Analytical Methods for the Newer Anticonvulsants

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Disclosure

- Relevant Financial Relationships
  - *Employee of MEDTOX Laboratories, Inc.*
- Off Label usage
  - *None*

Introduction: Anticonvulsants and TDM

Seizure Disorders and Anticonvulsants

- Seizure disorders currently affect more than two million individuals in the United States with 125,000 new cases diagnosed annually
- The frequency, intensity, and type of seizures are highly variable in this patient population
- Anticonvulsant drugs control seizure activity by minimizing or blocking the spread of excess electrical discharge in the brain.

TDM of Anticonvulsants

- Therapeutic Drug Management (TDM) of anticonvulsants is an important tool in the management of patients with epilepsy
- The goal is to optimize efficacy while minimizing adverse effects; this requires careful journaling of seizure activity, determination of anticonvulsant blood levels, and individualized dosing adjustments

What are the “Newer Anticonvulsants”?

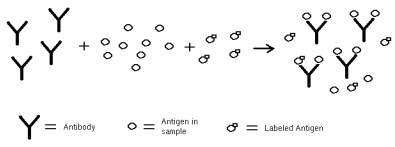
DRUG	US APPROVAL DATE	TRADE NAME
Phenobarbital	1912	
Phenytoin	1938	Dilantin
Primidone	1954	Mysoline
Ethosuximide	1960	Zarontin
Carbamazepine	1974	Tegretol
Clonazepam	1975	Klonopin
Valproic Acid	1978	Depakene
Felbamate	1993	Felbatol
Gabapentin	1993	Neurontin
Lamotrigine	1994	Lamictal
Topiramate	1996	Topamax
Tiagabine	1997	Gabatril
Levetiracetam	1999	Keppra
oxcarbazepine	2000	Trileptal
Zonisamide	2000	Zonegran
Pregabalin	2004	Lyrica
Lacosamide	2008	Vimpat
Rufinamide	2008	Banzel
Vigabatrin	Not yet approved in the US	Sabril (Canada)

Common Test Methods for Anticonvulsants

DRUG	Standard Testing Methodology
Phenobarbital	Immunoassay
Phenytoin	Immunoassay
Primidone	Immunoassay
Ethosuximide	Immunoassay
Carbamazepine	Immunoassay or Chromatography
Clonazepam	Chromatography
Valproic Acid	Immunoassay
Felbamate	Chromatography
Gabapentin	Chromatography
Lamotrigine	Chromatography
Topiramate	Immunoassay
Tiagabine	Chromatography
Levetiracetam	Chromatography
oxcarbazepine	Chromatography
Zonisamide	Chromatography
Pregabalin	Chromatography
Rufinamide	Chromatography
Lacosamide	Chromatography
Vigabatrin	Chromatography

Test Methods: Immunoassay

- Immunoassay
  - Methodology based on immune system principles – binding of antigens (drug/metabolite) and antibodies creates a measurable signal



Immunoassay Methods

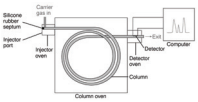
- Strengths:
  - Relatively inexpensive; easily automated, fast
  - No preliminary sample prep required
  - Can identify a broad spectrum of drugs/metabolites
- Weaknesses:
  - Cross-reactivity issues/specificity
  - Lack of FDA-cleared kits for the newer anticonvulsants

Immunoassay Methods

- Carbamazepine
  - FPIA demonstrates little or no cross-reactivity to the pharmacologically active 10,11 Epoxide
  - Reliance on IA only may limit understanding of therapeutic failures
- Topiramate
  - Assay license was limited to a single vendor until recently
  - Limited flexibility for instrument platforms

Chromatographic Methods: Gas Chromatography

- GC or (GLC): the stationary phase is a fluid with extremely high viscosity and the mobile phase is an inert gas
- Compounds are vaporized, carried through a column by the gas phase
- Separation is facilitated by degree of interaction with the stationary phase and volatility of the compound



Gas Chromatography

Strengths:

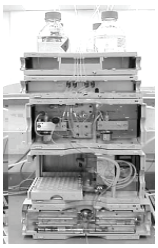
- Many choices for detector
  - Flame ionization, Electron Capture, NP, Atomic Emission, Flame-photometric, Thermal Conductivity, Photoionization, MS
- Temperature and Flow can be modified to enhance resolution
- Sharp peaks, excellent resolution

Limitations:

- Not amenable to thermally labile or non-volatile compounds
- With the exception of MS, detectors provide little structural information
- Derivatization may be needed to increase volatility or hide reactive groups
- Sample preparation required

Chromatographic Methods: High Performance Liquid Chromatography

- Silica-based stationary phases
- Mobile phases are liquids; solvents/buffers
- Properties of solid and liquid phases are utilized to effect separation of analytes in a mixture



HPLC

Strengths

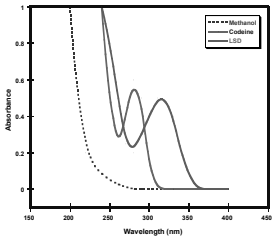
- Multiple variables can be employed to achieve resolution
  - Complex interactions can be exploited
- Amenable to non-volatile compounds
- Usually does not require derivatization

Limitations

- Low theoretical plate count compared to GC
  - More susceptible to interferences
- No universal detector
- Other than MS, detectors provide little structural information
- Sample prep required

UV Detectors

- UV Detectors have limited selectivity due to overlap of absorbance between compounds
- Not all compounds absorb light above background absorbance of the mobile phase

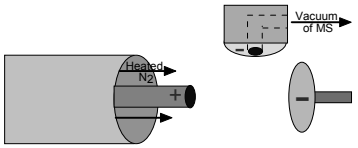


Chromatographic Instruments coupled to Mass Spectrometers

- Chromatographic methods separate sample extracts into components
- MS = mass spectrometry generates fragments that are characteristic of specific compounds. High degree of specificity and selectivity.

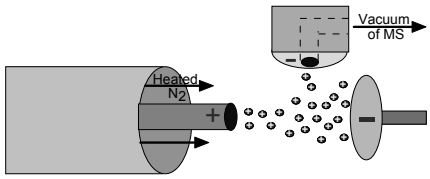
HPLC-MSMS

- The challenge: How do you connect a detection system that must operate in a vacuum, to a chromatographic system where analytes are dissolved into a solvent?
- Electrospray Interface



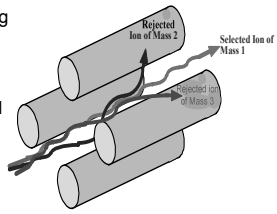
A high electrical potential (500 to 4000 volts) propels the charged droplets from the end of the capillary to the target. Ions must then be separated from the

Droplets prior to entering the vacuum of the MS

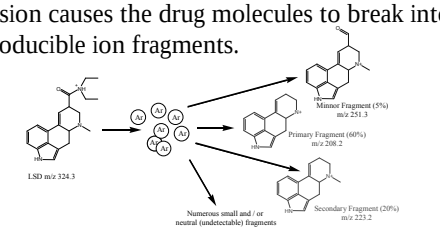


Quadrupole MS

- A quadrupole is a array of four parallel rods generating an electromagnetic field.
- By modifying the potential applied to the rods, drug ions can be selected based on their specific molecular weight. All other ions are ejected from the system.



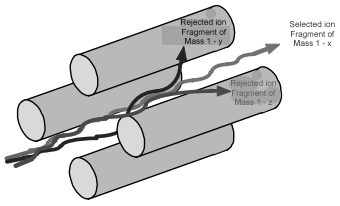
For tandem MS, drug ions selected in the first quadrupole are directed into a collision cell where they collide with gaseous argon. The energy of the collision causes the drug molecules to break into reproducible ion fragments.



Drug Ion Selected in the first Quadrupole

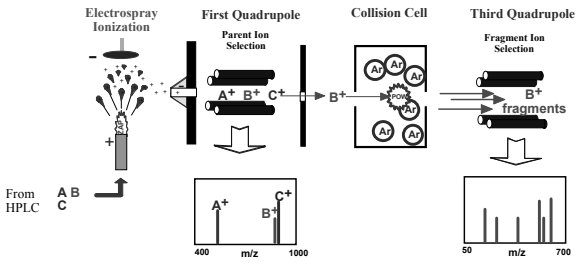
Ion Fragments Produced as a result of the molecular collision

The fragment ions are then directed into another set of quadrupoles where specific fragments are selected by weight to be sent to and counted by an ion detector.



Ion Fragments from the collision cell

Tandem Mass Spectrometry



Advantages of HPLC-MS/MS over Traditional HPLC-UV

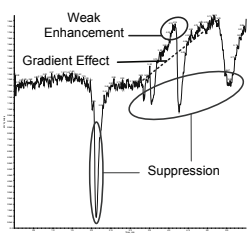
- **Reduced sample handling.** The potential for interference is reduced allowing more efficient and simplified sample clean-up steps.
- **Shorter run times**. LC-MS/MS can differentiate between co-eluting peaks if they differ in either molecular weight or fragmentation pattern allowing for faster chromatography.
- **Analytical panels**. Structurally or pharmacologically related compounds can be extracted together and analyzed in a single run.
- **Reduced sample volume requirements.** As the selectivity minimizes interferences and background, sensitivity is improved requiring smaller initial sample volumes.

Limitations/Disadvantages

- Expense
- Complexity/Technical requirements
- Depending on the application, can be overkill
- Ion suppression or enhancement
  - Other components in the mobile phase can alter the ionization of the analyte by affecting droplet formation, droplet evaporation, ion ejection
  - Polar compounds are more susceptible to ion suppression.

Ion Suppression

- If using isotopic internal standards
  - Ion suppression will appear as poor recovery.
  - If signal strength is adequate, quantitative performance is usually unaffected.
- If the analyte / internal standard ionize differently, ion suppression can be fatal to a quantitative assay.



Analysis of Anticonvulsants by HPLC-MS/MS

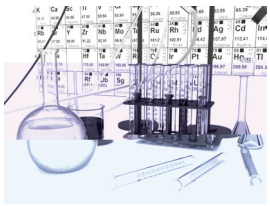
- The method:
  - A home brew method for the analysis of the basic antiepileptic drugs felbamate, lamotrigine, carbamazepine, carbamazepine-10,11-epoxide, gabapentin, pregabalin, levetiracetam and oxcarbazepine monohydroxy derivative (oxcarb MHD) in plasma
  - Allows simultaneous analysis of 8 drugs using less than 100 µL of sample for extraction and a 5 minute run time.

Method Principle

- Plasma samples are processed by protein precipitation using a combination of zinc sulfate and acetonitrile. The clarified sample is then analyzed by positive electrospray HPLC-MS/MS. Chromatography is performed using reverse phase chromatography.

Sample Preparation/Extraction

- 50 µl sample + i.s. + 10 µL of zinc sulfate
- 4000 µL of 50/50 Acetonitrile / water to each tube; Vortex samples for a minimum of 2 minutes.
- Centrifuge
- Transfer the supernatant into glass vials for analysis



Assay Dynamic Range

- Standards are defined to accommodate the appropriate therapeutic ranges
  - 6 standards
  - Felbamate, Levetiracetam, OxcarbMHD
    - 2.0 – 100 µg/ml
  - Carbamazepine, Gabapentin, Pregabalin, Lamotrigine
    - 0.5 – 25 µg/ml
  - Carbamazepine 10,11 Epoxide
    - 0.25 – 12.5 µg/ml

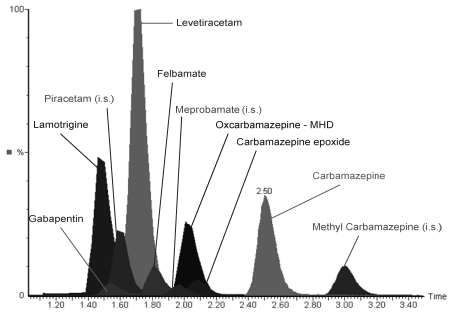
Chromatographic Method

- Prism RP 2.0 x 150 mm, 5µ (Thermo Hypersil)
- Mobile Phase, Isocratic Elution
  - 45% 10mM Ammonium acetate w/ 0.1% Formic acid, pH 3.0
  - 55% methanol
  - 0.40 ml/min
- Injection Volume 5.0 µl

Transitions Monitored

Compound	Transition	Dwell (sec)
Felbamate	239.10>117.10	0.1 - 0.2
Levetiracetam	171.20>126.20	0.1 - 0.2
Pregabalin	160.04>96.82	0.1 - 0.2
Gabapentin	172.20>154.19	0.1 - 0.2
Meprobamate (Internal Standard)	219.40>158.20	0.1 - 0.2
Piracetam (Internal Standard)	143.31>98.16	0.1 - 0.2

AED Method Chromatographic Overlay



Validation Performance Parameters

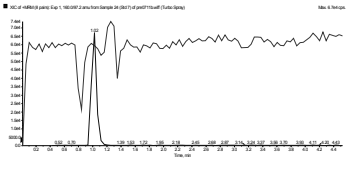
Inter-Run Precision and Accuracy	Target	CV	Accuracy
Felbamate	5	4.80%	95.80%
Levetiracetam	5	2.40%	98.60%
Oxcarb MHD	5	4.30%	93.00%
Carbamazepine	1.25	2.50%	96.80%
Gabapentin	1.25	5.00%	96.80%
Lamotrigine	1.25	3.20%	99.20%
Pregabalin	1.25	9.00%	90.90%
Carb epoxide	0.63	5.10%	93.70%

Validation Performance Parameters

Inter-Run Precision and Accuracy	Target	CV	Accuracy
Felbamate	80	4.10%	103.40%
Levetiracetam	80	2.90%	100.30%
Oxcarb MHD	80	4.20%	97.90%
Carbamazepine	20	3.20%	102.30%
Gabapentin	20	4.50%	99.30%
Lamotrigine	20	2.50%	98.60%
Pregabalin	20	6.70%	88.40%
Carb epoxide	10	5.20%	100.60%

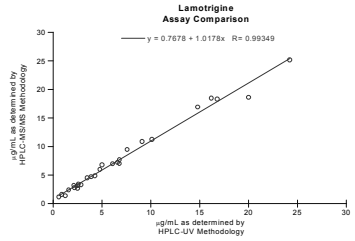
Validation Performance

- Recoveries
  - 88 – 110%
- Selectivity
  - No interferences noted
- Suppression



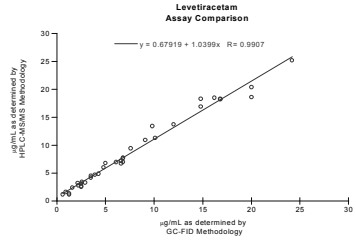
Correlation to Predicate Method

- Lamotrigine



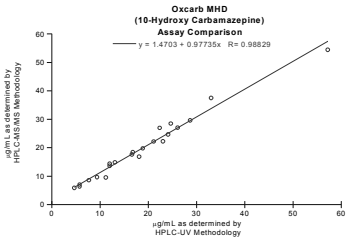
Correlation

- Levetiracetam



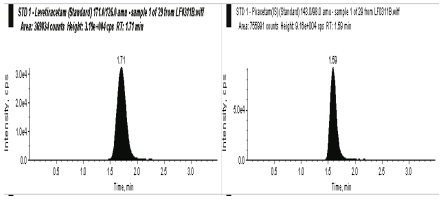
Correlation

- Oxcarb MHD

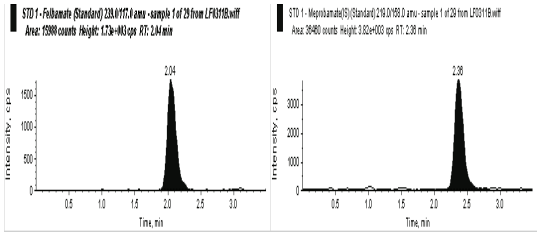


Extracted Chromatograms: Levetiracetam

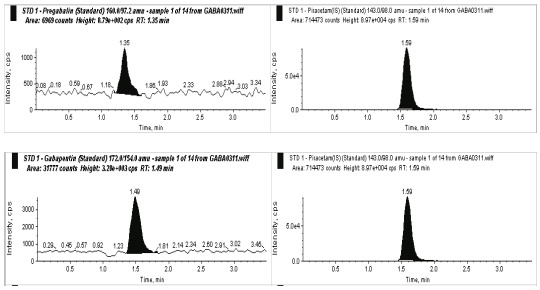
- Applied Biosystems Sciex 4000 AED LLOQ of 2 µg/ml



Extracted Chromatograms: Felbamate

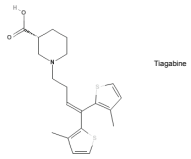


Extracted Chromatograms; 0.5 ug/ml



Tiagabine by HPLC-MS/MS

- **Linear Range** 5.0 – 500.0 ng/mL
- **LLOQ** 5.0 ng/mL
- **Accuracy** 96.0%
- **Precision (CV)** <10%



Tiagabine

Tiagabine

- Sample Preparation:
  - 200 µl sample + IS + buffer
  - SPE (mixed mode C8/cation exchange)
- HPLC Conditions:
  - Betasil-C18, 100 x 2 mm, 5 µm
  - Mobile Phase: 50% 10 mM ammonium acetate with 0.1% formic acid, pH 3.0: 50% acetonitrile
  - Flow Rate: 0.2 ml/min
  - Injection Volume: 10 µl

Transitions Monitored and Chromatography

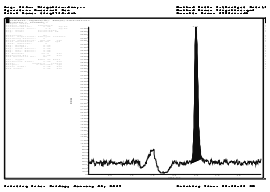
Tiagabine

Q1 Mass (amu)	Q3 Mass (amu)	Dwell (msec)
376.20	149.20	100.00

D_4-Haloperidol

Q1 Mass (amu)	Q3 Mass (amu)	Dwell (msec)
380.20	169.20	100.00

5 ng/ml Extracted



Zonisamide by HPLC-UV

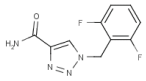
- **Linear Range 5 – 100** ug/mL
- **Accuracy 98 - 101**%
- **Precision (CV) 3 – 6%**

Zonisamide Methodology

- Sample Preparation
  - 250 ul sample + I.S. + 250 ul Phosphate Buffer
  - LIQ-LIQ Extraction
- Chromatographic Conditions:
  - Column: Agilent Eclipse XDB-C8
  - MP: 70% 10mM phosphate buffer, pH 3.0; 30% ACN
  - Flow rate = 1.8 ml/min;
  - Detector: UV @ 280nm

Rufinamide by HPLC-MS-MS

- **Linear Range** **0.2 – 30 ug/mL**
- **Accuracy** **91 - 105%**
- **Precision (CV)** **3 - 6%**



Rufinamide

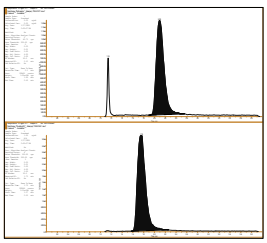
- Sample Preparation:
  - 100 ul sample
  - PPT; centrifuge
- Chromatography Conditions:
  - Column: Agilent SB-Phenyl 3.5µ column 100 x 2.1 m
  - Mobile Phase: 65% 10mM, ammonium acetate/formate buffer pH 3.0: 35% acetonitrile.
  - Flow Rate: 0.25 ml/min

MSMS Conditions

Transitions Monitored

- Rufinimide
  - 239.2>127.1 m/z
- Clonidine
  - 229.2>160.1 m/z

Rufinimide and IS; 0.2 ug/ml



Alternative Approaches to Anticonvulsant TDM

Rationale

- Levels of anticonvulsant compounds have traditionally been measured in serum or plasma prepared from whole blood collected by venipuncture.
- This requires access to an appropriate collection facility with trained phlebotomists, specimen preparation protocols to separate the serum from the remainder of the sample components, transportation of the specimen to a qualified laboratory under appropriate conditions and laboratory analysis to determine drug levels

Rationale, continued

- Individuals living in more remote areas or individuals who have difficulty providing samples (elderly, pediatric) may have more obstacles to overcome to ensure compliance with treatment/monitoring regimens.
- When treatment failure occurs, i.e. seizures are not controlled, measurement of drug levels may be delayed by hours/days/weeks from the time of the occurrence.
- Use of alternative matrices may provide a way to obtain samples more proximate to the occurrence and therefore test results may provide more relevant diagnostic information

Alternative Approaches

- Other matrices have been explored:
  - Saliva
    - Most studies conducted on older anticonvulsants; correlation to plasma fairly consistent
    - Free drug only
    - Specimen collection protocol may impact levels
  - Whole Blood Spots

Study Partner

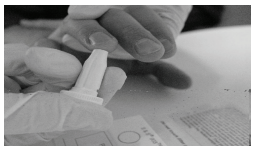
- The MN Epilepsy Group is a comprehensive epilepsy care center specializing in evaluation, treatment, consultations, research, and support services for adults, teens, and children with seizure disorders.
- Collaborated to enroll patients in the study and perform specimen collections.
- Subjects: adults with epilepsy currently being treated with one or more antiepileptic drugs

Study Design

- Antiepileptic Drugs

Anticonvulsant	*Routine testing methodology*	*FP testing methodology*
Carbamazepine and epoxide	HPLC	LCMSMS (ESI+)
Ethosuximide	IA	LCMSMS (ESI-)
Felbamate	GC	LCMSMS (ESI+)
Gabapentin	HPLC	LCMSMS (ESI+)
Lamotrigine	HPLC	LCMSMS (ESI+)
Levetiracetam	GC	LCMSMS (ESI+)
Oxcarbazepine (hydroxy metabolite)	HPLC	LCMSMS (ESI+)
Phenobarbital	IA	LCMSMS (ESI-)
Phenytoin	IA	LCMSMS (ESI-)
Topiramate	IA	LCMSMS (ESI-)
Valproic Acid	IA	LCMSMS (ESI-)
Zonisamide	HPLC	LCMSMS (ESI-)

Protocol

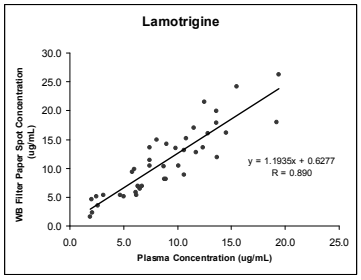


- Venipuncture samples are collected in the clinic according to the customary protocol. A lancet is then used to pierce the fingertip and two drops of capillary blood (approximately 120 µL) are collected onto a filter paper blood collection device.

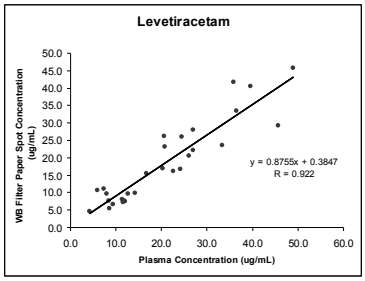
Testing Protocol

- Filter paper specimens are processed by using a hand punch to cut a 4.5mm circle from the dried filter paper blood spot.
- The filter paper circle is placed into a test tube, internal standards are added to the punch, and the analytes are solubilized into a mixture of acetonitrile and water.
- Quantitative standards and controls prepared in whole blood are spotted onto filter paper and processed in the same manner.
- Samples, standards, and controls are analyzed by two LC/MS/MS methods to quantify the included compounds.

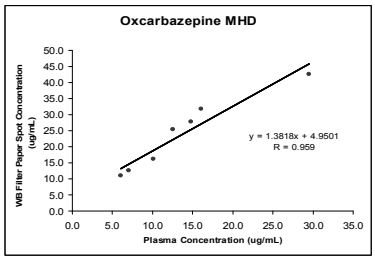
Lamotrigine Concentrations in Filter Paper Blood Spots vs. Venipuncture Plasma



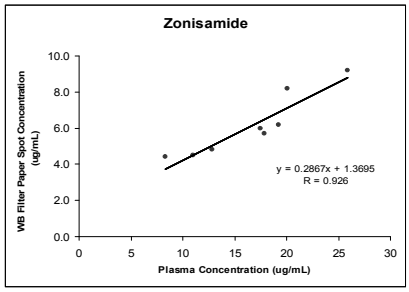
Levetiracetam Concentrations in Filter Paper Blood Spots vs. Venipuncture Plasma



Oxcarbazepine MHD Concentrations in Filter Paper Blood Spots vs. Venipuncture Plasma



Zonisamide Concentrations in Filter Paper Blood Spots vs. Venipuncture Plasma



Summary of Results

Anticonvulsant	*# of Comparisons*	*Correlation Coefficient*
Lamotrigine	41	0.890
Levetiracetam	29	0.922
Carbamazepine	10	0.876
Carbamazepine-10,11-Epoxide	8	0.992
Oxcarbazepine MHD	8	0.959
Felbamate	4	0.968
Topiramate	4	0.977
Valproic Acid	6	0.918
Zonisamide	8	0.926

Summary

- Most methods for analysis of the newer anticonvulsant drugs are “home brew”
- GC, HPLC, and HPLC-MS/MS all have applicability;
  - Each laboratory must evaluate the advantages and/or disadvantages in the context of laboratory needs
- The sensitivity of MS/MS techniques allows the exploration of alternate matrices and collection procedures for TDM purposes.

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