedure*

In the first step of the assays for morphine and for barbiturates, 0.03 ml of urine is

removed from a specimen in the front tube in the double rack. This volume is automatically transferred to the corresponding tube in the rear rack, along with 0.47 ml of the antigen-antibody reagent mix. Urine and reagent are effectively mixed by the force of the pump action. The reaction mix is allowed to incubate for one hour at room temperature.

Next, the racks are fed back through the automatic pipetting station, now set up to dispense 0.5 ml of the saturated solution of ammonium sulfate into the rear tube containing the reaction mixture. Because of the viscosity of the ammonium sulfate solution, the racks must be shaken briefly by hand to mix the reagents and precipitate the antigen-antibody complex. After a 10-min period, the tubes are centrifuged at 3000 *g* for 10 min.

Following centrifugation the racks are again processed through the automatic pipetting station, this time with the reaction mix in the front row of tubes and a clean row of tubes in the rear. The racks are assembled with the set of clean tubes before the centrifugation to avoid disturbing the sedimented material. The instrument is set to aspirate 0.5 ml of supernatant and transfer it to the rear tube, while simultaneously dispensing 1 ml of water into that tube to provide an adequate rinse of the despensing line.

Radioactivity in the tubes containing the supernatant and in appropriate standards is now determined in a gamma spectrometer. Usually three to four standards are run with each lot of 100 specimens to provide an average cutoff value (level of acceptance of a specimen as positive or negative) and to accommodate departures from the desired one-hour incubation time of the reaction mixture.

The actual counting time varied from the one minute suggested by the manufacturer so that a greater number of samples could be processed in a given period. The majority of the specimens were counted for 0.4 or 0.5 min.

Other variations from the original manufacturer's procedure concerned the antibody-antigen mix. The suggested 0.4-ml volume of the mix was diluted to 0.47 ml with saline for both morphine and barbiturate to provide a greater volume, since only 0.015 to 0.03 ml of urine is used. For both amphetamine and the combined morphine/barbiturate, 0.4 ml of the reagent mix was combined with 0.1 ml of urine.

The reagent volumes used in the various steps were selected to permit minimum amounts to be used without increasing the possibility of transferring radioactivity from one tube to the next. Such radioactive transfer is rare with these procedures. A second possible source of radioactive contamination results from the occasional pickup of some of the precipitate when the supernatant is being transferred to the counting tube. This usually results from either inadequate centrifugation or jostling of the tubes subsequent to centrifugation, and is indicated by inordinately high counts.

Urine volumes within the racked tubes should be limited to approximately 1 ml to avoid exceeding the capacity of the automatic pipetting station to adequately wipe the pipetting tip. Unless this is done, droplets on the sampling tip of the pipet may be transferred to the following specimen tube. For an extended discussion on the capabilities and potential problems of the automatic pipetting station, the literature of the manufacturer should be consulted.

## Results

### *Effect of Incubation Time*

The results of allowing the reaction mixture to incubate at room temperature are seen

in Table 1. In the morphine assay the reaction is not complete until about 6 h after mixing. Since this is too long for a mass screening program, a standard one-hour incubation period was chosen for all the RIA methods. Since the counts increase significantly between one and two hours, the one-hour time period must be strictly adhered to.

TABLE 1—*Reaction velocity at room temperature*[a] *and 37°C.*

| Time After Mixing | Room Temperature, cpm | 37°C, cpm |
|---|---|---|
| 10 min | 2196 | 2470 |
| 30 min | 2276 | 2607 |
| 1 h | 2406 | 2787 |
| 2 h | 2605 | 2985 |
| 3 h | 2714 | 3014 |
| 6 h | 2883 | 3158 |
| 24 h | 2815 | 2975 |

[a] 100 ng morphine per millilitre standards in the standard radioimmunoassay system were allowed to incubate for the times indicated before the addition of 0.5 ml of saturated solution of ammonium sulfate.

*Reproducibility of the Method*

Although the counting of any radionuclide is imprecise, the results shown in Table 2 indicate that the method is quite reproducible. The counts per minute (cpm) ± standard deviation are run through the procedure for at least 14 replicates. For instance, the percent standard error for the morphine counting would be $100/\sqrt{2095}$ or 2.2%. In terms of counts, this would be 46 cpm or slightly less than the observed standard deviation. These data show that the automatic pipetting station samples and dispenses with an acceptable reproducibility of about 0.5%.

TABLE 2—*Reproducibility of the radioimmunoassays.*[a]

| Drug | Average Counts per 0.4 min | Standard Deviation | Standard Error | Number |
|---|---|---|---|---|
| Morphine | 2095 | 58 | 15 | 14 |
| Secobarbital | 1632 | 58 | 15 | 14 |
| Amphetamine | 3001 | 96 | 21 | 21 |

[a] The listed drugs were run as replicates using the procedure described in the text.

Curves relating drug concentration to counts obtained are presented in Figs. 1, 2, and 3 for morphine, selected barbiturates, and amphetamine, respectively. These curves may vary somewhat depending upon the lot of reagent used and its age. The variation in reactivity of specific barbiturates with the reagent can be seen in Fig. 2.

Figure 4 shows the curves for morphine and secobarbital in the divalent morphine/barbiturate assay system. Ideally, the curves *should be* superimposable, and in fact *are*

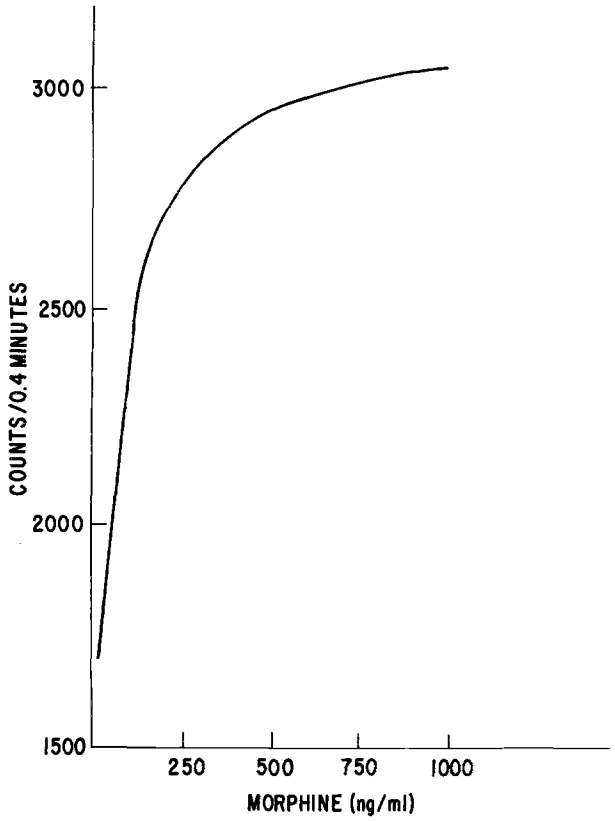


FIG. 1—*Concentration curve for morphine. Standards were added to pooled negative urine and diluted to the concentrations indicated.*

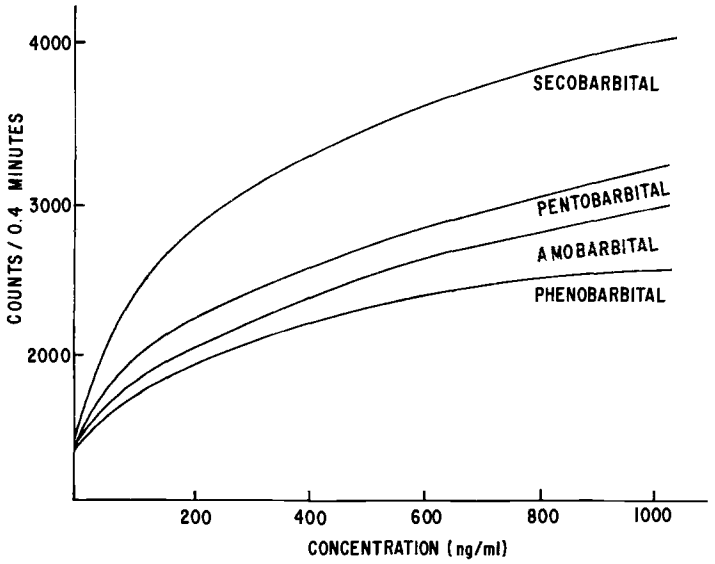


FIG. 2—*Concentration curves for barbiturates. Standards were added to negative pooled urine and diluted to the concentrations indicated.*

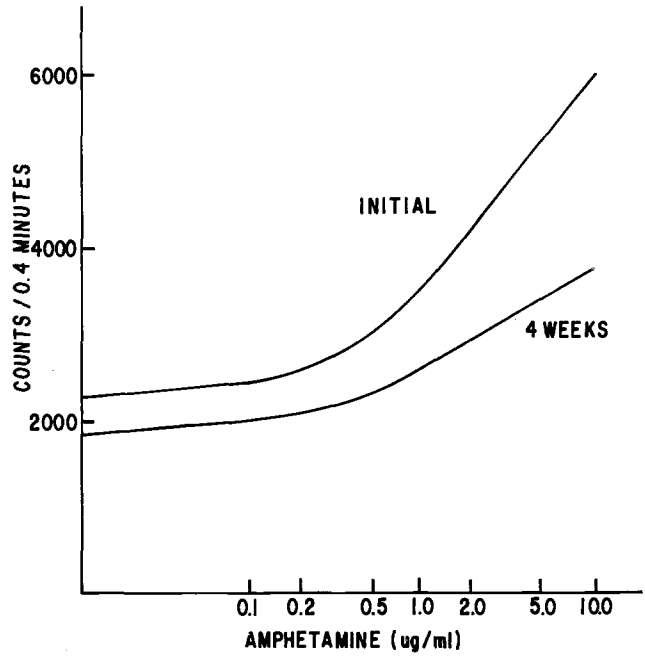


FIG. 3—*Concentration curve for amphetamine. Standards were added to negative pooled urine and diluted to the concentrations indicated. The curve labeled "initial" was run upon receipt of the initial reagent from the manufacturer. The other curve was run four weeks later.*

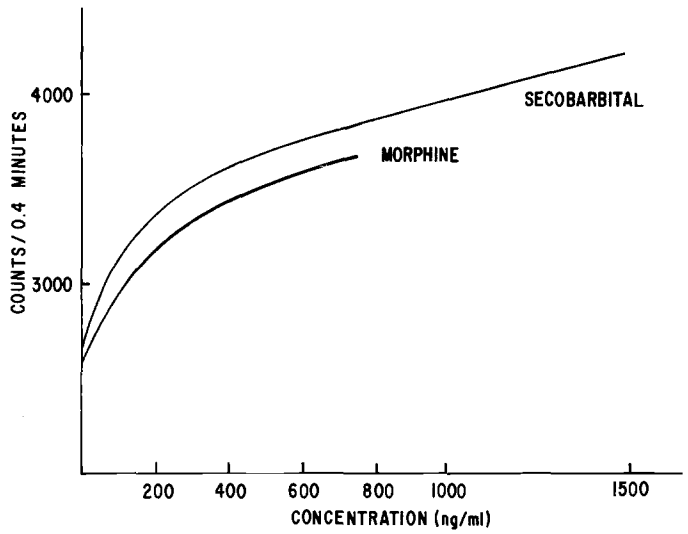


FIG. 4—*Concentration curves for morphine and secobarbital using the divalent reagent. Standards were added to negative pooled urine and diluted to the concentrations indicated.*

with certain batches of the reagent. The curves shown in Fig. 4 represent about the extreme in divergence that we have encountered with the divalent reagent. In practice, the cutoff we use for the divalent reagent is the average of the morphine and secobarbital standards.

*Shelf Life*

Limited shelf life experiments were conducted since the reagents were rarely used beyond four weeks. Results of shelf life determinations for secobarbital and morphine reagents over a period of six weeks indicate a linear decay rate with initial and final counts per 0.4 min of approximately 5000 and 4000, respectively.

*Specificity of the Assays*

Specificity studies were carried out to eliminate the possibility that drugs other than morphine, barbiturates, and amphetamine might interfere in the assays, and thus produce false positives.

These studies were done in two phases: in vitro and in vivo. For the in vitro experiments the specimen was a solution of the test drug in saline. A concentration of $5 \times 10^{-4}M$ was chosen to compare with a previous study made in relation to the free radical assay technique [6]. Drugs that did not interfere with morphine, barbiturate, or amphetamine radioimmunoassay are listed in Table 3. The in vivo drug containing urines were obtained from a pool of urines submitted to the Epidemiology Division for forensic examinations. The urines were positive for the drugs by chemical analytical techniques. Table 4 indicates the in vivo drugs that did not interfere with the radioimmunoassays. The radioimmunoassays for morphine and barbiturates have some cross-over reactivity, as is shown in Table 5. Similarly, a group of phenylethylamine compounds were found to react in the amphetamine assay system. These results are shown in Table 6.

Of the drugs that cross-react in the assay, only codeine would interfere at urine concentrations reasonably obtainable following therapeutic use, and in any program of drug abuse surveillance it is essential that codeine be detectable because of its abuse potential.

*Single Blind Studies*

In order to validate the semiautomated radioimmunoassay, a large number of samples was processed in a single blind evaluation; that is, the technician performing the assays did not know what the urines contained. The urines were placed in identical containers, coded and submitted in a random fashion. These specimens, containing known amounts of specific drugs, were independently prepared and furnished by the Drug Detection Quality Control Laboratory, Armed Forces Institute of Pathology, Washington, D.C.

Table 7 summarizes the results for a morphine single blind run. The "positives" were called when the cpm were equal to or greater than the cpm produced by a morphine standard of 100 ng/ml.

Using a 100-ng/ml standard, the findings (91.6% called positive) for the 100 ng/ml unknowns seem a little high but statistically possible. We cannot account for the nine false negatives at the 500-ng/ml level, except that it is likely they were the result of

TABLE 3—*drugs tested in vitro that did not interfere with morphine, barbiturate, or amphetamine radioimmunoassay.* [a]

| Morphine | | |
|---|---|---|
| aminophylline | diphenoxylate | neostigmine |
| amphetamine | ephedrine | nortriptyline |
| atropine | eserine | phenacetin |
| bufotenine | ethinamate | phenylisopropylhydrazine |
| caffeine | glutethimide | pilocarpine |
| chlordiazepoxide | hexobarbital | procaine |
| chlorpheniramine | homatropine | promethazine |
| chlorpromazine | hydrochlorthiazide | propanalol |
| chlorzoxazone | isoproterenol | propoxyphene |
| cocaine | lidocaine | quinine |
| dapsone | mecamylamine | reserpine |
| dextromethorphan | mephenesin | strychnine |
| diazepam | mescaline | zoxazolamine |
| diphenhydramine | methylphenidate | |
| **Barbiturate** | | |
| aminophylline | ephedrine | mephenesin |
| amphetamine | phenacetin | meprobamate |
| atropine | phenylisopropylhydrazine | mescaline |
| bufotenine | pilocarpine | methadone |
| caffeine | procaine | methylphenidate |
| chlordiazepoxide | promethazine | morphine |
| chlorpheniramine | propanalol | neostigmine |
| chlorpromazine | eserine | nortriptyline |
| chlorzoxazone | ethinamate | propoxyphene |
| cocaine | glutethimide | quinine |
| capsone | homatropine | reserpine |
| dextromethorphan | hydrochlorthiazide | scopalamine |
| diazepam | isoproterenol | strychnine |
| diphenhydramine | lidocaine | zoxazolamine |
| diphenoxylate | mecamylamine | |
| **Amphetamine** | | |
| aminophylline | eserine | neostigmine |
| atropine | ethinimate | nortryptiline |
| bufotenine | glutethimide | paraflex |
| chlordiazepoxide | hexobarbital | phenacetin |
| chlorpromazine | inversine | phenergan |
| codeine | isoproterenol | pilocarpine |
| dapsone | lidocaine | procaine |
| dextromethorphan | mephenesin | propanalol |
| dibenamine | mescaline | pyribenzamine |
| diphenoxylate | morphine | reserpine |
| ergotamine | | |

[a] Drugs were tested at a concentration of $5 \times 10^{-4} M$ against cutoff levels of 100, 200, and 2000 ng/ml of morphine, secobarbital, and amphetamine, respectively.

TABLE 4—*In vivo drugs that did not interfere with the morphine or barbiturate radioimmunoassay.* [a]

| | |
|---|---|
| ampicillin | chlorthiazide |
| ascorbic acid | dextromethorphan |
| aspirin | diazepam |
| caffeine | diphenhydramine |
| diphenylhydantoin | pentaerythritol tetranitrate |
| erthrityl tetranitrate | phenacetin |
| guanethidine | phenmetrazine |
| hydroxyzine | phenothiazine |
| lidocaine | phenothiazine sulfoxide |
| meprobamate | propoxyphene |
| methaqualone | thioridazone |
| norephedrine | trichlormethiazide |
| oxymetazoline | |

[a] The drugs listed were contained in clinical toxicologic specimens. Their presence were shown by chemical analyses. Concentrations of the above drugs varied, but all were in excess of $5 \times 10^{-4} M$.

TABLE 5—*The drugs listed below were equivalent to 100 ng morphine per ml in the assay at the concentration indicated.* [a] *Glutethimide gave a response equivalent to 100 ng secobarbital per ml at a concentration of 100 ng/ml.*

| Drug | Concentration, ng/ml |
|---|---|
| codeine | 90 |
| hydrocodone | 50 |
| meperidine | 125 |
| nalorphine | 150 |
| oxycodone | 50 |

[a] The drugs indicated were dissolved in saline and tested in the appropriate assay.

TABLE 6—*Interference of 10,000 ng/ml of phenylethylamine drugs with the amphetamine radioimmunoassay.* [a]

| Drug | Equivalent to, ng amphetamine/ml |
|---|---|
| ephedrine | 100 |
| mephentermine | 200 |
| methamphetamine | 500 |
| methoxamine | 100 |
| phenylephrine | 200 |
| phenylpropanolamine | 500 |
| pseudoephedrine | 100 |

[a] The drug concentrations are that of the free bases. The "equivalent to" refers to equivalent counts per minute; that is, 10,000 ng ephedrine per ml gives the counts per minute of 100 ng amphetamine/ml in the amphetamine radioimmunoassay system.

TABLE 7—*Morphine reagent single blind evaluation.*

| Morphine, ng/ml urine | Number of Specimens | Number Called Positive | % Called Positive |
|---|---|---|---|
| 50 | 305 | 87 | 28.5 |
| 100 | 310 | 284 | 91.6 |
| 125 | 230 | 230 | 100.0 |
| 250 | 220 | 220 | 100.0 |
| 500 | 1625 | 1616 | 99.5 |
| 750 | 220 | 220 | 100.0 |
| 1000 | 160 | 160 | 100.0 |

Number of negatives = 4300
Number of negatives called positive = 2 (0.05%)

manual recording or other administrative errors. Note that there were only two false positives out of 4300 negative specimens.

Table 8 shows the results for a barbiturate single blind evaluation. In this case, all 500-ng/ml samples were called positive using a 100-ng/ml cutoff. No false positives were seen in 2662 negative urines. The positive finds for those specimens presumably containing drug concentrations below the indicated threshold level cannot be adequately explained.

TABLE 8—*Barbiturate reagent single blind evaluation.*

| Secobarbital, ng/ml urine | Number of Specimens | Number Called Positive | % Called Positive |
|---|---|---|---|
| 50 | 50 | 50 | 100.0 |
| 100 | 101 | 101 | 100.0 |
| 250 | 199 | 198 | 99.5 |
| 500 | 148 | 148 | 100.0 |
| 1000 | 151 | 151 | 100.0 |
| 1500 | 151 | 151 | 100.0 |
| 2000 | 120 | 120 | 100.0 |

Number of negatives = 2662
Number of negatives called positive = 0

The results for the amphetamine radioimmunoassay are in Table 9. The cutoff used in this evaluation was 2000 ng/ml.

The results for the single blind evaluation of the (divalent) morphine/barbiturate assay are seen in Table 10. Of 2738 negative urines, seven were called positive; that is, they were false positives. Upon reassay with the monovalent barbiturate and monovalent morphine reagent, these seven positives were called negative. The cutoff used was the average for 100 ng/ml for morphine and secobarbital.

## Discussion

The semiautomated procedure described in this report has been used extensively by the drug abuse testing facilities within the U.S. Air Force. On the basis of our experience

TABLE 9—*Amphetamine reagent single blind evaluation.*

| Amphetamine, ng/ml urine | Number of Specimens | Number Called Positive | % Called Positive |
|---|---|---|---|
| 250 | 68 | 1 | 1.5 |
| 500 | 68 | 0 | 0.0 |
| 1 000 | 112 | 1 | 0.9 |
| 2 000 | 112 | 80 | 71.4 |
| 3 000 | 110 | 110 | 100.0 |
| 4 000 | 160 | 160 | 100.0 |
| 5 000 | 112 | 111 | 100.0 |
| 6 000 | 94 | 94 | 100.0 |
| 8 000 | 78 | 78 | 100.0 |
| 10 000 | 112 | 111 | 99.1 |

Number of negatives = 1016
Number of negatives screened positive = 2

TABLE 10—*Morphine/barbiturate divalent reagent single blind evaluation.*

| Morphine, ng/ml urine | Number of Specimens | Number Called Positive | % Called Positive |
|---|---|---|---|
| 50 | 140 | 2 | 1.4 |
| 100 | 141 | 58 | 41.1 |
| 200 | 143 | 143 | 100.0 |
| 300 | 155 | 155 | 100.0 |
| 500 | 138 | 137 | 99.3 |
| 750 | 139 | 137 | 98.6 |
| **Secobarbital, ng/ml urine** | **Number of Specimens** | **Number Called Positive** | **% Called Positive** |
| 50 | 133 | 80 | 60.2 |
| 100 | 140 | 135 | 96.4 |
| 200 | 146 | 145 | 99.3 |
| 300 | 143 | 143 | 100.0 |
| 500 | 120 | 119 | 99.2 |
| 750 | 144 | 144 | 100.0 |
| 1000 | 148 | 148 | 100.0 |
| 1500 | 132 | 132 | 100.0 |

Number of negatives = 2738
Number of negatives called positive = 7 (0.25%)

with over 400,000 urine specimens analyzed during the period of this study and the results of the concurrent internal and external quality control programs, we feel that the RIA methodology for mass screening of urine is acceptably reliable and rapid.

The major advantages have been a reduction in manual procedures and the elimination of large solvent volumes, hydrolysis procedures for morphine, and qualitative judgment steps requiring highly trained and experienced personnel. The latter is a result of the fact that a numerical cutoff value is used. The reduction in program personnel from

previous methodology is approximately 50%. The reduction in personnel, as well as the elimination of hoods and other apparatus, has reduced laboratory space requirements.

Technically, the advantages gained from the sensitivity of RIA methods must be balanced by the variation in specificity toward the different classes of drugs. For opiates and barbiturates the RIA methods have proved to be highly sensitive, but specificity is directed to the drug group (opiates or barbiturates), rather than to individual compounds. Because of less specificity the amphetamine procedure requires a higher cutoff value to reduce the possibility of interfering drugs. Consequently, GLC procedures for amphetamines are capable (with larger urine volumes) of detecting smaller quantities of the drug. This is counterbalanced by the advantages of the RIA methodology and in practice has not proved to be a burden on the confirmatory procedures.

Various cutoff values for the different drugs have been used throughout the studies reported. Those used were selected as a compromise to take advantage of the increased sensitivity of the methodology while meeting the detectability requirements of 5000 ng of amphetamine, 100 ng of barbiturates, and 500 ng of morphine in use at the time of these studies.

**Summary**

A rapid, semiautomated radioimmunoassay system for detection of morphine, barbiturates, and amphetamines is described. The assays are applicable to large drug abuse screening programs. The heart of the system is the automatic pipetting station which can accomplish 600 pipetting operations per hour. The method uses 15 to 30 μl of urine for the morphine and barbiturate assay and 100 μl for the amphetamine and combined morphine/barbiturate assays. A number of other drugs were tested for interference with the assays and the results are discussed.

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Division Chief
Epidemiology Division
USAF School of Aerospace Medicine
Brooks AFB, Texas 78235