

MDMA
(3,4-methylenedioxymethamphetamine)

DETECTION AND CONFIRMATION

IN EQUINE URINE

**A Procedure Developed
By**

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For

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Abstract

3,4-Methylenedioxyamphetamine (MDMA) is a psychotropic drug with hallucinogenic and stimulant properties. MDMA is chemically related to other designer drugs, one of which is a potent stimulant known as MDA (3,4-methylenedioxyamphetamine), and another is MDE (3,4-methylenedioxyethylamphetamine) a stimulant and hallucinogen. Both MDE and MDA will be studied along with MDMA, but the focus will be on MDMA and methods will be optimized for this drug. Chromatographic standards for MDMA, MDA, and MDE can be purchased from Alltech or Sigma.

The screening technique described in this SOP, is a thin-layer chromatography (TLC) method, however, MDMA can also be screened using the methamphetamine ELISA kits from either Neogen or Testing Components Corporation. The preferred TLC screening method is the Basic Urine (BU) extraction procedure because it is the best indicator of MDMA among the common TLC screening methods employed in equine drug testing laboratories. Note that all of the alkaline extracts tested (SEH, BU, IPU, and SPE) recovered MDMA to some extent. MDA, a metabolite of MDMA in humans, was also recovered in all of the alkaline extract tested. The pH of the BU method (~11), is not optimum for recovery of MDMA or MDA, but a cleaner extract is obtained at this pH (~11) which makes the visualization by TLC easier. The optimum pH for extracting MDMA and MDA is ~7.5

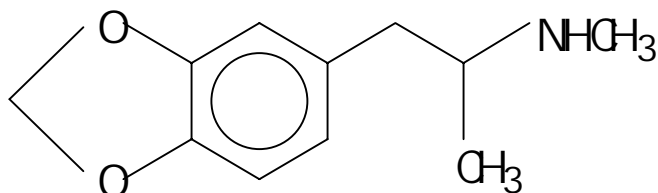
Introduction

MDMA is referred to as a “designer drug” and is produced in clandestine laboratories. Use of designer drugs, especially MDMA, has become wide spread among recreational users throughout the United States. The most common street names for MDMA are “Ecstasy”, “Adam”, and “X”. MDMA is usually seen as a pressed pill, in capsule form, or as a powder. Administered routes (for humans) include swallowing, snorting, smoking, and by IV injection. A standard dose (for humans) is approximately 2 milligrams per kilogram of body weight, which will vary depending on the desired effect. Peak effects after oral administration (human) will be reached in 1 to 1 ½ hours and last for 4 to 6 hours. If MDMA affects horses the same way it affects humans, the euphoria from an MDMA “high” coupled with the stimulant effect could make MDMA an effective drug in enhancing the performance of race-horses.

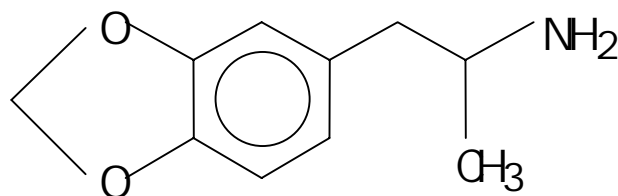
Clinical uses of MDMA have been limited to a small number of therapists and psychiatrists who believe that MDMA increases perceptions of self-insight and empathy. Therapists have used low doses of MDMA in themselves to become more in tune with their clients. Monks have used similar low doses (40 to 60 mg) to aid them in meditation.

The FDA has not approved MDMA for clinical use. The Drug Enforcement Agency (DEA) lists MDMA in the Schedule 1 category, which are drugs with no medical use and a high potential for abuse.

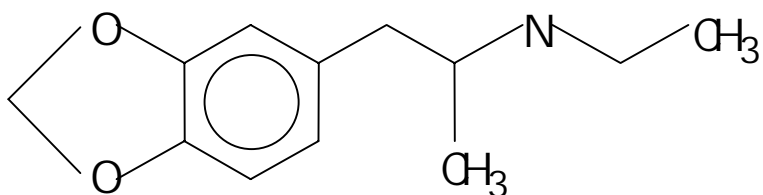
CHEMICAL STRUCTURES OF MDMA, MDA, & MDE



3,4-METHYLENEDIOXYMETHAMPHETAMINE
(MDMA)



3,4-METHYLENEDIOXYAMPHETAMINE
(MDA)



3,4-METHYLENEDIOXYETHYLAMPHETAMINE
(MDE)

Scope

This SOP is proposed for detection of MDMA in equine urine by thin-layer chromatography (TLC) and confirmation by gas chromatography / mass spectroscopy (GC/MS).

The limit of detection for MDMA in equine urine by thin-layer chromatography (TLC) is approximately 100 ng/mL using the basic urine (BU) extraction method. The BU method is preferred to other liquid-liquid extraction methods, because the TLC visualization for MDMA is most pronounced from this extract. T-1 (3:200, NH₄OH:CH₃OH) is the solvent system of choice for TLC plate development because the R_f value of MDMA and MDA are in an area normally clear of background interference. FES overspray (see page 21, *Spray Reagents*) produces the most intense chromogenic reaction for visualization. MDMA and MDA (at 100 ng/mL) were also visible on the BU Davidow plate (BU extraction followed by TLC plate development with Davidow) after Dragendorff's followed by sodium nitrate over-sprays (see page 21, *Spray Reagents*). The limit of detection for confirmation by GC/MS is ~10 ng/mL using the BU extraction (9 mL, no TLC) or by the solid phase extraction method (10 mL, no TLC). The GC/MS was run in full-scan mode.

Principle

MDMA is an alkaline drug and was extractable in all of the alkaline media examined. Of all the extracts examined, the BU (basic urine) was chosen for recovery because this is a very simple extraction to perform and it produced equal and often better results than the other methods examined.

Derivatization with *N*-Methylbis(trifluoroacetamide) (MBTFA) before GC/MS analysis was performed to enhance chromatography and produce ions sufficiently unique to clearly identify the target drug(s). Unique ions also enhance the usable sensitivity (signal to noise ratio) of the GC/MS instrument.

Limitations

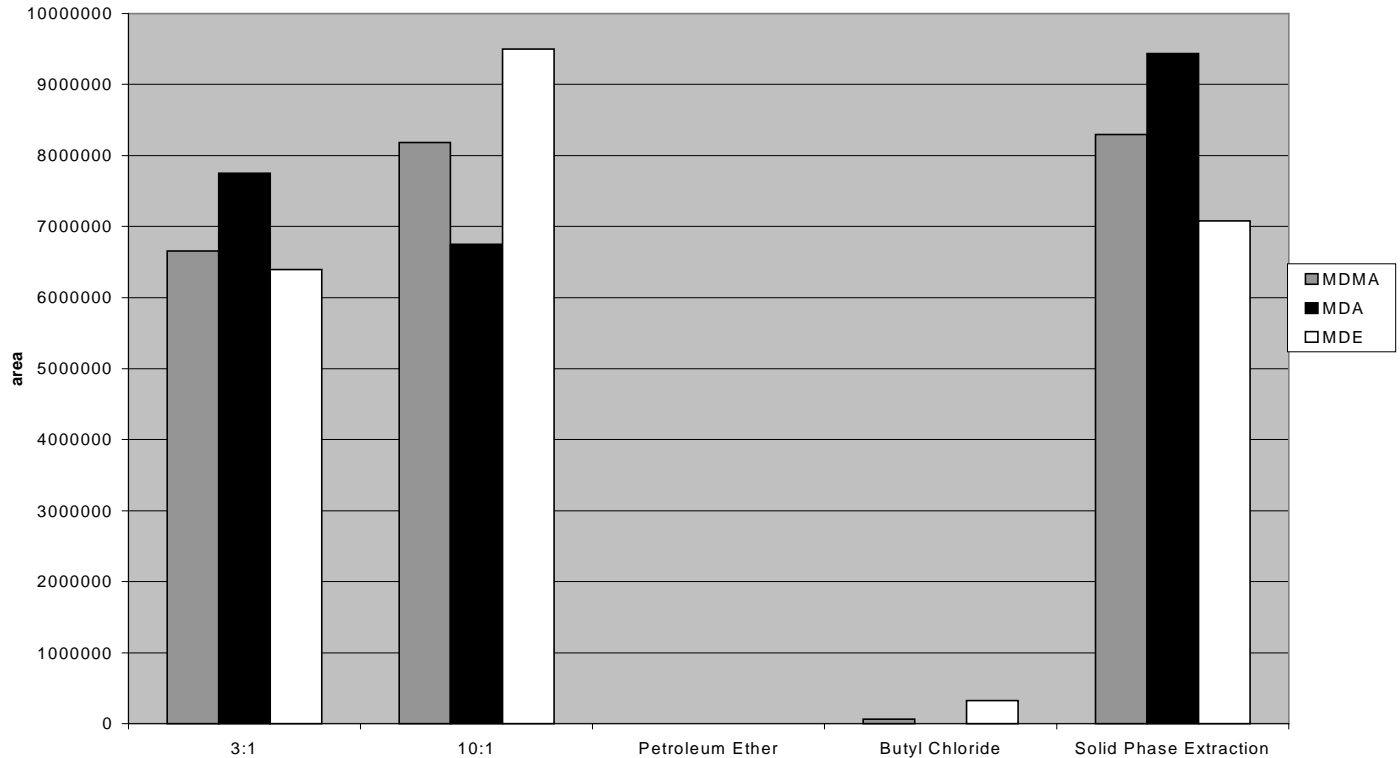
Due to its volatility, analysis of MDMA by GC/MS after TLC can be problematic. The portion of the TLC plate that is sprayed with FES (see page 21, *Spray Reagents*) to visualize the MDMA will need to be heated. When this is done the remainder of the plate gets hot as well, and the MDMA on the unsprayed portion will be greatly diminished or lost. Recovery of MDMA after TLC for GC/MS analysis is poor even without heating. Adequate sensitivity for GC/MS analysis (~10 ng/mL) can be achieved from the "raw residue" after a BU extraction (9 mL of urine was used).

Standards

MDE (3,4-methylenedioxyethylamphetamine) 1 mg/mL in methanol catalog # 014543
MDA (3,4-methylenedioxyamphetamine) 1 mg/mL in methanol catalog # 014603, and
MDMA (3,4-methylenedioxy-methylamphetamine) 1 mg/mL in methanol cat# 014093
were obtained from Alltech (800-437-3784).

Portions of these solutions were used to spike blank urine to yield urine concentrations of 0, 2, 5, 10, 25, 50, 100, and 500 ng/mL.

Effect of Solvent on Recovery of MDMA, MDA, & MDE



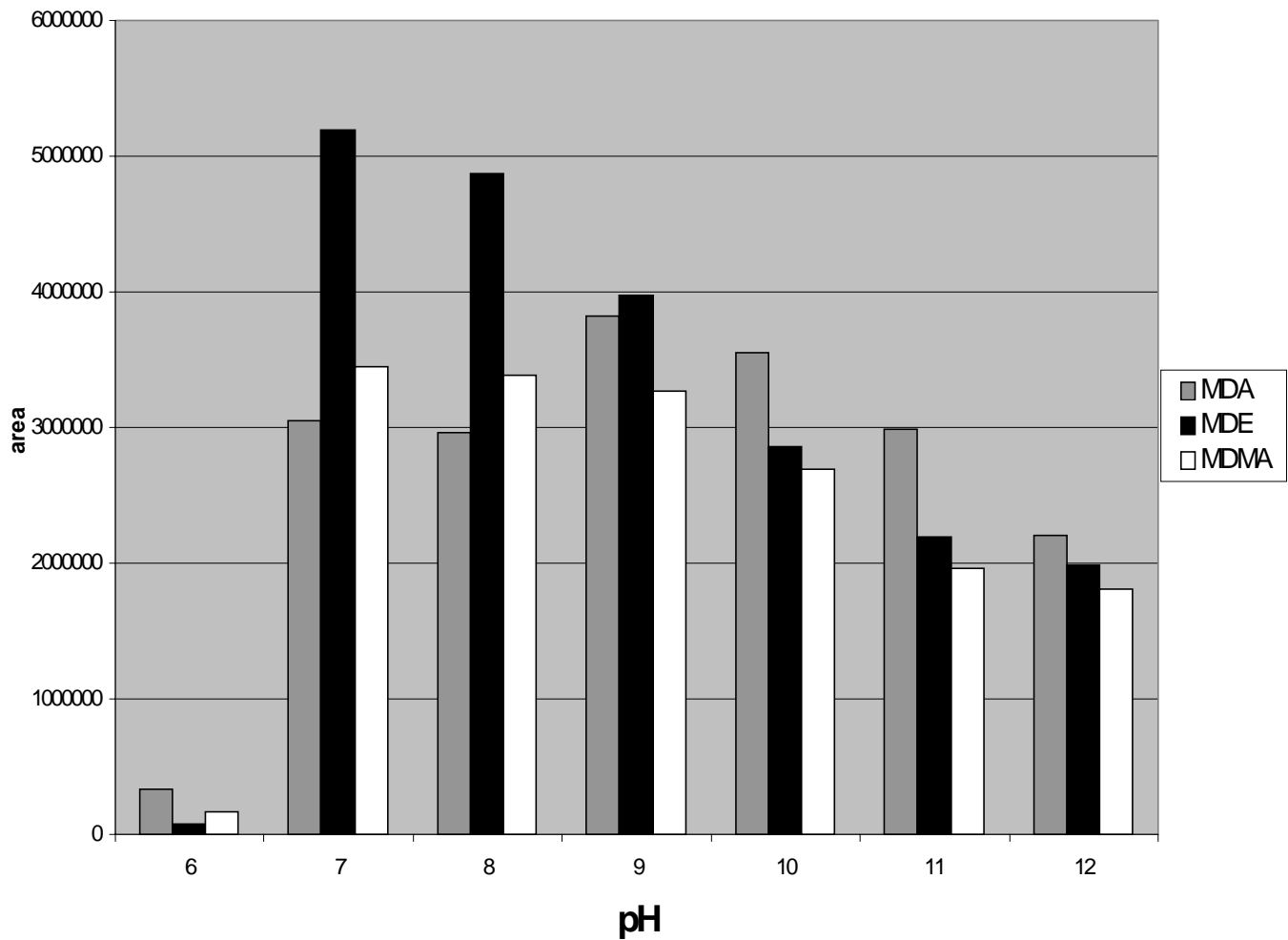
Above chart shows extraction efficiency of different solvent systems (extracted from urine in carbonate buffer at pH 8). The chart also shows a comparison of the BU method to that of the solid phase extraction method with respect to recovery. In each extraction, equal volumes of urine were used; the “raw residue” from each extract was derivatized and analyzed by GC/MS.

3:1 = 3:1 dichloromethane:isopropyl alcohol (DCM:IPA)

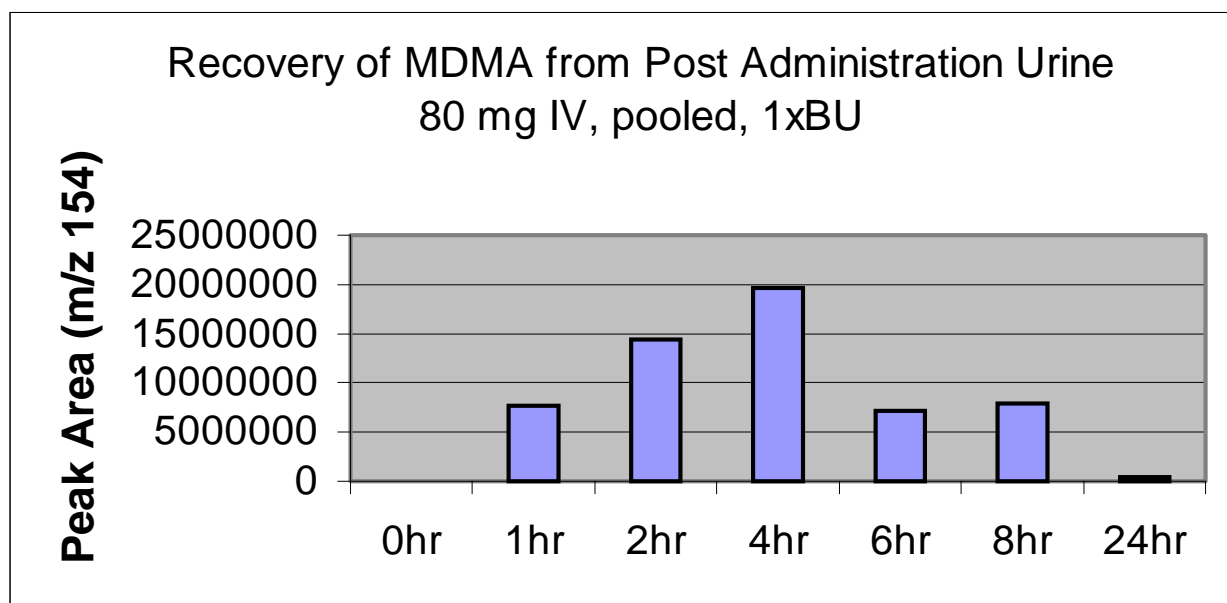
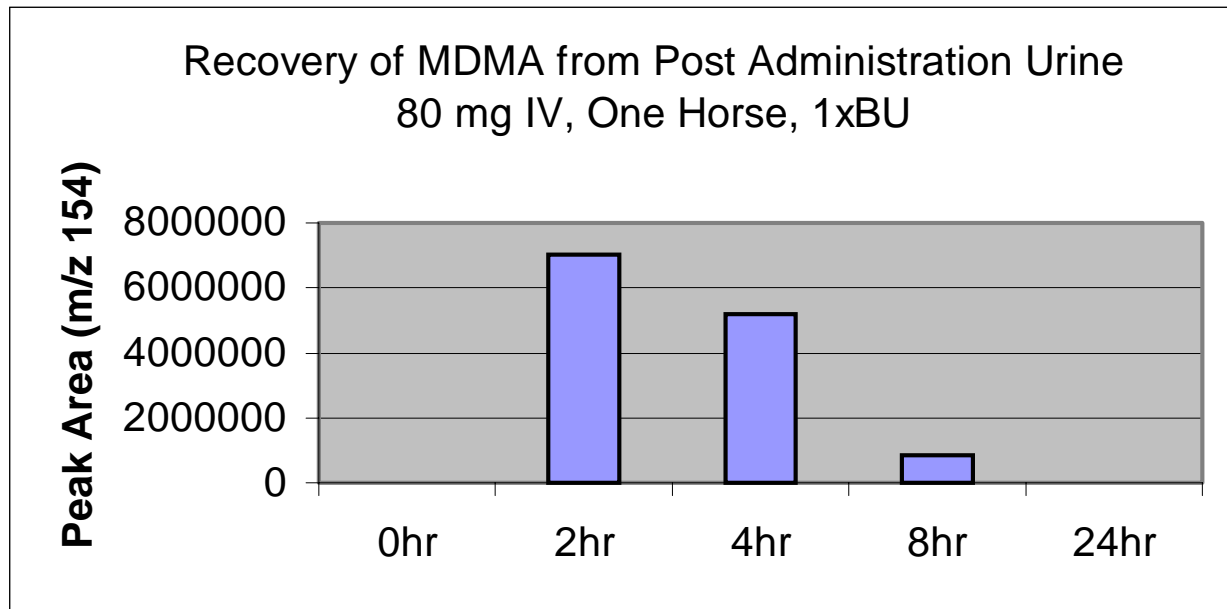
10:1 = 10:1 DCM:IPA

The chart below shows extraction efficiency versus pH (extracted from urine in carbonate buffer; the solvent was 3:1 dichloromethane:isopropyl alcohol). The “raw residue” from each extract was derivatized and analyzed by GC/MS.

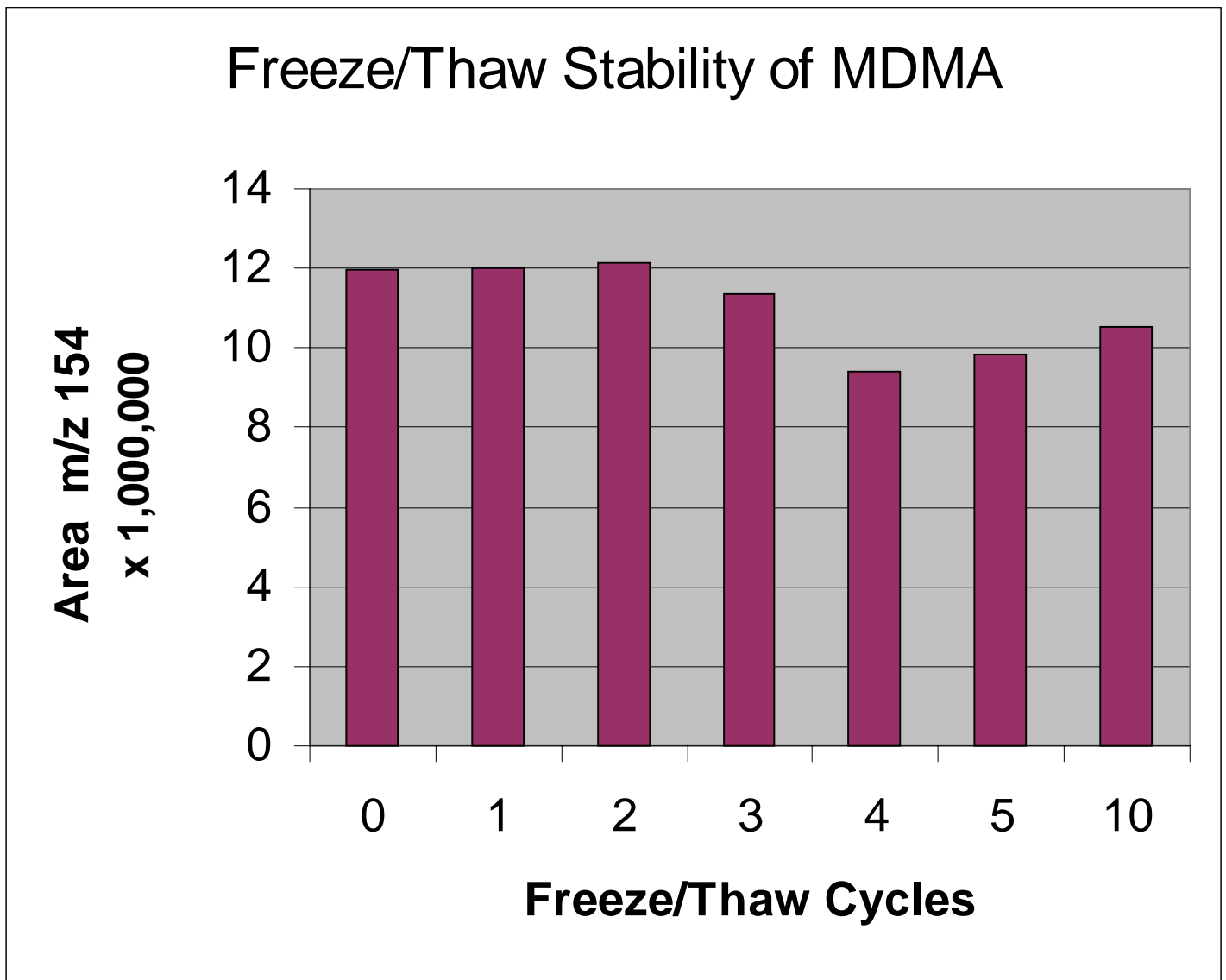
Effect of pH on Recovery of MDMA, MDA & MDE



The chart below shows the relative concentrations of MDMA recovered from post administration urine samples. Two separate studies were conducted. Samples were collected from zero through 24 hours post administration of MDMA in equine. Using the BU method, a 9 mL urine sample from each time point was extracted. The "raw residue" from each extract was then derivatized with MBTFA and injected on a Hewlett Packard 5890/5971 GC/MS in full scan mode. The 154 ion was used to calculate the relative areas.



Equine urine spiked with MDMA was divided into seven separate aliquots. Each sample was extracted using the BU method after the number of freeze/thaw cycles indicated on the graph below. The residue from each extract was reconstituted in methanol and preserved at -4°C until all aliquots were extracted. The methanol was then evaporated, the residue derivatized, and analyzed by GC/MS. The graph shows adequate stability for MDMA in urine even after numerous (10) freeze/thaw cycles.



METHODOLOGY

References

Thin-layer chromatography (TLC) methods:

NASRC Quality Assurance Program (1982-1988)

ARCI Quality Assurance Program (1988-1995)

The TLC methods are modification (or verbatim) acquired from the above two programs.

Solid Phase Extraction (SPE) Method:

The solid phase extraction method used in this SOP was derived from the application manual provided by United Chemical Technologies, Inc. for extraction of drugs using Clean Screen® solid phase extraction columns.

Definitions

GC/MS:	Gas Chromatography / Mass Spectroscopy
SOP:	Standard Operating Procedure
TMS	Trimethylsilyl
MBTFA	N-methyl- <i>bis</i> (trifluoroacetamide)
Dav	Davidow development solvent (34:4:2 CH ₃ COOCHC ₂ H ₅ : CH ₃ OH: NH ₄ OH)
T-1	T-1 development solvent (3:200, NH ₄ OH:CH ₃ OH)
Drag	Dragendorff's overspray reagent (see page 21, <i>Spray Reagents</i>)
Nitrite	Sodium nitrite overspray reagent (5% sodium nitrite in water)
Cupric	Cupric chloride overspray reagent (see page 21, <i>Spray Reagents</i>)
FES:	FES overspray reagent (see page 21, <i>Spray Reagents</i>)
Mand	Mandeline's overspray reagent (see page 21, <i>Spray Reagents</i>)
DCM	Dichloromethane
IPA	Isopropyl alcohol
3:1	Dichloromethane:isopropyl alcohol
10:1	Dichloromethane:isopropyl alcohol

Procedures

BASIC URINE EXTRACTION AND THIN LAYER CHROMATOGRAPHY FOR DETECTION OR CONFIRMATION OF MDMA AND MDA

APPARATUS

- 1 E.M. Science, silica gel, Fluorescent, Thin Layer Chromatography Plates
- 2 Capillary plate spotter
- 3 Hot plate with small fan
- 4 16 x 125 mm screw top test tubes with caps
- 5 Automatic pipettor with 10 mL graduated pipettes
- 6 Rotorack mixer for test tubes
- 7 Centrifuge
- 8 Vacuum aspiration apparatus
- 9 Disposable 15 x 85 mm tubes
- 10 Light box with 254 nm and 365 nm ultra-violet light
- 11 Water bath
- 12 Hot plate

REAGENTS

- 1 Carbonate/bromothymol blue buffer
- 2 3:1 dichloromethane/isopropyl alcohol
- 3 2N NaOH
- 4 Dichloromethane:methyl alcohol (9:1)
- 5 Davidow's solvent (34:4:2 CH₃COOCHC₂H₅: CH₃OH: NH₄OH)
- 6 T-1 solvent (3:200, NH₄OH:CH₃OH)
- 7 Dragendorff's reagent (see page 21, *Spray Reagents*)
- 8 Sodium nitrite solution 5X (see page 21, *Spray Reagents*)
- 9 Cupric chloride solution 25% (see page 21, *Spray Reagents*)
- 10 Mandelin's reagent (see page 21, *Spray Reagents*)
- 11 FES reagent (see page 21, *Spray Reagents*)

STANDARD STOCK SOLUTIONS

3,4-methylenedioxyamphetamine (MDMA); 1 mg/mL (Alltech Lot # 193-9071)

3,4-methylenedioxyamphetamine (MDA); 1 mg/mL (Alltech Lot # 187-9041)

3,4-methylenedioxyethylamphetamine (MDE); 1 mg/mL (Alltech Lot # 183-9034)

EXTRACTION

Base Urine Extract (BU)

- 1 Add 9.0 mL urine to a 16 x 125 mm screw top tube.
- 2 Make entry into "Reagent Addition" log book.
- 3 Add 2.0 mL carbonate/bromothymol blue buffer and 4.0 mL 3:1 DCM:IPA.
- 4 Cap tube and rotorack for 15 minutes. As samples are rotoracking add a few drops of 2N NaOH to any sample that has a yellowish organic layer. (Organic layer should be clear).
- 5 Centrifuge at 1500 rpm (550 rcf) for 5 minutes. Aspirate aqueous (upper) layer to waste.
- 6 Make entry into "Sample Handling" log book.
- 7 Transfer organic layer to a clean 15 x 85 mm tube.
- 8 Concentrate to dryness in 50°C water bath under a gentle stream of nitrogen.
CAUTION: Boiling points are low (MDE 85-95°C, MDMA 110-110°C, MDA 157°C).

TLC

Dissolve extract residue with 2 to 3 drops 9:1 DCM:MeOH. Spot the entire residue equally on 2 TLC plates. Cool plates prior to development.

Plate #1 - Davidow

- 1 Spot MDMA, MDA, and MDE as standards.
- 2 Develop in Davidow (use 40-50 mL/tank) for 4 cm. Dry plate well.
- 3 Observe with 365 nm UV light. Indicate fluorescence with =.
- 4 Observe with 254 nm UV light. Indicate quenching with | |.
- 5 Spray with Dragendorff's. Record colors and R_f values.
- 6 Spray several times with sodium nitrite (wait a few seconds between sprays for color reaction to develop). Record colors and R_f values.
- 7 Spray with cupric chloride. Record colors and R_f values.

Plate #2 - T-1

- 1 Spot MDMA, MDA, and MDE as standards.
- 2 Develop in T-1 (use 40-50 mL) for 4 cm. Dry plate at room temperature under a small fan.
- 3 Observe with 365 nm UV light. Indicate fluorescence with =.
- 4 Observe with 254 nm UV light. Indicate quenching with | |
- 5 Spray with FES. Heat on hot plate until standards are visible. Record colors and R_f values.
- 6 Spray with Mandelin's. Record colors and R_f values (MDMA gives blue-green color).

Note: Depending on concentration, MDMA, MDA, and MDE may react with each of the above spray reagents.

**BU EXTRACTION OF MDMA AND MDA FROM EQUINE URINE
(PREPARATION FOR GC/MS ANALYSIS)**

MDMA and MDA were confirmable by GC/MS as low as ~10 ng/mL using the "raw residue" (no TLC) from the above BU extraction method. A 9 mL urine sample was used in preparation for GC/MS confirmation. The urine residue was reconstituted in 20 µL ethyl acetate plus 20 µL MBTFA in a sealed vial and heated for 20 minutes at 70°C.

SOLID PHASE EXTRACTION OF MDMA AND MDA FROM EQUINE URINE SPECIMENS (PREPARATION FOR GC/MS ANALYSIS)

EQUIPMENT

- Solid Phase Extraction (SPE) Columns, ZSDUA020 (United Chemical Technologies, Inc.)
- Vacuum Manifold with stopcocks, variable vacuum control, gauge and collection rack for test tubes
- Adjustable volume Automatic pipettor with disposable tips (100 μ L tips)
- pH paper (broad range 0 to 14 and narrow range 6 +/- 0.5)
- Vortex mixer
- Heating bath
- Vials, Test tubes (100 mm screw top tubes are used to collect elution solvent, see elute alkaline drugs)
- Centrifuge
- Horn sonicator

REAGENTS

Water (deionized)

Methanol (HPLC grade)

Hydrochloric acid (ACS reagent grade)

Sodium Hydroxide (ACS reagent grade)

Methylene chloride (HPLC grade)

Ethyl acetate (HPLC grade)

Acetic acid (glacial) (ACS reagent grade)

Ammonium hydroxide (ACS reagent grade)

Sodium phosphate monobasic (NaH_2P_0_4) (ACS reagent grade)

Sodium phosphate dibasic (Na_2HP_0_4) (ACS reagent grade)

Isopropyl alcohol (HPLC grade)

MBTFA (with 1% TMCS)

pH 5 acetate buffer (see solution F, next page)

Patella vulgata enzyme solution (5,000 units per mL of deionized water)

Solutions

A. 0.1M Phosphate Buffer, pH 6 (100 mL)

Weigh 0.17g of Na_2HPO_4 and 1.21g NaH_2PO_4 into 80 mL of deionized (DI) water. Dilute to 100 mL and mix. Adjust pH to 6.0 \pm 0.1 with saturated monobasic sodium phosphate (lowers pH) or saturated dibasic sodium phosphate (raises pH). Store in glass at 5° C. Stable for 1 month.

B. 1.0M Acetic Acid (50 mL)

To 40 mL of DI water pipette 2.86 mL of glacial acetic acid. Dilute to 50 mL and mix. Store in glass or plastic at 25° C. Stable for 6 months.

C. Methylene Chloride:Isopropyl Alcohol (80:20) with 2% Ammonium Hydroxide (100 mL)

Into a 10 mL screw top tube add 8 mL of methylene chloride and 2 mL of isopropyl alcohol. Mix. Remove 200 μL of this solution and add 200 μL of ammonium hydroxide. Mix well. Cap tightly. Prepare fresh daily.

D. 2 N NaOH

Dissolve 80 g sodium hydroxide into 1 liter of DI water.

Use caution: dissolution is exothermic.

E. 1 N HCl

In a 1000 mL volumetric flask containing approximately 800 mL DI water, add 83.3 mL concentrated HCl. Add distilled water to 1000-mL mark and mix.

F. pH 5 Acetate Buffer

Dissolve 328 g of sodium acetate in three liters of deionized water. Add 66 mL glacial acetic acid. Dilute to 4 liters with water.

PROCEDURE

1. MANIFOLD PREPARATION

Wipe the tips on the ports of the extraction manifold lid with methanol. The same port must be used for all stages of preparation of a given urine aliquot. Plug all unused ports.

2. SPECIMEN PREPARATION

A. Short Enzyme Hydrolysis and Sonication

Add 10 mL urine to a clean 100 mL beaker.

Make entry into "Solid Phase Retest" log book.

Add 4.0 mL pH 5 acetate buffer (F) and 2.0 mL *Patella vulgata* enzyme solution (5,000 units per milliliter).

Sonicate urine with horn sonicator for 1.5 min. at full power. Turn up power slowly over the first 10 to 15 sec.

Incubate in 65°C water bath for 3 hours. Cool.

B. ADJUST PH AND CENTRIFUGE

Check the pH of the specimen with pH paper. Adjust pH to 6.0 +/-0.5 using drop-wise addition of 2 N NaOH (D) or 1 N HCl (E) as needed.

Centrifuge as needed: precipitates, urine still viscous, too much foam, suspended particulates, etc. will clog SPE column. Unless urine is very clean and clear it is a good idea to centrifuge (5 minutes at 1500 rpm).

3. SPE COLUMN PREPARATION

Place a Clean Screen[®] solid phase extraction cartridge (Catalog ZSDAU020 or equivalent) into the vacuum manifold port referred to in step 1 above. Sequentially pass the following through the column:

3 mL methanol

3 mL deionized water

2 mL pH 6 phosphate buffer (A).

Important: Turn off vacuum as soon as water reaches the top of the sorbent bed to prevent column from drying.

4. SPECIMEN APPLICATION

Load the specimen into the column reservoir. Loosen the flow valve to reduce vacuum. Draw the specimen slowly through the column.

Important: It should take at least two (2) minutes for the specimen to pass through the SPE column.

5. Column Rinse

Sequentially pass the following through the column:

- 3 mL deionized water
- 2 mL 1.0 M acetic acid (B)
- 3 mL methanol

6. ELUTE ALKALINE DRUGS

Place a rack with new labeled 100 mm screw top test tubes into the vacuum manifold. Pass through the column and collect:

5 mL methylene chloride:isopropyl alcohol (80:20) with 2% ammonium hydroxide (C)

7. RECONSTITUTE AND INJECT

Evaporate the eluent @ 40°C under a steady stream of nitrogen. Reconstitute with 20 µl ethyl acetate plus 20 µl MBTFA. Cap tightly and heat at 70°C for 20 minutes.

Inject 1 or 2 µl of the solution into GC/MS.

GC/MS Conditions:

Splitless for 0.8 min. at a Head Pressure of 15 psi
Column: 25 meter HP-5, 0.33 µm film, 0.2 mm ID
Initial Temp: 80°C 1.51 min. hold
Program Rate: 20°C/min.
Final Temp: 275°C 13.74 min. hold

GC/MS was performed in full scan EI mode over the mass range of 40-350 amu.

Some significant ions in order of abundance:

MDMA monoTFA	154, 162, 135, 110, 77, 289	Retention time: ~9.4 min.
MDA monoTFA	135, 162, 77, 136, 140, 275	Retention time: ~8.6 min.
MDE monoTFA	168, 162, 140, 135, 77, 303	Retention time: ~9.6 min.

Quality Assurance

TUNING Proper tuning of the GC/MS instrument is required before analyzing standards controls and samples for the confirmation of any drug by GC/MS. Tuning to EPA 625 DFTPP tuning requirements was used in the development of this method. Other tuning compounds and requirements may be used depending on the standard operating procedures of your facility.

CONTROLS When performing a confirmation of any drug at least one positive control should be used to assure that the method is working and that a reasonable recovery is achieved. MDMA (10 ng/mL) in urine, extracted by this method, should be easily detectable by GC/MS (as well as meeting criteria for calling a positive; see below). A positive control is a screened negative urine that is spiked with the drug (MDMA) under analysis.

A negative control should also be used to assure that the drug under analysis is not being picked up as a contaminant during handling and extraction of samples and controls. A negative control is a urine sample that was previously screened and found to be negative for the presence of the drug under analysis. The control negative should show no signs of the drug under analysis via extracted ion profiles. Extracted ion profiles for the control negative should be generated under the same conditions as the reagent blanks (see explanation of how this is performed under "Reagent Blanks").

REAGENT BLANKS A reagent blank is the same derivatizing reagent and/or solvent which is used to dissolve/ derivatize the extract from the sample and controls. Where these blanks are used is shown below (under: "Suggested Sequence of Analysis"). Reagent blanks should show no sign of the presence of the drug under analysis. This verification is achieved by using extracted ion profiles of three of the major ions of the drug under analysis and scaling the view of these ions to the same scale as the extracted ion profiles of the sample, control positive or standard. The reagent blank ion profiles should be scaled to the smaller of the sample, control positive or standard which it is adjacent to, according to the run order (see "Suggested Run order for Analysis", below).

CRITERIA FOR CALLING A POSITIVE GC/MS analysis of drugs and their metabolites gives several different kinds of information which are used in the determination of a "positive" test result. The two most important are the mass spectrum and the chromatographic retention time. Final determinations on positive samples are made based on the weight of all evidence and not solely on one or two parameters.

A. Mass Spectral Criteria

FIT: The mass spectrum of the sample is compared to the standard spectra in the computer library. The computer calculates how well the sample mass spectrum matches the library and displays this in terms of a FIT number. The instruments used in this method development were an ion trap system (ITS40) and a quadrupole system (HP 5890/5971). A FIT of >899 is the goal to call a positive when using the ITS40. A fit quality of >89 is the goal to call a positive when using the HP data system (i.e. 90% or better for either system).

B. Chromatographic Criteria

Retention Time: A comparison is made between the retention time of the sample and that of the standard or the control positive. The goal for percent difference between the retention times is $\pm 2\%$.

C. Supporting information

While the retention time and FIT are the primary considerations in calling a positive test result, a number of criteria should be examined to support the initial call. Another factor that should be examined is the peak ratios of major ions. Generally, the peak ratios (percent relative abundance) of the largest 5 to 6 peaks in the mass spectrum of the drug under analysis are compared to the corresponding peaks in the mass spectrum of the standard or control positive. A goal of $\pm 30\%$ is set for corresponding peaks.

Suggested Sequence of Analysis

DFTPP: Instrument must pass tuning requirement

Standard: The standard or standards for the drug or drugs under investigation.

Blank: A reagent blank of the same matrix as used for samples, standards, and controls.

Control negative: Blank urine extracted at the same time as the sample(s), using the same procedure.

Sample: Extract of urine containing suspect(s).

Blank: A reagent blank of the same matrix as used for samples, standards, and controls.

Sample: Other samples with same suspect(s) may be analyzed here, each followed by a reagent blank.

Blank: A reagent blank of the same matrix as used for samples, standards, and controls.

Control positive: Blank urine that was spiked with the drug(s) that is being confirmed, or urine which was previously confirmed to have that drug(s) present.

Suggested Sequence of Analysis (continued)

Blank: A reagent blank of the same matrix as used for the samples, standards, and controls.

Standard: The standard or standards for the drug or drugs under investigation. In confirmations performed at this facility, the run order will include (after the control positive) another reagent blank followed by another injection of the standard. The second injection of the standard is to assure that the performance of the instrument has not significantly changed since the beginning of the analysis sequence.

NOTE: The sequence of analysis used as well as other criteria for calling a positive test result will depend on the standard operating procedures of the facility performing the analysis.

Reagents for MDMA SOP

TLC Analysis

Extraction Solutions

Bromothymol Blue/Carbonate Buffer (BU)

Dissolve 424 g anhydrous sodium carbonate or 496 g sodium carbonate monohydrate in 3 liters of distilled or deionized water. Dissolve 800 mg bromothymol blue in a minimum volume of methanol (ca. 5 mL) and add to the sodium carbonate solution. Shake well. Add 1 liter of deionized water Shake well. Filter before use.

Developing Solvents

- **Davidow:** Ethyl acetate 3400 mL
Methanol 400 mL
Ammonium hydroxide 200 mL
- **T-1:** Methanol 4000 mL
Ammonium hydroxide 60 mL
(Easiest to add 60 mL ammonium hydroxide to a new 4 liter reagent bottle of methanol.)

Spray Reagents

- **Dragendorff's Spray**

Mix equal amounts of Solution A and Solution B.

Solution A: 9.4 g Bismuth Subnitrate dissolved in approximately 600 mL DI-H₂O. Add 306 mL Glacial Acetic Acid. Bring to 1 liter with DI-H₂O. Mix for several minutes and filter.

Solution B: 112.1 g Potassium Iodide in 1 liter DI-H₂O.

- **Sodium Nitrite Spray**

5% solution of sodium nitrite in DI-H₂O.

- **Cupric Chloride**

200 g cupric chloride in 600 mL DI-H₂O. Add 200 mL methanol.

- **FES - Phenothiazine Spray**

Dissolve 2.0 g ferric chloride in 600 mL ethanol. Slowly add 200 mL concentrated sulfuric acid. (Danger: Exothermic reaction! Add acid to ethanol with cooling and stirring. Use proper face and eye protection.)

- **Mandelin's**

Heat 800 mL concentrated sulfuric acid in an Erlenmeyer flask in a 60°C water bath about 1 hour. Add 4.0 g Ammonium Metavanadate, slowly. Heat for an additional hour.