

TOPIRAMATE: DETECTION AND CONFIRMATION IN EQUINE URINE

A Procedure Developed

By

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Abstract

Topiramate (TOP) is a sulfamate-substituted monosaccharide (2,3:4,5-di-O-(1-isopropylidene)-*B*-D-fructopyranose sulfamate) with anti-epileptic/anti-convulsant activity. TOP is marketed by Ortho-McNeil Pharmaceutical as Topomax[®]. It is one of several newly approved drugs, which is structurally distinct from other anti-epileptic drugs (AEDs) (figs 1,2). Topiramate can be used in adjunct therapy with other AEDs or as an effective AED by itself. The prescribed dosage regimen in humans results in peak urinary concentrations of approximately 50 ug/ml. The following method was developed with this expected urinary concentration in humans as being reflective of a significant plasma concentration of TOP in mind.

The screening method is by thin-layer chromatography (TLC) following acidic extraction (AU) into an organic solvent. Topiramate spotted TLC plates developed in 4:4:2 (chloroform: cyclohexane: acetic acid) were visualized with Folin-Ciocalteu reagent. Confirmation of the methyl derivative of parent TOP is by gas chromatography/mass spectrometry (GC/MS) in full scan electron impact (EI) mode. The following method will examine procedures, alternative possibilities, stability of the analyte, and ruggedness of methodology for information in characterizing possible detection of topiramate in test samples.

Introduction

AEDs such as phenytoin have traditionally been used for the management and prophylaxis of “tying up” (chronic intermittent rhabdomyolysis or exertional rhabdomyolysis) in the equine athlete (ref 15). Several new, structurally unrelated, AEDs (fig 2) have recently been approved by FDA and may eventually find usage in equine therapeutics (ref 5,6,7,9). These compounds are structurally unrelated to traditional AEDs (fig 1) as well as each other. Topiramate has been shown to be effective in adjunct therapy with other AEDs, and by itself (ref 5,6,8,9,12) in controlling partial onset seizures (ref 5,8,12,14). Topiramate has been reported to increase the urinary output of phenytoin when both are combined in a multi-drug therapeutic regimen (ref 8,9,1).

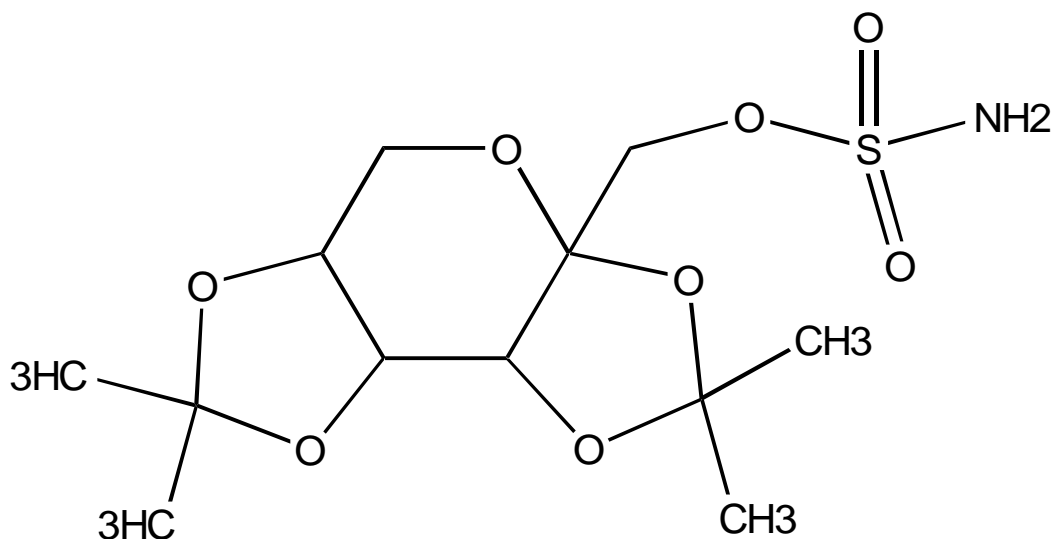


Figure 1

The monosaccharide backbone of topiramate provides few chemical properties amenable to detection by many standard analytical methods. The sulfamate functionality negates the detection properties inherent normally in primary amines. A TDx compatible FPIA assay is available from Oxis International Inc. (page 16,fig. 3), and the methyl derivative described in this SOP is amenable to instrumental screening by GC or GC/MS. This SOP describes a rapid screening procedure for TOP by thin-layer chromatography and subsequent confirmation of the methyl derivative by gas chromatography/mass spectrometry.

Common Substructure for the Structurally Related AntiConvulsant Drugs

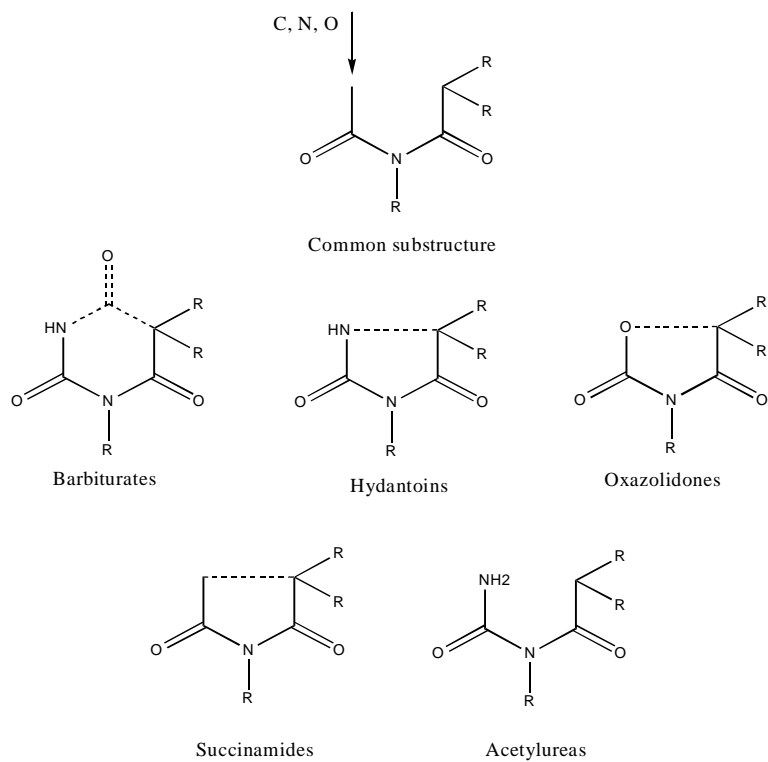
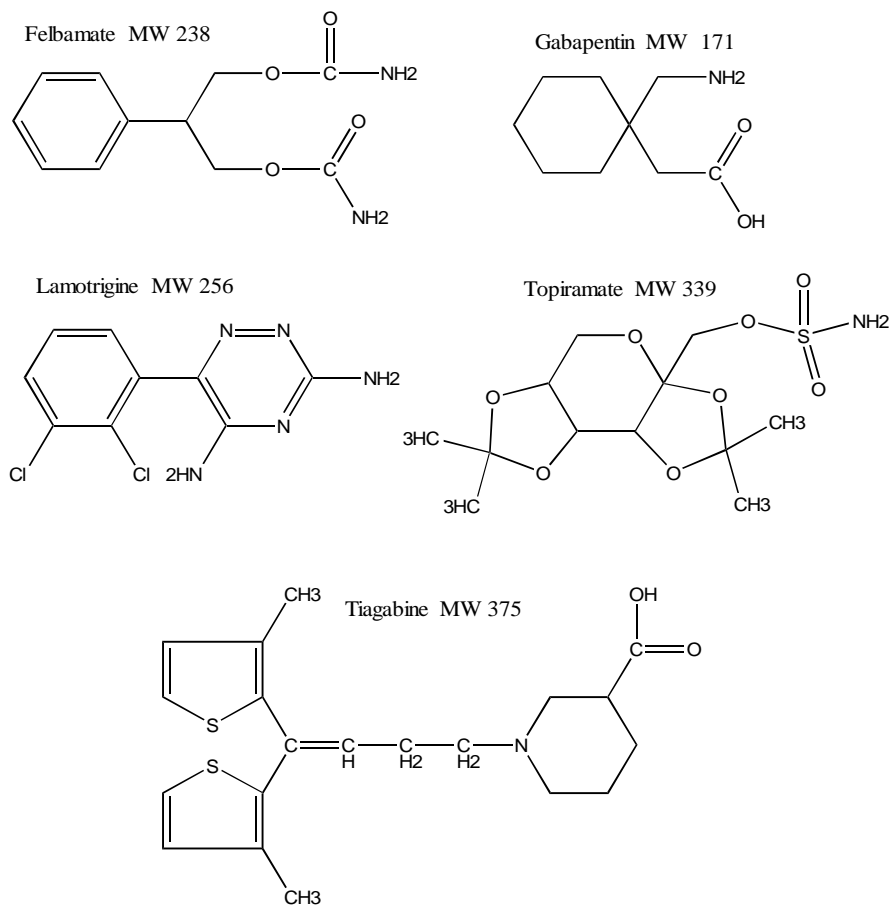


Figure 1

New AntiConvulsants (Structurally Non-Traditional)



Scope:

This SOP details screening and confirmation of parent topiramate at concentrations greater than 1 ug/ml. Extraction procedure comprises acidification of urine, extraction into an organic solvent, and washing of the solvent extract with aqueous sodium bicarbonate. Screening of TOP is accomplished by thin-layer chromatography (TLC) in 4:4:2 (chloroform: cyclohexane: acetic acid) solvent system with visualization by Folin-Ciocalteu overspray. Confirmation is achieved by gas chromatography-mass spectrometry (GC/MS) of the dimethyl derivative of topiramate in full scan EI mode.

Limitations:

The chemical properties of TOP confer few characteristics that enhance TLC detection of its urinary concentrations expected after therapeutic administrations. Ultraviolet coefficients of extinction are poor, negating detection by short-wave UV. The sulfonyl group negates the chemical properties of the primary amine that normally respond to Fluram, Ehrlichs, Ninhydrin, and Dragendorff visualization reagents. The bicarbonate wash is used to enhance TLC detection by reducing normally occurring co-eluting background in urine samples. The limit of detection (LOD) on TLC appears to be a sufficient threshold to ensure that residual urinary concentrations of TOP that are of any pharmacological relevance are excluded from further investigation. Methylation is required to inhibit thermal degradation and rearrangement of TOP under GC/MS analysis conditions.

Adminisistration:

1000 mg Oral

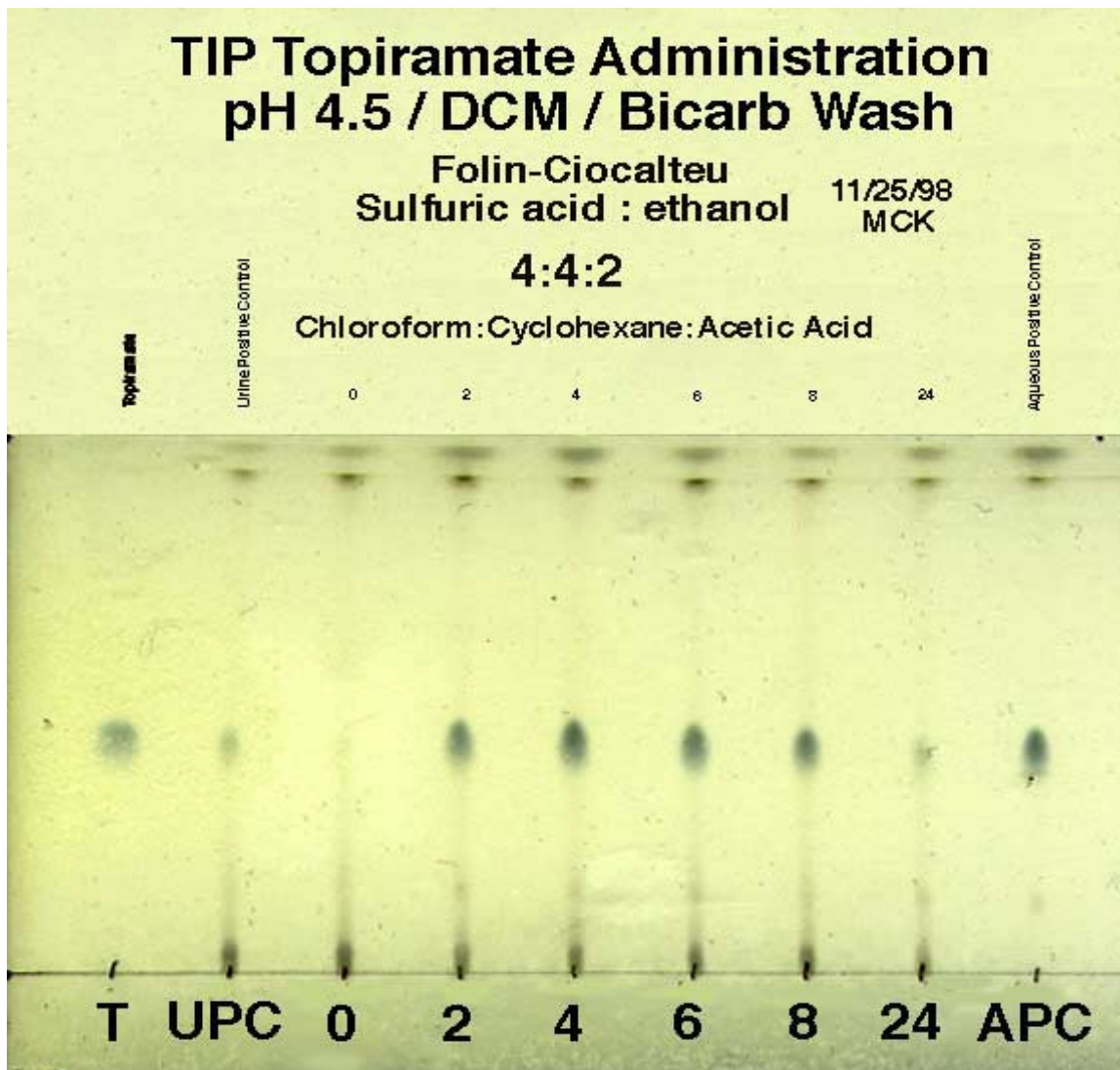
Extraction and Thin-layer Chromatographic Analysis**Extraction:**

Be sure to include PC and NC sample with each set.

1. 2 ml pH 4.5 buffer (Formula #59)
5 ml DCM
1 ml urine (or plasma, if blood only)
2. Cap, rack, centrifuge, aspirate top layer to waste
3. Add 4 mls aqueous sodium bicarbonate (Formula #76)
4. Cap, rack, centrifuge, and aspirate top layer to waste.
5. Pour DCM over into "pour over" tube and dry at 65°C
6. Reconstitute with 3 drops of DCM.

TLC:

1. On a 10x20 cm TLC plate
2. Spot sample (and NC, PC, and standards) on each plate.
3. Spot 3 QA standards: acepromazine, caffeine, and meclufenamic acid on each plate.
4. Develop TLC plate in 4:4:2 (chloroform : cyclohexane : acetic acid)
5. **Spray sequence for Topiramate plate developed in 4:4:2:**
 - a) Evenly spray with Folin-Ciocalteu Reagent (Sigma), followed by Sulfuric Acid:Ethanol 1:1
 - b) Gently warm the TLC plate on a hot plate. Record observations
 - c) Topiramate was observed as blue-grey spots at rf 0.4
 - d) Topiramate was detected through 24 hours as known samples, but 8 hours safely as an unknown.



Analysis and Confirmation By Gas Chromatography-Mass Spectrometry

General Scope:

The following confirmation procedure for TOP is applicable following presumptive screening for positive results, or as a batch instrumental screening procedure, which may encompass a wide population of target analytes. This procedure is geared toward detecting and confirming urinary concentrations of TOP within 36 hours following recommended therapeutic doses.

Related General SOP's:

Working Acid Urine (AU/W) PETRL SOP #103.0.

Methyl Iodide Derivatization for GC/MS Analysis PETRL SOP #131.0

Basic Principles:

Liquid/liquid extraction followed by GC/MS analysis of the methyl derivative.
Procedure materials, reagents and formulas (pages 8-10).

Extraction and Derivatization Procedure:

1. Obtain positive control urine, negative control urine, and sample urine.
2. Label 16x125 mm screw-top tubes with the appropriate identification.
3. Add 1 ml of urine to their respective tubes.
4. Add 2 ml pH 2 buffer, and 5 ml dichloromethane (DCM).
5. Rotorack tubes for 10 minutes.
6. Centrifuge (2500-3000 rpm x 10 min.), and decant the lower DCM phase to clean, labeled 16x125 mm screw-top test tubes. Evaporate to dryness.
7. Add 300 ul acetone, 30 ul methyl iodide, and 10 mg potassium carbonate to each tube.
8. Cap and heat at 65°C for 30 minutes.
9. Decant solvent carefully to clean, labeled 16x125 mm screw-cap test tubes. CAUTION: DO NOT TRANSFER ANY SOLID MATERIAL.
10. Evaporate to dryness.
11. Reconstitute with 50 ul ethyl acetate for GC/MS analysis.

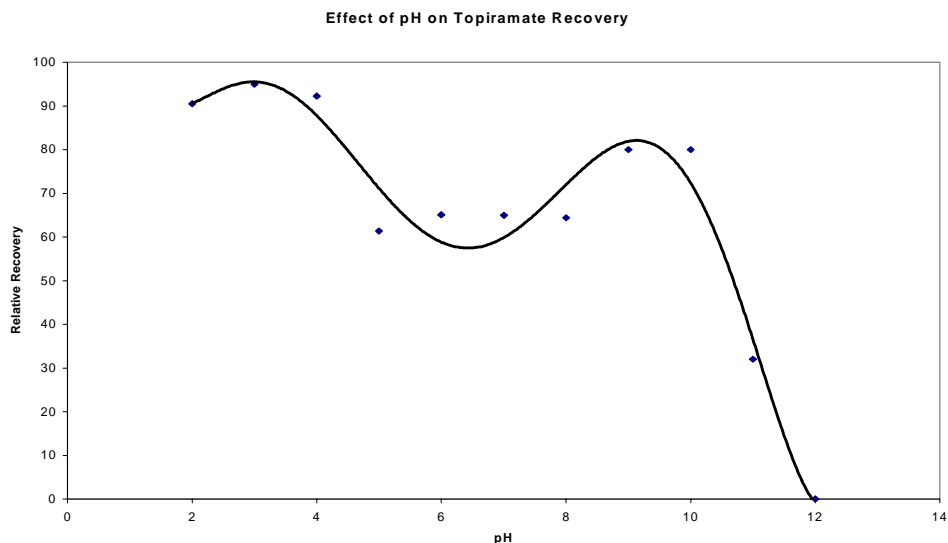
GC/MS Conditions:

Instrument: Detector: Hewlett-Packard 5970 MSD
 Chromatograph: Hewlett-Packard 5890 GC
 Column: Capillary-25M, BPX-5, 5 μ (SGE)
 Conditions: Initial Temperature: 65°C (1 minute hold)
 Program Rate: 30°C/min
 Final Temperature: 320°C (3.5 minute hold)
 Electron Multiplier: 400 volts above tune voltage
 Scan Mode/Range: full scan EI, 46-446 amu
 Monitor Ions: 220, 338 mono-methyl Topiramate
 108, 352 di-methyl Topiramate

Ruggedness and Stability Information

Extraction pH range:

Topiramate extracts into organic solvents over a wide pH range, thus it may be co-detected in a variety of procedures used for other target analytes.



Interference:

It should be noted that the methyl derivatives of oxyphenbutazone will produce spectra similar to those of the methyl derivatives of topiramate^(pages 12,13). The comparison of the mass spectra for these dimethyl derivatives is given in the appendix. Although a monomethyl derivative of TOP may be observed, Oxyphenbutazone rarely forms only a monomethyl species, with the reaction quickly proceeding completely to the dimethyl species. These respective potential interferences are chromatographically distinct with retention times differing by 1 to 3 minutes on typical capillary columns. The monomethyl topiramate spectrum is presented for identification purposes^(page 13).

Solvent Selectivity:

Common polar organic solvents (dichloromethane, ethyl acetate, dichloromethane-isopropanol (10:1 and 3:1)) efficiently extract topiramate.

Non-polar solvents such as diethyl ether, petroleum ether, cyclohexane, and hexanes exhibited poor recovery of TOP.

Limits of Detection:

Thin-Layer Chromatography – Limit of detection by SOP extraction, development in 4:4:2 (chloroform: cyclohexane: acetic acid), and visualization with Folin-Ciocalteu overspray: ~ 1 ug/ml

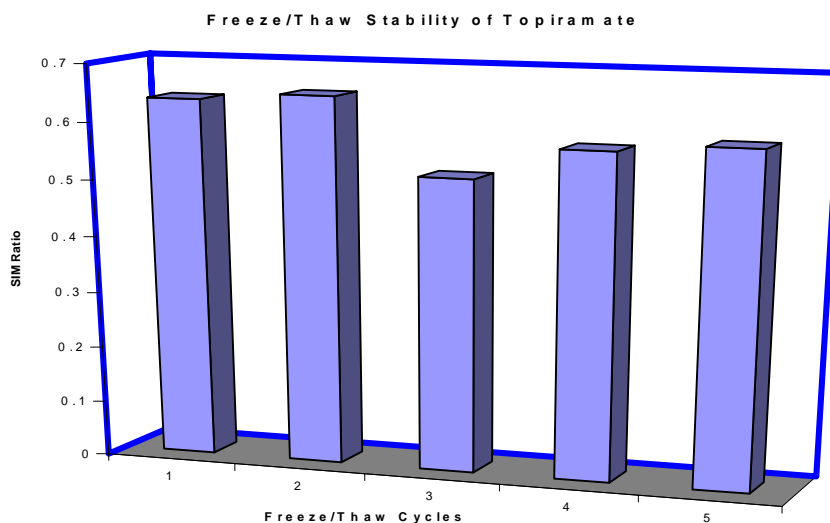
Gas Chromatography - Mass Spectrometry – Limit of detection of the dimethyl derivative ~ 10 ng/ml: Limit of confirmation of the dimethyl derivative: ~ 25 ng/ml

Topiramate Chemical and Thermal Degradation:

When topiramate is underivatized, the unprotected sulfamate group is thermally unstable to chromatographic injector and column temperatures. Topiramate may be isolated in a range of preparative chemistries; and, the urine concentration following therapeutic administration is usually greater than 1 microgram per milliliter. The spectra of these degradation products are presented as an aid to diagnosis of unknown spectra, which may be co-detected during analyses of other suspected compounds^(pages 14,15).

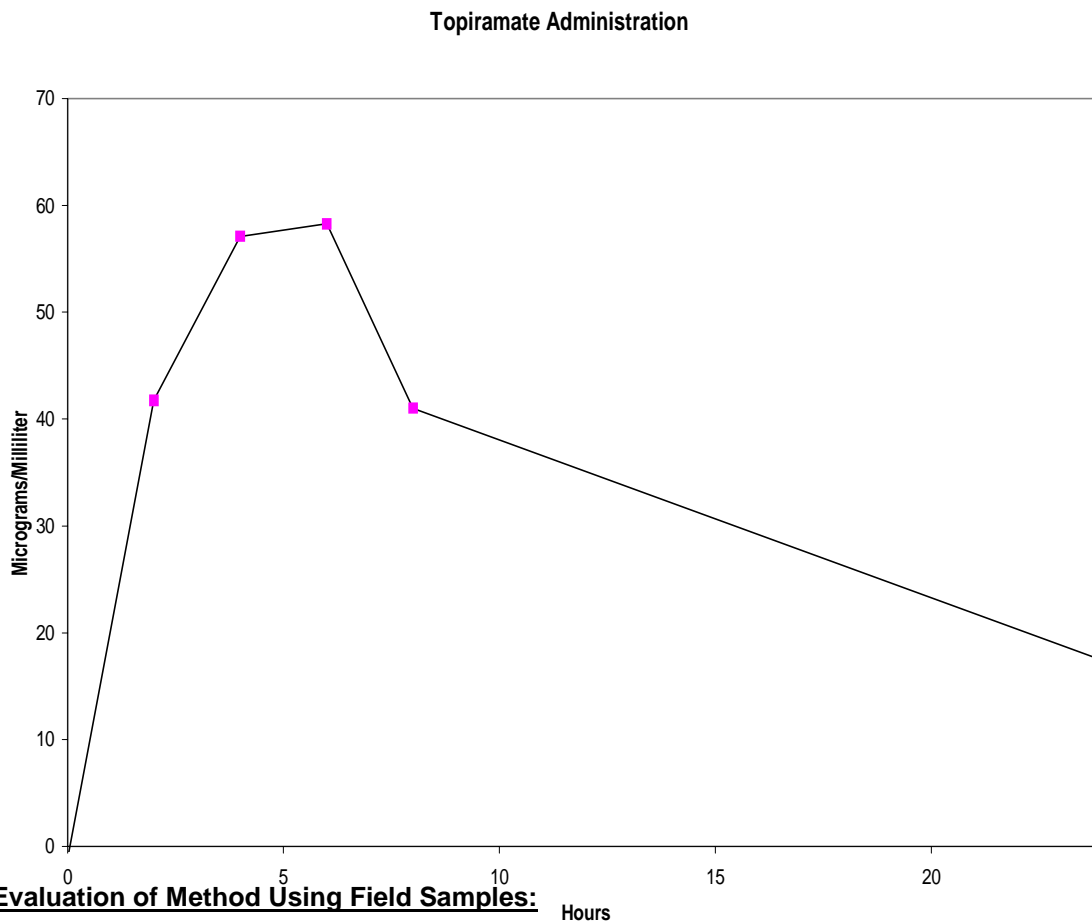
Effect of Freeze/Thaw cycles on Stability:

A TOP spiked urine sample was frozen as five separate aliquots. An aliquot was extracted by the GC/MS extraction procedure and stored at 0°C as the organic solvent extract until all 5 freeze/thaw cycles were similarly extracted. The solvent was then evaporated, samples derivatized, and analyzed by GC/MS. Topiramate was stable when subjected to all freeze-thaw cycles.



Semiquantitation of Topiramate in Administration Urine Samples:

TOP produced typical clearance characteristics, following an oral administration (1 GM, PO). Urine concentrations of TOP were greater than 10 micrograms per milliliter at 24 hours post administration.



Approximately 300 urine samples from official races conducted in Pennsylvania; and, 12 positive phenytoin urines were screened by the GC/MS SOP we have presented and found to be negative for topiramate (down to the LOD of ~10 ng/ml). However, TOP administration urine samples were found to be positive when subjected to this SOP for TOP.

Reagents and Formulas

Dichloromethane	Fisher	D143-4
Acetone	Fisher	A40-4
Methyl Iodide	Sigma	I-8504
Sodium Bicarbonate	Fisher	S-223
Potassium Phosphate (monobasic)	Fisher	P285-3
Folin Ciocalteu (phenol reagent)	Sigma	F-9252
Chloroform	Fisher	C298-4
Cyclohexane	Fisher	D143-4
Acetic Acid	Fisher	A38 ^C -212
Phosphoric Acid	Fisher	A-242

TLC Solvent 4	4:4:2 (Chloroform:Cyclohexane: Acetic Acid)	1000 ml	400 ml Chloroform (CHCl ₃) 400 ml Cyclohexane 200 ml Acetic Acid
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TLC Plates

Merck Kieselgel 60 F₂₅₄ 20x20 cm

#1.05715

Formula #58 - pH 2.0 Buffer

Procedure for 800 ml

Prepare a saturated solution of monobasic potassium phosphate (KH₂PO₄) by adding KH₂PO₄ to 1 liter of dH₂O while stirring until no more will go into solution and a precipitate remains. The pH of this solution should be 4.5 if it is saturated.

Decant supernatant into a clean reagent bottle and adjust pH to 2.0 with Phosphoric Acid (H₃PO₄).

Formula #59 - pH 4.5 Buffer

Procedure for 1000 ml:

Prepare a saturated solution of monobasic potassium phosphate (KH₂PO₄) by adding KH₂PO₄ to 1 liter of dH₂O while stirring until no more will go into solution and a precipitate remains. The pH of this solution should be 4.5 if it is saturated.

Allow the saturated solution to stand a minimum of 12 hours at room temperature and decant the clear solution into a clean reagent bottle.

Formula #76 - Sodium Bicarbonate Buffer (NaHCO₃) 0.1 N

For use in PCFIA base buffer, NU extraction, AU back-extraction or clean-up.

Procedure for 1000 ml:

Dissolve 8.40 g NaHCO₃ into dH₂O and bring to 1000 ml volume.

Acid Urine (AU/W) Working Protocol (Red)**PETRL SOP 103.0****Extraction:**

Be sure to include PC and NC sample with each set.

1. 2 ml pH 2 buffer
5 ml DCM
3 ml urine (or plasma, if blood only)
2. Cap, rack, centrifuge (2500-3000 rpm x 10 min.), aspirate top layer to waste.
3. Transfer into a clean tube and dry at 65°C
4. Reconstitute with 2 drops of DCM.

TLC:

1. Two plates:
1 in 90:25:5 and 1 in 85:10:5(Davidow),
2. Spot 1/5-1/4 sample on each plate.
3. Spot 3 QA standards: acepromazine, caffeine, and meclofenamic acid on each plate.
4. Spot sulfa and furosemide standards on 90:25:5 plate.
5. Spot bromfenac on 85:10:5 plate
6. **Spray sequence for 1st plate developed in 90:25:5:**
 - a) Check wet under UVL for (+) furosemide reaction, mark
 - b) Dry plate and observe under UVL and UVS. Mark
 - c). Fluram (Formula #31) Dry. Observe under UVL.
If no fluram (+) (sulfa drug is fl+), and/or unconfirmed lasix, then proceed to step "e".
 - d). To confirm lasix or sulfa:
Spray with Modified Ehrlich's (Formula #27) --> Observe.
HCl --> heat. Wait. Observe. (Heat until furosemide sample is dark purple with light yellow background).
 - e). Spray with Liebermann's (Formula #36) --> observe.
 - f). To detect phenylbutazone(**bute**), spray with Mandelin's (Formula #38) --> observe
Oxyphenylbutazone (OPB) is yellow, R_f is 0.7, bute is red, R_f ≈0.8).
 - g). Lightly warm on hot plate --> observe under UVL (Some NSAID's fluoresce).
7. **Spray sequence for 2nd plate developed in 85:10:5:**
 - a). Dry plate, and observe under UVL and UVS. Mark
 - b). Fluram-->observe
 - c). Dragendorff-->observe
 - d). NO₂ -->observe
 - e). Allow plate to develop at room temp for 15-30 minutes as bromfenac will develop a distinct blue spot within that time period
Note:(spot will continue to darken for up to 4 hours)
8. *Some drugs detected by AU: diclofenac, furosemide (Lasix), sulfa, phenylbutazone, OPB, naproxen, bromfenac and other NSAID's.*

Methyl Iodide Derivatization for GC/MS Analysis

PETRL SOP 131.0

General:

The method involves the methylation of labile protons. This derivatization has the following advantages over more standard methods such as "MethElut" derivatization:

1. Decreased thermal decomposition
2. More rigorous and complete derivatization
3. Decreased glass adsorption

Materials:

1. Methyl Iodide (redistilled)
2. Acetone
3. Anhydrous Potassium Carbonate (K_2CO_3)
4. 16 x 125 mm screw cap tubes

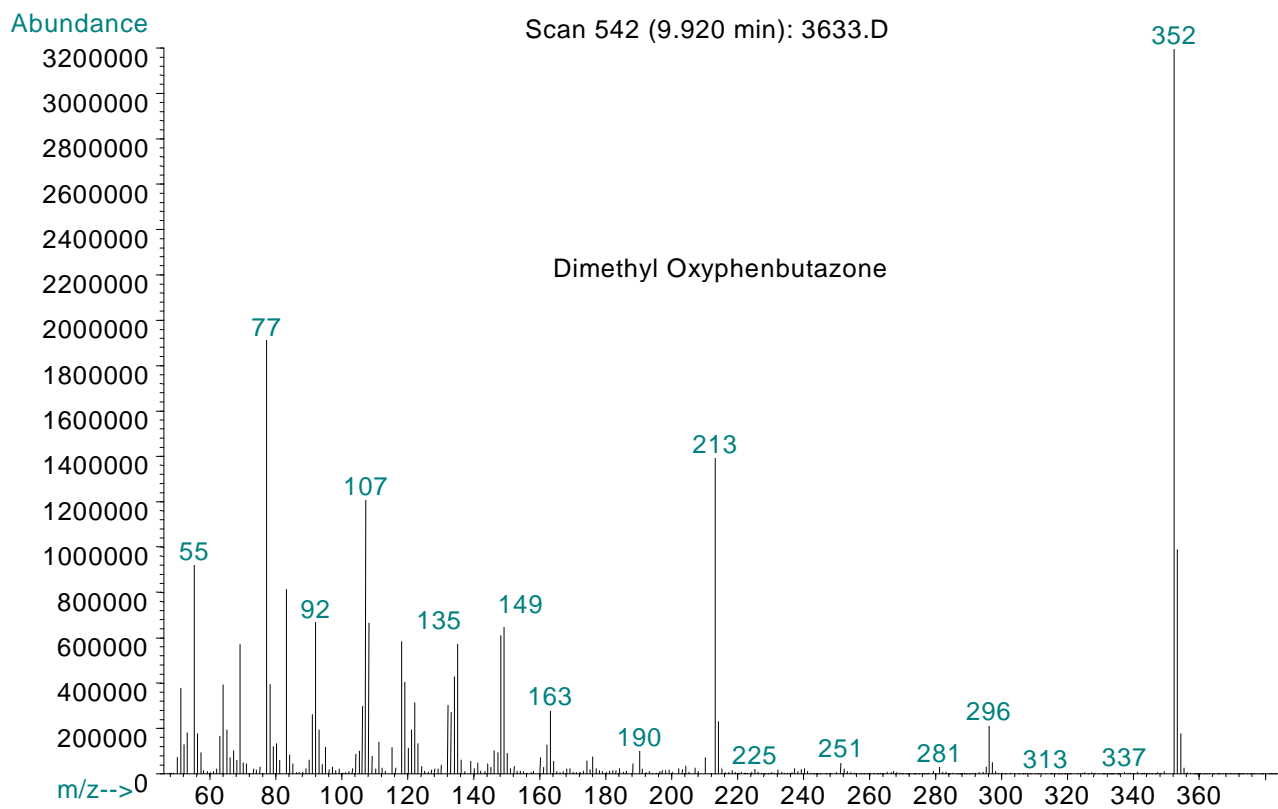
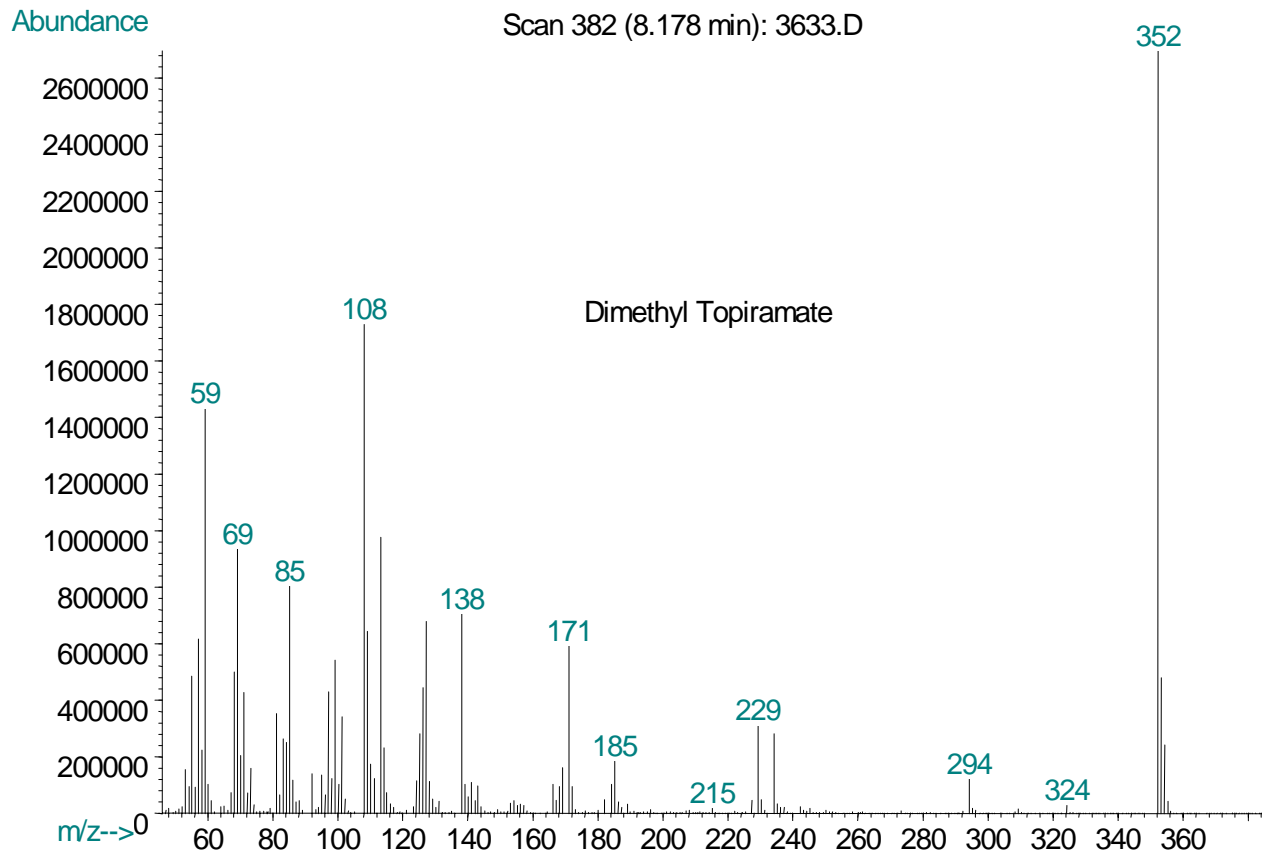
Procedure:

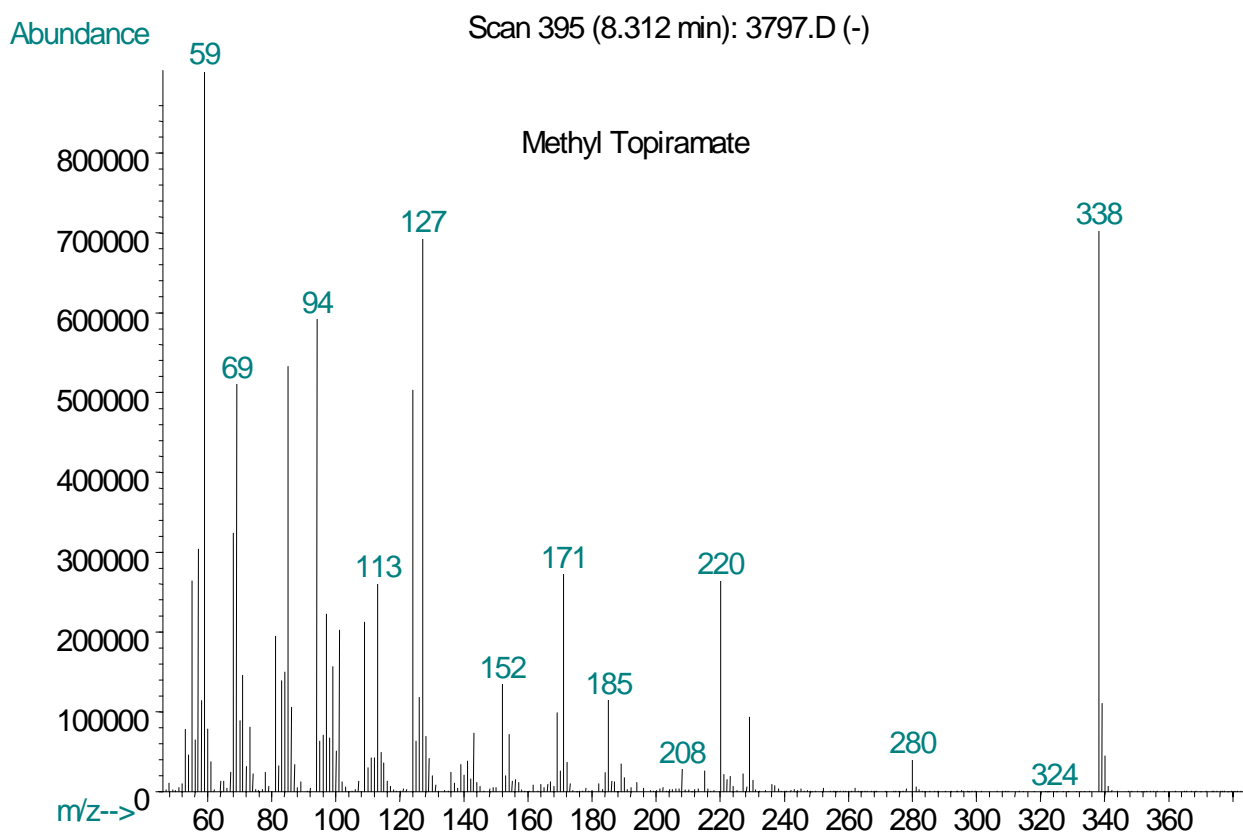
1. Extract drug from sample matrix using the described extraction procedures and a 16 x 125 mm screw cap tube as a sample transfer tube.
2. To the dry residue in the screw cap tube, add:
 - 300 μ l acetone
 - 30 μ l methyl iodide
 - 10 mg potassium carbonate
3. Cap and heat in a water bath @ 65°C for 30 min.
4. Cool, transfer acetone to a clean dry 16 x 125 mm screw cap tube.
5. Evaporate to dryness.
6. Add 50 μ l ethyl acetate for GC/MS analysis

Topiramate References

1. **Merck Index 12th Ed.**, Ref # 9686, pg. 1629
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13. Focus on Topiramate: B. Green: <http://www.publinet.it/pol/focus7.htm>
14. Topiramate (Systemic): <http://health.phillynews.com/pharmacy/vol2/203085.asp>
15. "Tying UP" or Chronic Intermittent Rhabdomyolysis: J. Beech <http://www.midatlantichorse.com/NewBolton.htm> : enclosed
16. **Martindale Pharmacopoeia**: enclosed
17. **Physicians Desk Reference**: enclosed

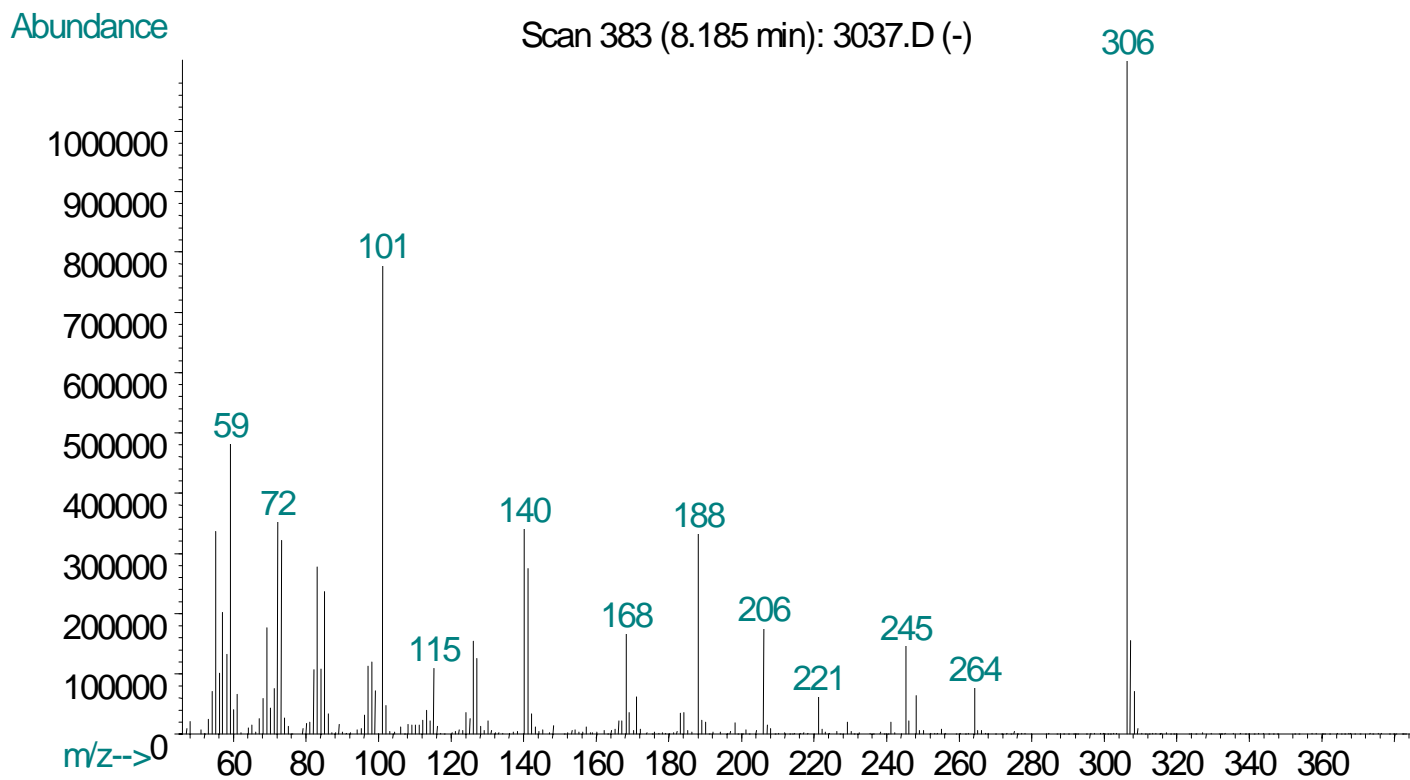
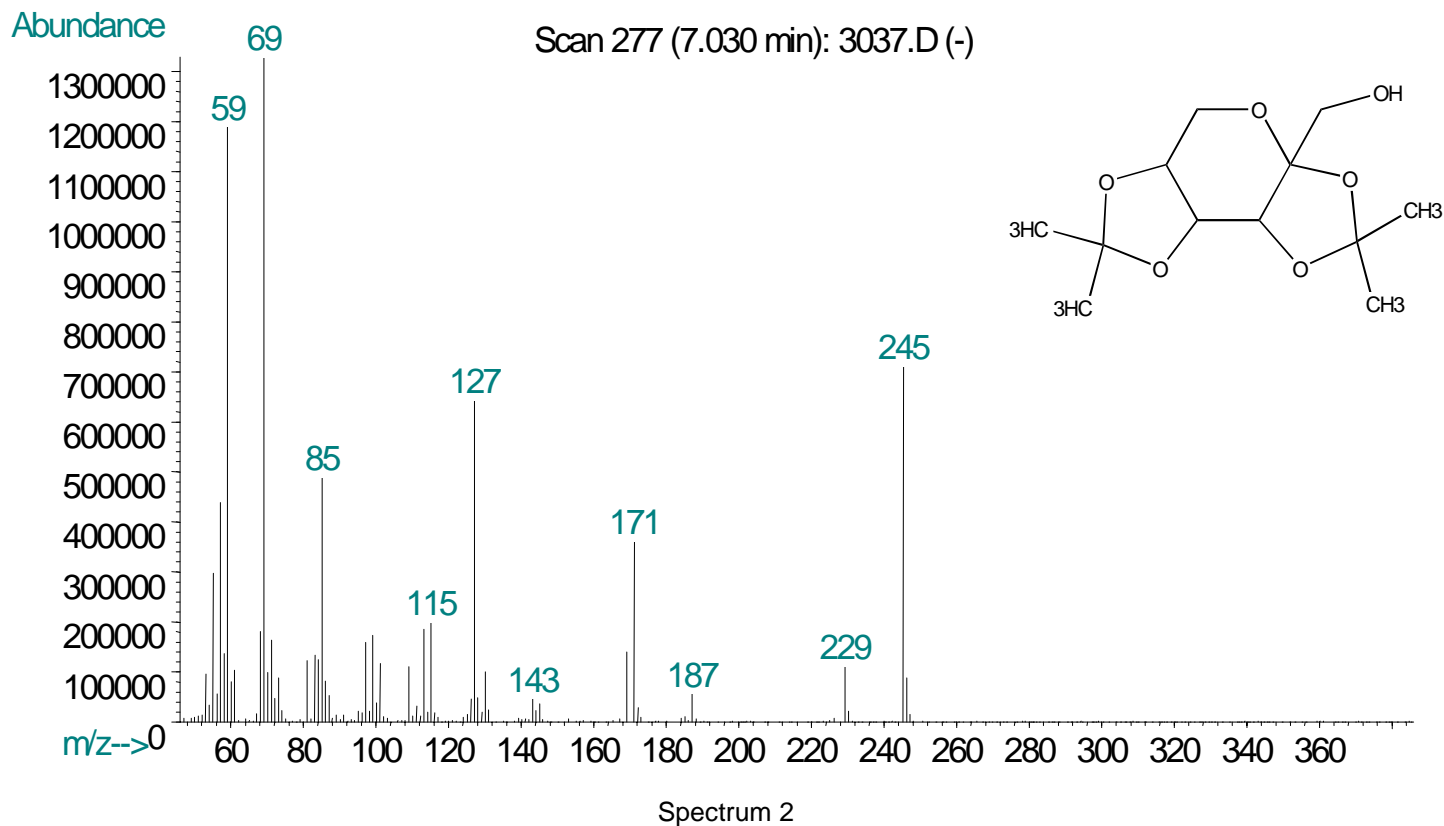
APPENDIX



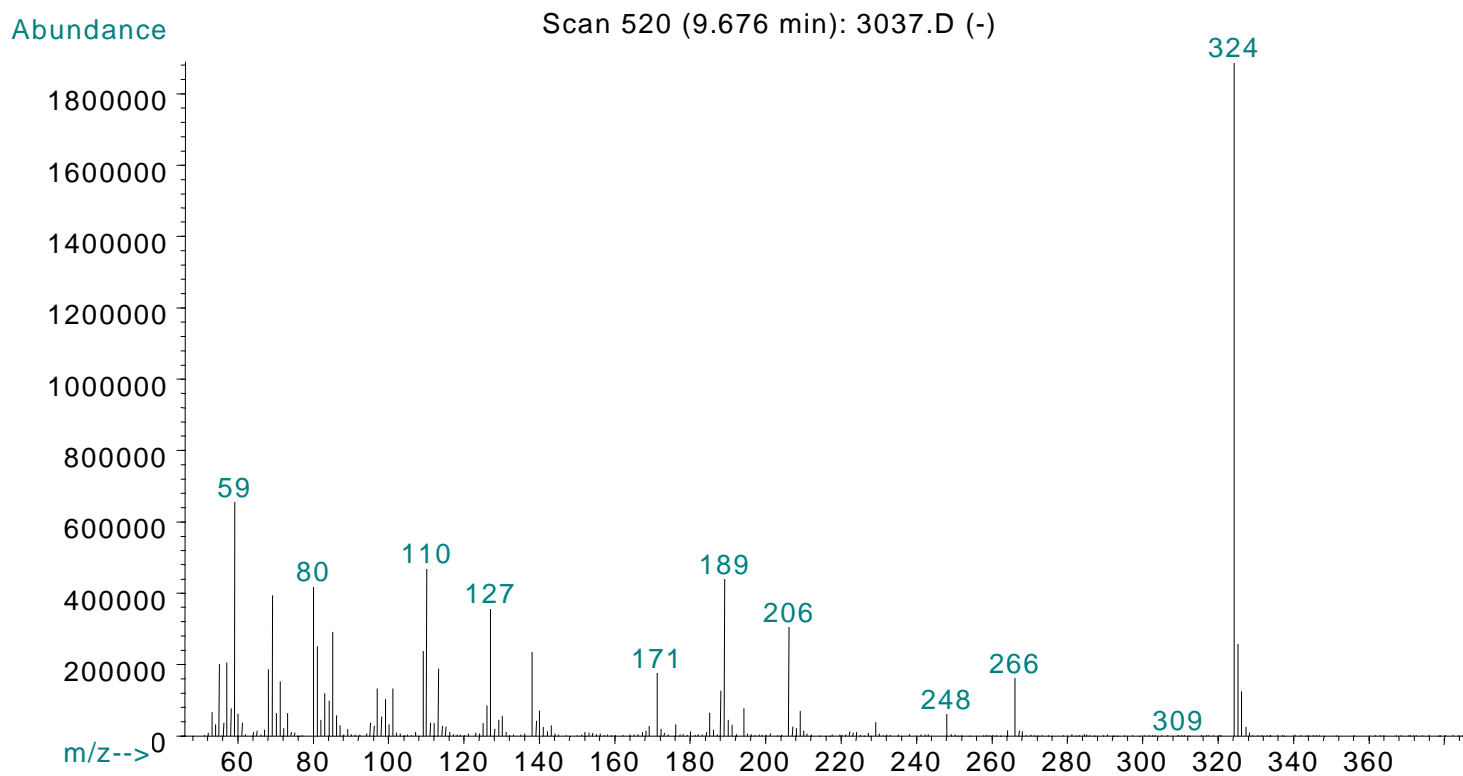


The following degradations all involve elimination and addition reactions of the sulfamate group. Spectrum #1 is most likely the reported 2,3:4,5-bis O-(1-methylethylidene)-*b*-D-fructopyranose ^{spectra 1}(ref 2). These artifacts are produced both thermally and chemically. Pretreatment of standard or urine with increasing concentrations of NaOH produces increasing amounts of this base degradation compound (Spectrum #1) relative to the other presented spectra. If a sample contains TOP, these compounds may appear as artifacts or interferences during neat GC/MS analysis for other compounds.

Spectrum 1



Spectrum 3



Spectrum 4

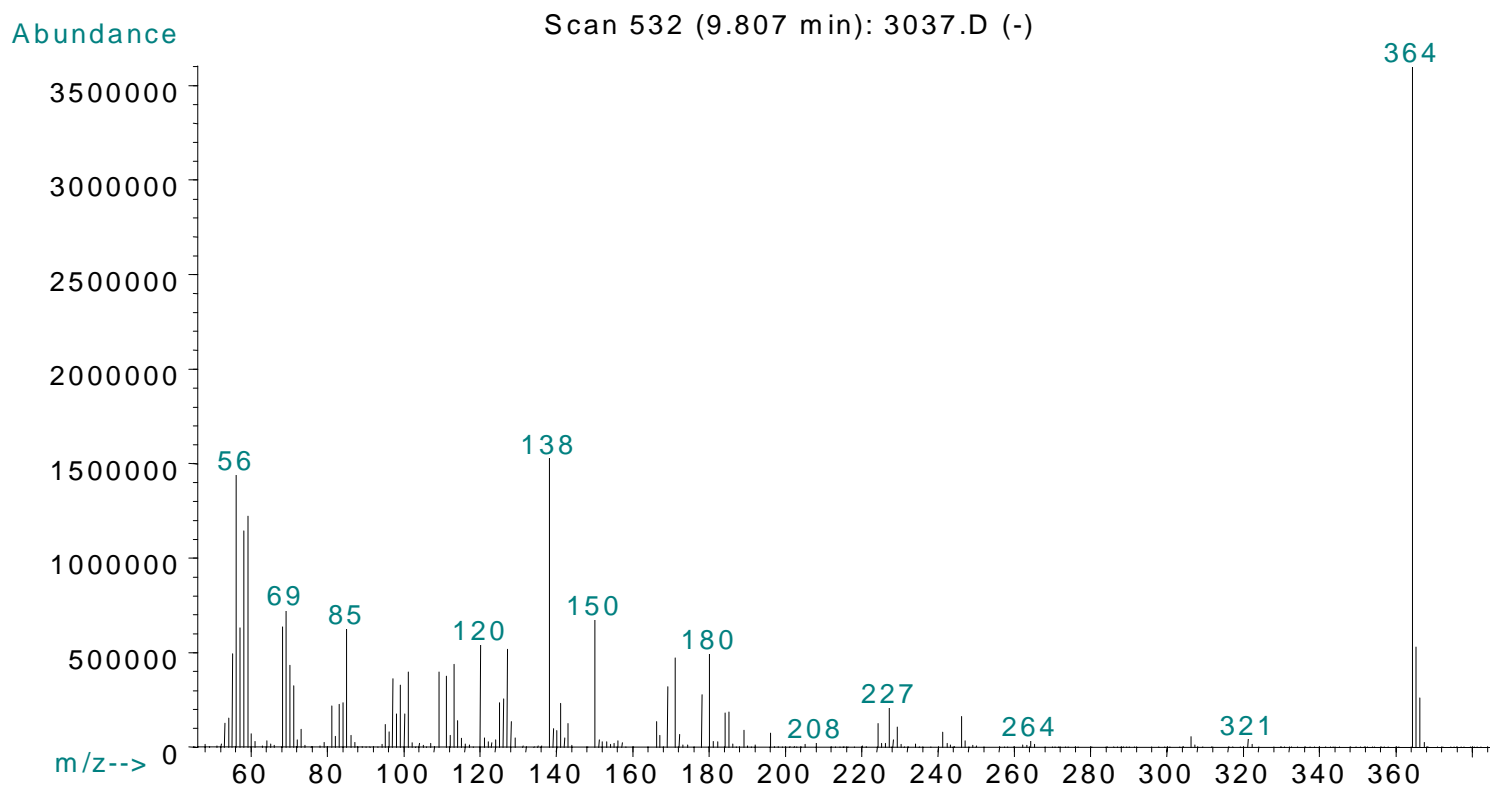


Figure 3

Source of TDx/TDxFLx compatible TDM (therapeutic drug monitoring) FPIA kits.

OXIS International, Inc. **United States**

Voice: 800-547-3686
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Email: info@oxis.com
WWW: <http://www.oxis.com>