Thin-layer Chromatography and Enzyme Immunoassay of \(L\)-Alpha-Acetyl Methadol and Methadone Metabolites in Urine

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We describe a thin-layer chromatographic technique suitable for isolating at least four metabolites of \(L\)-\(\alpha\)-acetyl methadol. The metabolites are discussed in terms of their color reactions to chromatography spray reagents and their appearance in the urine during various time intervals after dosage. Methadone is detected by the presence of the unmetabolized drug and two of its metabolites in the urine. We present a simple means of distinguishing methadone-containing specimens from those that contain \(L\)-\(\alpha\)-acetyl methadol. Results of an immunoassay for methadone applied to specimens from patients receiving \(L\)-\(\alpha\)-acetyl methadol or methadone are discussed. The method is suitable as a routine screening procedure for most laboratories that use thin-layer chromatography.

Additional Keyphrases: toxicology • screening • drug abuse

\(L\)-\(\alpha\)-Acetyl methadol (LAAM) has been considered as a substitute for methadone in the maintenance of narcotics addicts (1, 2). The possibility of its large-scale use is currently being evaluated in an extensive cooperative study between the Veterans Administration and the Special Action Office for Drug Abuse Prevention. There are numerous advantages to the use of LAAM. Because it is long acting, fewer visits to the clinic are required and the need for take-home doses is eliminated. The drug can be administered by a nurse or other authorized person, and thus an existing program can be expanded to accommodate more patients without increasing the number of staff members. The patient is benefited by becoming less involved in drug-seeking behavior and by being able to spend his time more advantageously in attending school or supporting his family. A decreased traffic in illicit methadone benefits the community.

A first need in the cooperative study was for an analytical method that would provide information on the extent of drug abuse by patients participating in the program. We have applied a thin-layer chromatographic method based on Davidow’s procedure (3), by which specimens may be analyzed for LAAM metabolites and a wide range of other drugs. The thin-layer method is complemented by an enzyme-immunoassay (4–7) that is sensitive to 0.5 \(\mu g\) of methadone hydrochloride per milliliter of urine.

Materials and Methods

Apparatus

Thin-layer chromatography apparatus. This includes glass plates coated with a 250-\(\mu\)-layer of silica gel G-60 and divided glass tanks with covers (all supplied by Brinkmann Instruments, Inc., Westbury, N. Y. 11590). The plates are activated for 30 min at 105 °C and stored in a desiccator before use.

Repipet, 50 ml, for extracting solvent (Lab Industries, Inc., Berkeley, Calif. 94710).

Wide-mouth glass centrifuge tubes, 50 ml, with an etched area for labeling (Corning Glass Works, specially supplied by Aremco Scientific, Sepulveda, Calif. 91343).

Culture tubes, 50 ml, with Teflon-lined caps (Corning).

"Pipetman" repipet, for microliter volumes, 0–200 \(\mu l\) (Cole Scientific Co., Canoga Park, Calif. 91303).

Reagents

All solvents (Malinckrodt Chemical Co., Los Angeles, Calif. 90058) and chemicals of reagent grade were used without further purification. Extracting solvent and Dragerhoff reagent were prepared according to Davidow et al. (3). The remaining solutions were modified and prepared as follows:

Ammonium chloride buffer. Add 220 g of ammonium chloride (Malinckrodt) to 560 ml of distilled water, mix until the solution has reached room temperature, and add 110 ml of strong (27%) ammonia
solution. The pH of this solution should be 9.1 ± 0.05; if necessary, adjust with hydrochloric acid or ammonium hydroxide. Store the solution in a stoppered bottle and check the pH daily.

Developing solvent. Ethyl acetate:methanol:strong ammonia solution (17:2:1 by volume). The use of strong ammonia solution instead of concentrated ammonium hydroxide (38% NH₃) facilitates the separation of unmetabolized methadone from its metabolite.

Ninhydrin spray reagent. Dissolve 0.5 g of ninhydrin in 100 ml of n-butanol. Store refrigerated and protected from light.

Iodoplolate spray reagent. Prepare according to Davidow et al. (3) with chloroplatinic acid (J. T. Baker Chemical Co., Phillipsburg, N. J. 08865). We have found that the Baker reagent to be the most sensitive and stable. Store refrigerated. Results are better when this reagent is prepared one day in advance. Discard unused solution after four days.

Standard stock solution. Methadone hydrochloride, 10 mg per milliliter of distilled water.

Minimum working standard (1 μg/ml final concentration). Dilute 200 μl of the methadone stock solution to 5 ml with distilled water.

Maximum working standard (12.5 μg/ml final concentration). Dilute 2 ml of the methadone stock solution to 4 ml with distilled water.

Enzyme Multiplied Immunoassay Technique (EMIT)² Apparatus

The apparatus for EMIT described by Schneider et al. (5) is used without modification. The reagents are supplied by the Syva Corporation.

Methods

Extraction and chromatography. Add 25 μl of each of the working standard solutions to separate culture tubes containing 10 ml of drug-free urine and mix. Add 1.0 ml of the NH₄Cl buffer to all of the tubes to be used in the assay. Using one of the standard tubes as a visual reference, add about 10 ml of urine unknowns to each tube. Dispense extracting solvent to fill the tubes, using a setting of 28 to 31 ml on the Repipet. Cap the tubes and shake them on an automatic shaker at 280 strokes per minute for 1 min. Centrifuge at 1200 rpm for 5 min and aspirate and discard the aqueous (upper) phase. Dry the organic phase by filtering it through coarse filter paper into a 50-ml conical centrifuge tube. Add a small chip of a crushed boiling granule and evaporate the extract in a water bath at 70 °C. Use a Pasteur pipet to wash the sides of the tube with about 1 ml of methanol. Evaporate the alcohol, reconstitute the residue in two drops of methanol by rolling the warm tube gently at an angle, and spot the extract on a chromatography plate. Develop the plate to 10 cm above the point of application (about 25 min) and allow it to dry in a fume hood.

Color development. Heat the plate at 70 °C for 10 min and spray it with ninhydrin while it is still warm. Mark any pink or purple spots and expose the plate to short-wave ultraviolet light in a portable darkroom for 4 to 5 min. Spray the plate with the iodoplolate reagent, allow it to dry and spray with Dragendorff reagent.

EMIT procedure. The procedure of Schneider et al. (5) is followed without modification.

Results

Thin-layer Chromatography

The characteristic color reactions and mobilities (Rₚ) of four detectable metabolites of LAAM and three detectable metabolites of methadone are summarized in Table 1. Urine specimens from methadone patients contain a spot that corresponds to the methadone standard, which is detected at Rₚ 0.68, a major metabolite at Rₚ 0.78 and a minor, less frequently detected metabolite at Rₚ 0.90. After spraying with the ninhydrin reagent and exposing to ultraviolet light, the intense pinkish-purple reaction and subsequent reddish appearance of the metabolite at Rₚ 0.78 is very characteristic of methadone. The Table demonstrates that the LAAM metabolite at the same Rₚ does not react with ninhydrin.

The number of detectable LAAM metabolites in urine appears to depend on the time interval between ingestion of the drug and collection of the specimen. Kaistha and Jaffe (8) detected the presence of two metabolites in urine by thin-layer chromatography. The four metabolites that we have detected appear at various times up to 72 h after the ingestion of LAAM. The metabolite that is reactive to ninhydrin plus ultraviolet light at Rₚ 0.53 is detected up to about 24 h, but it is not always observed. We have not determined the optimum collection time for isolating this metabolite as yet, but we have observed that it is always detected in the presence of the metabolite at Rₚ 0.60. The metabolite at Rₚ 0.60 is the major extractable metabolite during the first 24 h and its concentration gradually decreases as the final metabolites at Rₚ 0.68 and Rₚ 0.78 begin to be detected. These metabolites are detected up to at least 48 h after ingestion of the drug. Table 1 illustrates that unmetabolized LAAM is detected at Rₚ 0.78, but we do not think that the corresponding spot that is detected in chromatography of the urine of LAAM patients is this compound because of its late appearance in the urine. This finding is consistent with the observation of Kaiko and Inturrisi (9) that less than 2% of the drug is excreted unmetabolized.

The metabolite detected at Rₚ 0.60 is the most characteristic metabolite of LAAM, but its detection depends on the hour of specimen collection after the dose, and it may not be detected in urine of a patient

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² "EMIT" is a registered trademark of the Syva Corp., Palo Alto, Calif. 94304.
taking LAAM. During the later stages of metabolism, the only observable difference between LAAM-containing urines and methadone-containing urines is the nonreactivity of the LAAM metabolite at RF 0.78 to the ninhydrin spray reagent and ultraviolet light. This demonstrates the importance of marking the ninhydrin-reactive spots when specimens from both types of patients are being analyzed.

Other Drugs

This procedure has also proven itself to be an effective screening method for many drugs that are commonly detected in the urine specimens of persons on a methadone maintenance regimen. By doubling the urine and buffer volume and splitting the extract for chromatography on two chromatography plates, barbiturates and related compounds may be detected with use of the same single-solvent development system and barbiturate detection reagents (3) on the second plate. We have encountered little or no interference with the detection of methadone or LAAM by other drug metabolites.

Enzyme Immunoassay

The EMIT assay is based on a competitive-binding process between an antibody–drug–enzyme complex and an antigen (the drug in the urine). The enzyme in this complex is inactive because of steric hindrance by the large antibody molecules located near the active site. When antigen is present, some antibody is made unavailable for binding to the drug–enzyme. The amount (activity) of unbound enzyme, measured spectrophotometrically, is proportional to the amount of drug in the urine. The method is rapid because urine specimens can be tested directly without any previous extraction or other treatment. The assay is useful for the direct analysis of specimens that contain compounds that may interfere with the thin-layer chromatographic determination of methadone but do not interfere with the EMIT assay.

The results of the EMIT assay for methadone in urine specimens from methadone patients and LAAM patients are presented in Table 2. We have observed that the EMIT assay is consistently positive when unmetabolized methadone is detected by thin-layer chromatography and negative when it is not detected. The immunoassay is negative when either of the two initial LAAM metabolites at RF 0.53 and RF 0.60 are detected and positive when the final two metabolites are present. This cross-reactivity is evidence that the immunoassay is not sensitive only to free methadone.

Discussion

Several authors have described metabolites of LAAM (8-12) in urine, and much research is currently being devoted to the elucidation of their structure and activity. Methadone and LAAM appear to

Table 1. Detection of Urinary Metabolites of L-α-Acetyl Methadol (LAAM) and Methadone by Thin-Layer Chromatography

<table>
<thead>
<tr>
<th>Reagent</th>
<th>LAAM&lt;sup&gt;c&lt;/sup&gt; 0.78</th>
<th>Urines from patients on LAAM</th>
<th>Methadone&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Urine from patients on methadone</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>NR</td>
<td>0.53, 0.60, 0.68, 0.78</td>
<td>0.68</td>
<td>0.53, 0.60, 0.68, 0.78</td>
</tr>
<tr>
<td>II</td>
<td>Or</td>
<td>0.53, 0.60, 0.68, 0.78</td>
<td>0.68</td>
<td>0.53, 0.60, 0.68, 0.78</td>
</tr>
<tr>
<td>III</td>
<td>Or/Brn</td>
<td>0.53, 0.60, 0.68, 0.78</td>
<td>0.68</td>
<td>0.53, 0.60, 0.68, 0.78</td>
</tr>
</tbody>
</table>

<sup>a</sup> NR, no reaction; Or, orange; Brn, brown; Pu, purple; Pk, pink; Rd, red.
<sup>b</sup> I, Ninhydrin; II, ninhydrin plus short-wave ultraviolet light; III, iodoplatinate/Dragendorff.
<sup>c</sup> These data were obtained by extracting L-α-acetyl methadol·HCl or methadone·HCl from drug-free urine and thin-layer chromatography as described in the Methods section. The method described is sensitive to as little as 1 μg of L-α-acetyl methadol per milliliter of urine.
<sup>d</sup> If the concentration of methadone and its metabolites in the urine is high, this spot will appear faintly purple when sprayed with reagent I (and II). We assume that unmetabolized methadone and a metabolite which is detected only in the presence of high concentrations of methadone are superimposed.

Table 2. EMIT Assay for Methadone

<table>
<thead>
<tr>
<th>Urine specimen</th>
<th>RF of metabolite detected at time of assay</th>
<th>EMIT Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methadone</td>
<td>0.68</td>
<td>Positive</td>
</tr>
<tr>
<td>Urines from patients on methadone</td>
<td>0.68, 0.78, 0.90</td>
<td>Positive</td>
</tr>
<tr>
<td>LAAM&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.78</td>
<td>Negative&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Urines from patients on LAAM</td>
<td>0.53, 0.60, 0.78</td>
<td>Negative</td>
</tr>
<tr>
<td>Methadone&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.68</td>
<td>Positive</td>
</tr>
</tbody>
</table>

<sup>a</sup> Drugs were added to drug-free urine and treated as described in the Methods section.
<sup>b</sup> Unmetabolized methadone (RF 0.68) appears only rarely and appears to depend on the time interval between dosage and specimen collection. The probability of detecting unmetabolized methadone decreases as this interval increases.
have at least two active metabolites in common: normethadonol and methadonol (9, 12–15). Although the metabolic pathway for the formation of normethadonol appears to be a minor one, this metabolite is reportedly one of the most active (14). If normethadonol or methadonol are responsible for the cross-reactivity to the EMIT assay for methadone, they are not present during either the early stages of LAAM metabolism or the later stages of methadone metabolism. The specific compound or compounds responsible for the cross-reactivity have not been determined, but our preliminary gas chromatography–chemical ionization-mass spectrometry data indicate that the EMIT assay is probably not sensitive to acetylmethadonols until they lose their acetate groups.

The thin-layer chromatographic procedure we describe has provided important information on the isolation and detection of some of the metabolites of methadone and LAAM, and has demonstrated that LAAM is metabolized to a number of compounds that are detected in the urine at different times after a dose. These results are consistent with reports that the long-acting properties of the drug are attributable to its extensive metabolism to numerous active compounds (11, 12).

We thank the Finnigan Corp., Sunnyvale, Calif. 94086, for kindly providing the GC/CIMS data, and Mrs. Marilee C. Buher and Mr. Roger A. New for the preparation of the samples for GC/CIMS. We also wish to acknowledge the expert technical assistance of Miss Rita Johnston during the early stages of method development. This work was supported by the Special Action Office for Drug Abuse Prevention, Washington, D. C. 20420; project number SADA3AC723-5526.

3 The LAAM metabolite at RF 0.60, isolated by preparative chromatography and removed from the silica gel with three washes of methanol, was analyzed by gas chromatography–chemical ionization-mass spectrometry. Preliminary data indicate the presence of at least two acetylmethadonols. Apparently, the EMIT assay for methadonol is not sensitive to the methadone backbone when it is attached to an acetate group. However, it does appear to become sensitive to one or more of the de-esterified metabolites (12), which have structures more similar to methadone and its metabolites. Similar studies of the methadone metabolite detected at RF 0.78 are underway, but the data are not available at the time of publication. The pyridone derivatives described by Sullivan et al. (16, 17) may be components of the spot. Such compounds would not be likely to cross-react with the immunosassay for methadone.

References