TECHNICAL REPORT NO. LWL-CR-07B73

DETECTION OF DRUGS BY AN ENZYME METHOD

Final Report

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U. S. ARMY LAND WARFARE LABORATORY
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FOREWORD

This report is submitted in compliance with contractual requirements as directed by the U.S. Army Land Warfare Laboratory, Aberdeen Proving Ground, Maryland, under Contract No. DAAD05-73-C-0145.

The Franklin Institute Research Laboratories wishes to acknowledge the collaborative assistance given by Mr. Harold H. Rosen of the Biological Sciences Branch during this project.

Principal Investigator for the program at The Franklin Institute Research Laboratories was Ms. Tamar Der O Hannessian, Research Chemist, Materials & Physical Sciences Department. Other personnel contributing significantly to the program were Dr. Peter Francis, Director of the Materials & Physical Sciences Department and Mr. William Collins, Director of the Electrical Engineering Department.

ABSTRACT

Under Work Assignment No. 1 of Contract DAAD05-73-C-0145 by The Franklin Institute Research Laboratories (FIRL) for the U.S. Army Land Warfare Laboratory, a study was conducted using the standard Army "G" Agent Detector Kit, ABC-M30A1 as a means for detecting drugs (heroin).

The objective of this study was to establish the sensitivity of the enzyme inhibition method for morphine detection and to determine a procedure for rapidly cleaving the glucuronide.

CONTENTS

FIGURES

1. INTRODUCTION

There is, at present, a great variety of tests which detect morphine and other drugs in body tissues and fluids, especially the urine, but they all involve procedures to be performed in laboratories requiring the use of specialized techniques and apparatus. The aim of this study was to apply the kit available for "G" agent detection to morphine, and also to find a simple and fast way of splitting the glucuronide of morphine which would be compatible with the enzyme test. The splitting of the glucuronide would make more morphine available in the test.

Since morphine glucuronide was not available commercially, an attempt was made at synthesizing morphine glucuronide.

2. CONCLUSIONS

Morphine did inhibit cholinesterase and could be detected in urine using the M30A1 Agent Detector Kit. However, the method as used in the kit was not sensitive enough to directly detect that quantity of free morphine generally present in the urine of addicts. To utilize the kit either the morphine in addicts' urine would have to be concentrated by extraction or the morphine glucuronide split to raise the level of free morphine. Another alternative would be to utilize the inhibition method in a manner different from the kit, which might prove to be more sensitive.

No clear-cut conclusion can be arrived at concerning the attempts to split the glucuronide. The analytical methods used were such that the background levels in urine were high, and increases in the level of free morphine could not be measured. However, in no case did any hydrolysis method tried raise the free morphine level in addicts' urine to a point that could be detected by the M30A1 kit.

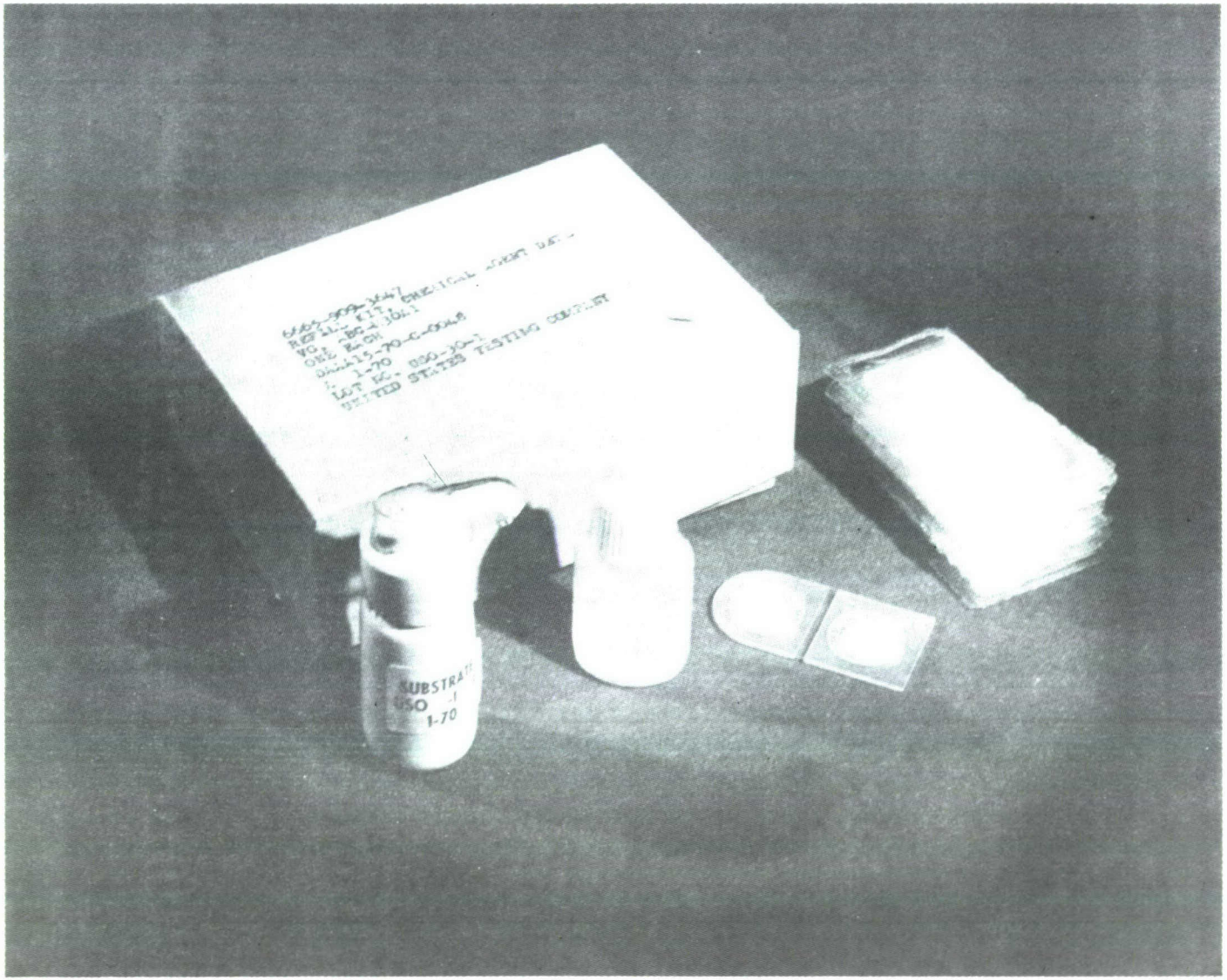

Figure 2-1. Photograph of the M30A1 Kit

3. THEORETICAL

3.1 G-AGENT DETECTION KIT--M30A1

The Army M30A1 G-Agent Detector Kit utilizes the inhibition of cholinesterase as the basis for agent detection. In the kit a paper disc impregnated with cholinesterase is wetted with buffer solution and exposed to the toxic agent. The substrate, dichloroindophenol acetate, is then added to the disc. If no inhibitory agent is present, the enzyme cleaves the substrate, yielding a blue color. The absence of color indicates the presence of the agent. One purpose of this study was to determine whether this kit could be applied to the detection of morphine.

3.2 MORPHINE AS AN INHIBITOR OF CHOLINESTERASE

The inhibitory action of morphine on cholinesterase has been established (1) and was confirmed in this study utilizing the kit described above. From observation of color development versus time and substrate concentration, it is proposed that the inhibition of cholinesterase by morphine is reversible (unlike the organophosphates which inhibit cholinesterase irreversibly [2]). This proposition is developed more fully in the experimental section of this report.

3.3 CLEAVING THE MORPHINE GLUCURONIDE

Most of the morphine ingested by humans is excreted in the urine.* Of this, only about 5 percent appears as free morphine, the balance being conjugated as the glucuronide. Any test for morphine in the urine would, therefore, be enhanced by splitting the glucuronide. This was another portion of the effort described herein.

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*Krantz and Carr, Pharmacologic Principles of Medical Practice, p. 556.

3.4 SYNTHESIS OF MORPHINE GLUCURONIDE

To facilitate the study of the cleavage of glucuronide, it was necessary to obtain this substance. None was found available, and most syntheses (3) or extractions from natural sources (4) were too time consuming, complex, or uncertain to be accomplished in the short time allotted to this study. Several unsuccessful attempts were made to produce the glucuronide directly from morphine and glucuronice acid and are described below.

4. SELECTION OF ANALYTICAL TECHNIQUE AND INITIAL EXPERIMENTS

4.1 EXTRACTION AND SPECTROPHOTOFLUOROMETRY

The level of free morphine as excreted in urine is too low to be detected; therefore it was extracted into a smaller volume of solvent and used in subsequent analyses.

The urine samples (3 ml aliquots) were made basic (pH9) with 20% supersaturated sodium borate and extracted with 9 ml of 5% isobutanol in chloroform. The extracts were washed with 0.01% sodium borate and the morphine extracted with 1 ml 0.1N H_2SO_4. The aqueous extracts were in turn washed twice with chloroform. 0.4 ml of the acidic extract was pipetted into a quartz 10 x 10 mm cuvette. 1 ml of saturated sodium borate was added and the fluorescence measured in an Aminco Bowman spectrophotofluorometer with excitation at 290 nm and emission at 430 nm. The reading was adjusted to zero with the blank, and 1 drop of 0.19 mg/ml potassium ferricyanide was added, mixed, and the fluorescence measured again. An increase in the fluorescence indicates morphine (5).

4.2 THIN LAYER CHROMATOGRAPHY

10 ml urine samples were extracted and concentrated prior to chromatography. The extraction procedure involved adjusting the pH of the samples to 9 with NH_4OH and extraction with 9:1 chloroform-isopropanol. The organic layer was separated, evaporated to dryness and the residue dissolved in 1 ml ethanol. The extracts were spotted on Silica Gel G plates (1.5" x 3.5") and developed in Davidow's solution: ethylacetate-methanol-conc. ammonium hydroxide (255:30:50). The plates were subsequently dried at 120° C for 20 minutes, cooled to room temperature for three minutes and sprayed with iodoplatinate reagent. A blue spot indicates morphine (Rf = 0.3).

4.3 SPECTROSCOPY

Morphine has a broad absorption peak at 285 nm and the absorbance can be quantitated against a standard solution. The Bausch and Lomb Spectronic 505 was used with 1 cm cells.

4.4 INCUBATION WITH GLUCURONIDASE

The glucuronidase enzyme was used with addicts' urine samples to hydrolyze the morphine glucuronide and increase the level of free morphine. The enzyme was obtained from Worthington Biochemicals. A 20 mg/ml aqueous solution was prepared and 0.5 ml was used to hydrolyze 5 ml of addicts' urine samples. The pH was adjusted to 4.5 with sodium acetate buffer, and the samples incubated at 37° C overnight. All runs were accompanied by an incubation mixture containing urine from control subjects, phenolphthalein glucuronide and glucuronidase, to make sure that the enzyme maintained its activity under the given conditions.

4.5 ACID HYDROLYSIS

As an alternative method to incubation with glucuronidase, urine samples were autoclaved with acid to cleave the glucuronide (7-9) and raise the level of free morphine. 3 ml of urine samples were autoclaved with 0.3 ml conc. HCl or HI at 15 psi. The duration was varied from 15 minutes to 2 hours. All runs included urine samples from control subjects to which morphine tartrate was added. The purpose was to make sure that morphine was not affected by the conditions used.

4.6 SYNTHESIS OF MORPHINE GLUCURONIDE

Synthesis of morphine glucuronide was attempted with morphine base and glucuronic acid as starting materials. The following solvents were tried: dioxane, acetone, ether, benzene, dimethyl sulfoxide (DMSO) and methanol. Of these only the last two could solubilize both components. But DMSO could not be used because it was found to interact with the glucuronic acid in the presence of hydrogen chloride. Methanol was first

dried over anhydrous sodium carbonate and then filtered into a round bottom flask with two inlets. The morphine and the glucuronic acid were then added (0.5 gm of each) and dissolved partially in the methanol. A capillary tubing was fitted in one inlet through which gaseous hydrogen chloride was bubbled into the reaction mixture. The outlet was fitted with a desiccant to prevent any moisture from getting in. After three days at room temperature and with magnetic stirring, the reaction mixture was centrifuged. The precipitate was divided into two. One part was dissolved in water and the other dissolved in more methanol. The supernate from the reaction mixture and the two solutions were spotted on Silica Gel G plates and developed in butanol-acetone-acetic acid-5% ammonia-water (45:15:10:10:20) (10). After drying, the plates were sprayed with the iodoplatinate reagent.

5. EXPERIMENTAL

5.1 SENSITIVITY OF CHOLINESTERASE TO MORPHINE

Since the primary objective was to apply the "G" agent detector kit to morphine, it was necessary to establish the feasibility of the test and to determine its sensitivity.

Using morphine as the inhibitor of cholinesterase at a concentration of 1% caused almost complete inhibition. When diluted to 0.1%, inhibition was only partial but the inhibited disc could still be distinguished from the uninhibited. Morphine concentrations lower than 0.1% failed to produce an inhibition detectable by the kit.

It was also observed that in the presence of higher substrate concentration, the inhibition was less obvious and also in time, the color on the inhibited discs became the same as the uninhibited. These observations supported the reported fact that the inhibition due to morphine was reversible and competitive. This would necessitate high concentrations of the inhibitor to compete with the substrate and produce an inhibitory effect.

The test was modified by using vials in which 5 µl of the substrate was dried. 0.1 ml of buffer was added followed by 10 µl of cholinesterase solution (210 units/ml). Thus, by lowering the concentration of the substrate, the method seemed to become more sensitive; so that a 0.01% morphine tartrate solution did produce a detectable degree of inhibition.

Thus, the test kit as available at present would not be suitable for detecting morphine in addicts' urine.

An attempt was made to raise the level of free morphine in addicts' urine by splitting the glucuronide, so that it could be detected by the M30A1 kit. Urine samples obtained from addicts and shown to be morphine positive by extraction and thin layer chromatography were autoclaved with

hydrochloric acid or incubated with glucuronidase as described earlier. When subsequently used on the kit, no significant inhibitory action could be observed (Table 1).

5.2 SPECTROSCOPY AND TLC

Both were found to be very useful and relatively sensitive methods of showing the presence of morphine in water, but they had their drawbacks. Spectrophotometry could not be applied to extracts from urine samples due to very high baseline readings. Thin layer chromatography, on the other hand, worked well with urine extracts but was only qualitative.

5.3 HYDROLYSIS AND ANALYSIS OF MORPHINE GLUCURONIDE

First, it was necessary to extract the urine samples and determine the morphine content. Since the only source of morphine glucuronide was the urine samples obtained from addicts, success in evaluating any hydrolytic method would depend on the efficiency of the extraction procedure and the sensitivity of the analytical method used to quantitate the free morphine. A great variety of extraction procedures have been described, the most suitable of which was found to be the method described by Santinga (5) which involved raising the pH to 9 and extraction with 5% isobutanol in chloroform. As for the quantitative measurements, the most sensitive was spectrophotofluorometry.

The procedure used was according to P.H. Santinga (5). As low as 0.2 µl/ml of morphine tartrate in water could be detected by this procedure (Table 2); but when urine samples were used, the method failed. This was due to the very high readings obtained with urine blanks (containing no morphine) so that the sensitivity of the instrument had to be kept very low to keep the readings on scale. Thus, when the potassium ferricyanide solution was added, no increase in fluorescence was observed.

0.5 mg morphine tartrate added to 3 ml non-addict urine, on the other hand, could be detected by the above method. So, at least 0.5 mg/3ml

Table 1. Urine on Cholinesterase Discs Before and After Incubation with Glucuronidase

Urine Sample	Color after 5 Minutes
1	C darker than I
2	C lighter than I
3	C same as I
4	C lighter than I
5	C same as I
6	C very slightly darker than I
7	C same as I
8	C lighter than I
10	C same as I
11	C darker than I
12	C lighter than I
13	C same as I
Control 1	C slightly lighter than I
Control 2	C same as I

C = before incubation

I = sample incubated with glucuronidase

Table 2. % Transmittance of Morphine Tartrate

Concentration µg/m	% Transmittance Initial	% Transmittance Final
200	16.5 x .001	49.0 x .01 38.0 x .03*
100	13.0 x .001	48.0 x .01
50	12.5 x .001	40.0 x .01
20	11.5 x .001	27. x .01
10	11.0 x .001	61.0 x .003
5	10.5 x .001	34.5 x .003
2	10.5 x .001	44.0 x .001
1	13.0 x .001	32.0 x .001
0.5	15.0 x .001	22.0 x .001
0.2	13.0 x .001	17.0 x .001

*After two more drops of potassium ferricyanide.

of morphine would be needed in the urine before it can be detected by the method (Table 3).

Hydrolysis of morphine glucuronide was repeated using acid or enzyme hydrolysis as described in Sections 4.4 and 4.5. The length of the acid hydrolysis varied from 15 minutes to 2 hours. Enzyme incubations were performed at 37° C overnight. The urines were then extracted and assayed spectrophotofluorometrically. Both treatments failed to produce levels detectable by spectrophotofluorometry (Table 4). To show that morphine was not destroyed by autoclaving, 0.5 mg of morphine tartrate was added to 3 ml water and subjected to the same hydrolytic and extraction procedures. A sharp increase in fluorescence was observed (Table 4).

Hydriodic acid was also used (Table 5). It was found that hydriodic acid was not a suitable hydrolyzing agent because when added to solutions of morphine, it affected the morphine so that it could not be measured. This was also true for solutions which were not autoclaved.

5.4 MORPHINE GLUCURONIDE SYNTHESIS

As described in Section 4.6, the synthesis was attempted with morphine base and glucuronic acid as starting materials. Thin layer chromatography was used to check the progress of the reaction. Both the supernatant liquid and the precipitate present in the reaction mixture showed only morphine. Absence of other spots indicated that the reaction had not progressed.

5.5 SUMMATION OF RESULTS

Since no morphine glucuronide could be made available either from manufacturers or by our own attempts at synthesis, evaluation of hydrolytic procedures was limited to the work done on addicts' urine and no conclusions could be drawn.

As to the sensitivity of the M30A1 kit, a 0.1% or 1000 µg/ml morphine solution is required to show a detectable inhibition. This level is much higher than the level of morphine and morphine glucuronide excreted

Table 3. Spectrophotofluorometric Determination of Morphine Extracts from Urine

Sample	% Transmittance	
	Initial	Final
Control	23 x .003	24 x .003
2	57 x .003	57 x .003
3	24 x .01	49 x .01
4	14 x .01	14 x .01
5	50 x .01	48 x .01
6	29 x .01	29 x .01
8	48 x .01	47 x .01
Control + 50 µl Morphine Solution	29 x .003	43 x .003

Table 4. Spectrophotofluorometric Determination of Morphine Extracts from Sample Autoclaved with HCl

Sample	% Transmittance	
	Initial	Final
Water + 0.5 mg Morphine Tartrate Solution	44 x .001	60 x .01
Control Urine + 0.5 mg Morphine Tartrate Solution	53 x .003	30 x .01
1	44 x .01	42 x .01
2	27 x .01	26 x .01

Table 5. Spectrophotofluorometric Determination of Morphine Extracts from Samples Autoclaved with HI

Sample	% Transmittance	
	Initial	Final
Water + 50 µl Morphine Tartrate Solution	13 x .001	15 x .001
2	54 x .01	52 x .01
3	45 x .01	44 x .01

in the urine of addicts. Therefore, a modification of the test should be sought to enhance the effect of morphine on cholinesterase and raise the sensitivity of the test.

During the progress of this study, a hemagglutination-inhibition test for morphine became available commercially, under the brand name HI-M-Test. This made use of the high specificity and sensitivity of antigen-antibody reactions. Tried in our laboratories, the test gave a distinct positive result with all the addicts' urines available. The method was also quite fast (60-90 minutes) and very convenient for mass screening. One drawback was the fact that the reagents did not have a long shelf life (about 2 weeks).

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