LC-MS/MS QUANTITATION IN DRUG DISCOVERY & DEVELOPMENT

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Presentation Outline

- Bioanalysis and Metabolites in Drug Discovery and Development
- Case Study
  - Quantification of Phthalates in Urine
- Conclusion

What is Bioanalysis?

- **Bioanalysis employed for the quantitative determination of drugs and their metabolites in biological fluids, plays a significant role in the evaluation and interpretation of bioequivalence, pharmacokinetic and toxicokinetic studies** **(Viswanathan c.t. et al. Workshop/conference Report, AAPS Journal 2007; 9 (1) Article 4)**

Metabolites and Bioanalysis In Drug Development

- Identify and profile animal metabolites

In vitro | In vivo (cold/C^{14})

- Identify and profile human metabolites

In vitro | In vivo (cold/C^{14})

- Bioanalytical methods

P	Non-GLP method	GLP method
M		

GLP: Good Laboratory Practice P: Parent M: Metabolite(s)

The cost to bring a drug to the market is about $1.3 billion (US FDA, 2004)

MS Instrumentation of Choice

- Bioanalysis
  - Triple Quadrupoles
    - Most of the Quantitative Assays are Performed in Multiple Reaction Monitoring (MRM) Mode
- Metabolite Identification
  - Ion Traps
  - Q-Tofs
  - Triple Quadrupoles

Bioanalysis in Drug Discovery Phase

Scope

Determine Concentrations/Exposures in Pharmacokinetic (PK), Pharmacodynamic (PD) Studies

Requirements and Challenges

- Significant Throughput for the Screening Across Different Chemical Series
- Generic Assays are Preferred/Automated Method Development
- No Stable Isotope Internal Standard
- Unknown Compound/Metabolite Stability
- No QC Samples to Verify Method Performance

Bioanalysis in Preclinical Phase

Scope

- **Determine Concentrations/Exposures in Toxicokinetic Studies Using Validated Assays to Evaluate Drug Safety**
- **Requirements and Challenges**
  - Full Validation Required
  - Stability in Matrix Investigated
  - Highly Regulated: Conducted According to Good Laboratory Practice (GLP) and Most Activities Should Follow Standard Operation Procedures (SOP)
  - More Work for Troubleshooting and Validation to Produce Rugged and Robust assay
  - Time Constraint: Validation Completed Before the Actual Study Starts
  - Long-Term Use for Routine Analysis
  - The Bioanalytical Assay Becomes a Part of Regulatory Submission (e.g. IND)

Bioanalysis in Clinical Phase

Scope

- **Determine Concentrations/Exposures in Clinical /Bioequivalence Studies (Phase 1 - Phase 3)**
- **Requirements and Challenges**
  - Assay Adjusted for Human Matrices (Plasma, Serum, Urine)
  - Ultra-Sensitive Assay May be Required
    - Novel Target is Pursued
    - Microdosing
  - Long-Term Usage is Desirable: Preferable to Use the Same Assay Through Phase 1 – Phase 3 clinical studies
  - Assay Flexibility with Regards to Concomitant Medications or Background Interferences
  - Fast Turn Around for First in Man Studies

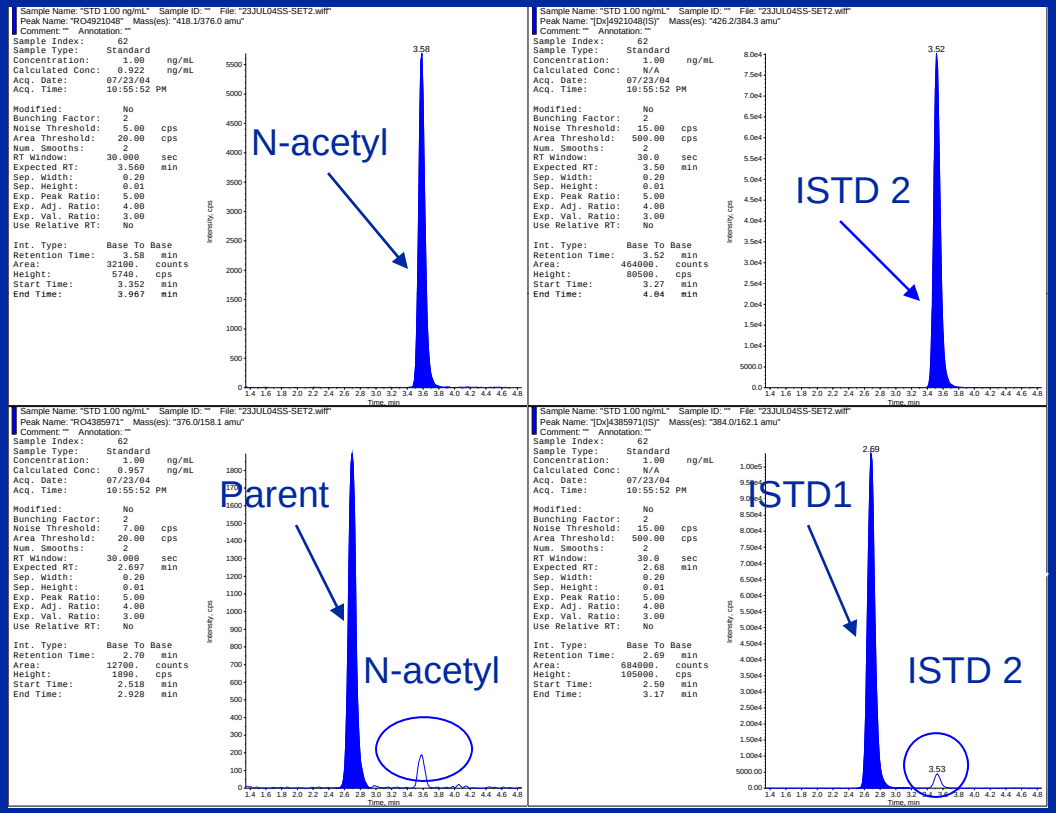
Analytical Approaches for Bioanalysis

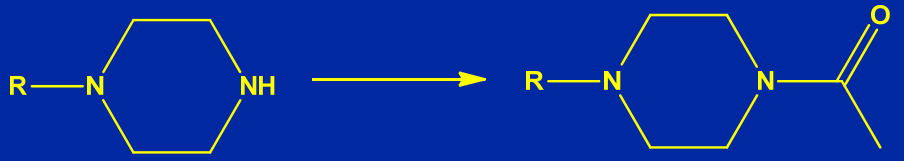
- **LC/Ultraviolet (UV)/Fluorescence**
  - Due to Low (if any) Specificity of Detectors Not Much in Use for Over a Decade
- **LC/MS**
  - Most of the Assays are Performed Using Triple Quadrupole Instruments Operating in Selected Reaction Monitoring (SRM) Mode
- **Ionization Techniques**
  - APCI
    - More appropriate for Poorly Ionized Analytes
    - Less Matrix Effect
  - ESI
    - With Biological Matrices ESI is Prone to Ionization Suppression/Enhancement
    - Sample Purification is Required

Liquid Chromatography in Bioanalysis

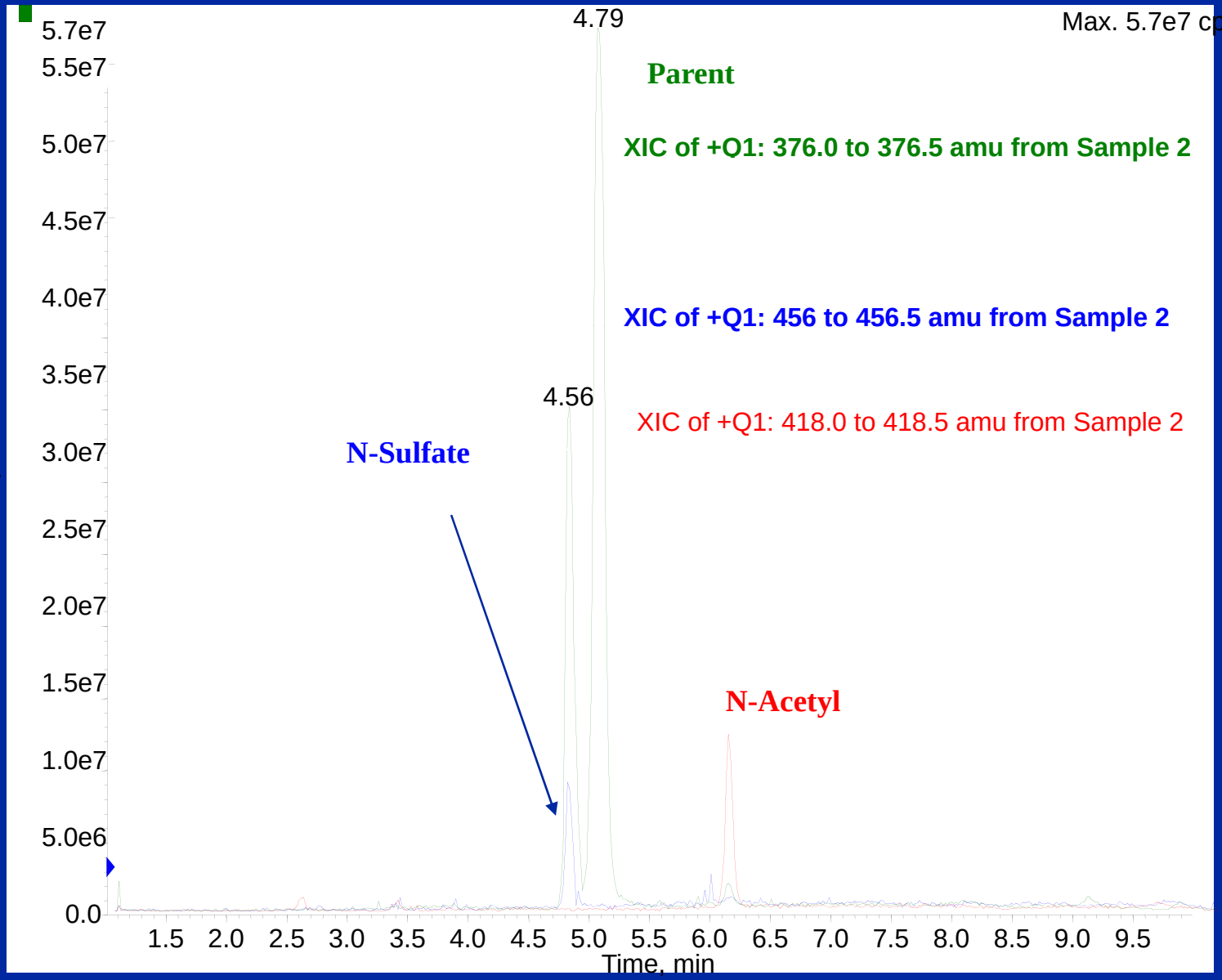
- Role of Chromatography for Bioanalysis Changed
- Baseline Separation is Not Required Due to High Selectivity of MRM
  - LC Remains an Important Method for
    - Concentration of Analytes During Injection or
    - Minimize Matrix Suppression
- Only in Selected Cases Separation of Analytes is Required
    - Low Level of Detection
    - Phase II Metabolites

Phase II Metabolites Interference





Phase II Metabolites Interference



Sample Preparation Techniques in Bioanalysis

- **Goal**
  - Increase Sensitivity and Selectivity
  - Minimize Ion Suppression
  - Concentrate Sample
- **Most Popular Techniques**
  - Protein Precipitation (PPT)
  - Liquid-Liquid Extraction (LLE)
  - Solid Phase Extraction (SPE)
    - On line
    - Off line

Case Study

Scope

- Develop Bioanalytical Method for Quantification of Urinary Phthalate Metabolites

Assay Requirements

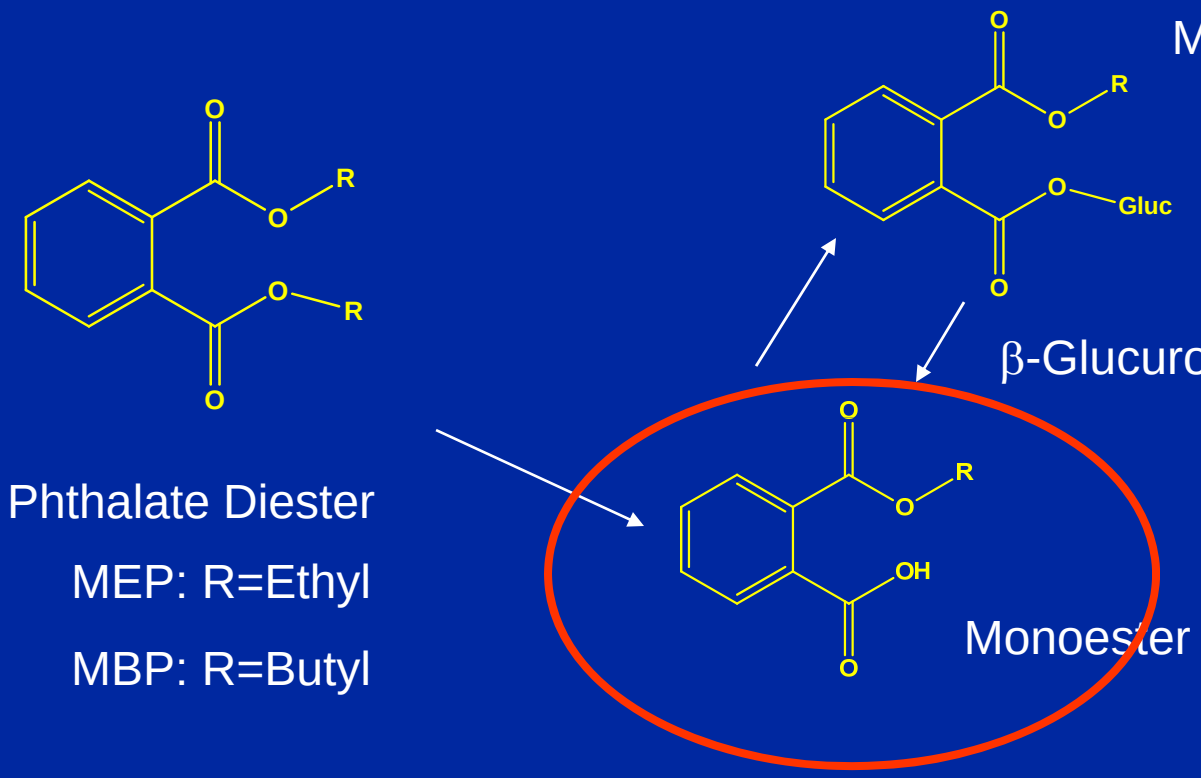
- Urine
- Two Analytes
  - Total Monoethyl Phthalate (MEP) (Free and Glucuronidated)
  - Total Monobutyl Phthalate (MBP) (Free and Glucuronidated)
- Stable Isotope Labeled Internal Standards
- MEP and MBP Glucuronides are not available
- Lower Limit of Quantification LLOQ=5 ng/mL
- LC-MS/MS

Background

- People are routinely exposed to phthalates because of their wide use as industrial solvents and plasticizers
- Diethyl and dibutyl phthalates are widely used in perfumes, cologne, soap, shampoo, nail polish and cosmetics
- Some phthalates and their metabolites are responsible for reproductive and developmental toxicities in animals
- Phthalate monoesters and their respective metabolites used as urinary or serum biomarkers of phthalate exposure

Phthalate Metabolism

- In Humans, Phthalate diesters are metabolized to their respective monoesters which are partially glucuronidated
- Excreted through Urine and Feces



Challenges of the method

- Develop Hydrolysis procedure to determine total monoester amount
  - Free monoester+congugated monoester
- Possible Matrix effect
- Needs to be pre-concentrated and purified before injection
  - Off line SPE
  - On-line SPE

On-line SPE Procedure Implemented

Silica based monolithic column for sample pre-concentration/purification

Column: Chromolith Flash RP-18e column (4.5x25mm, Merck KGaA, Germany)

Back-flush to Analytical column – MS

Column: Eclipse Plus, Phenyl Hexyl 2.1x150mm, 5um (Agilent)

Mobile Phase:

A (0.1% Acetic Acid in water);

B (0.1% Acetic Acid in Acetonitrile)

Adopted from literature: Kato K. et al. Anal. Chem., 2005, 77

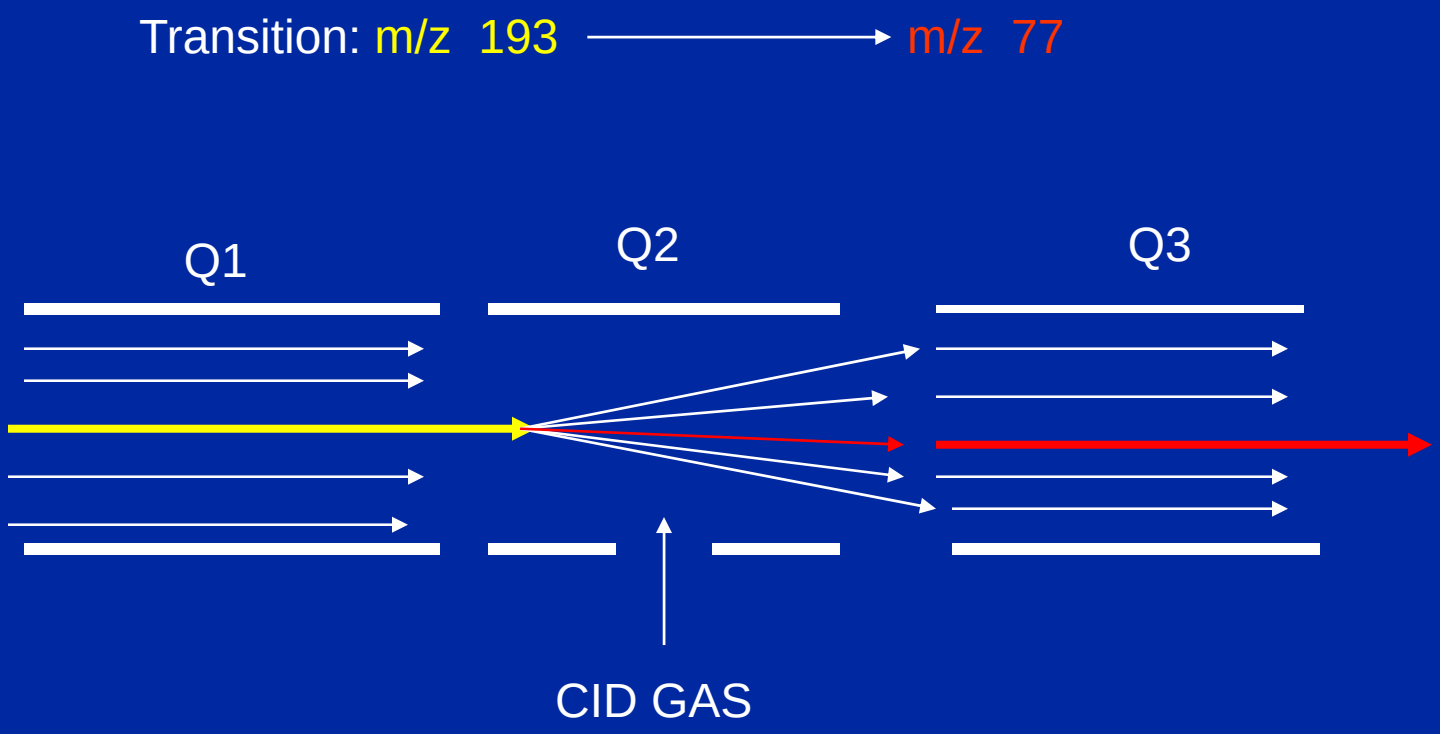
LC-MS Conditions

- Mass spectrometer: Triple Quadrupole (Micromass Quattro)
- Ionization: ESI-
- Mode: MRM

- Injection Volume
  - 50uL

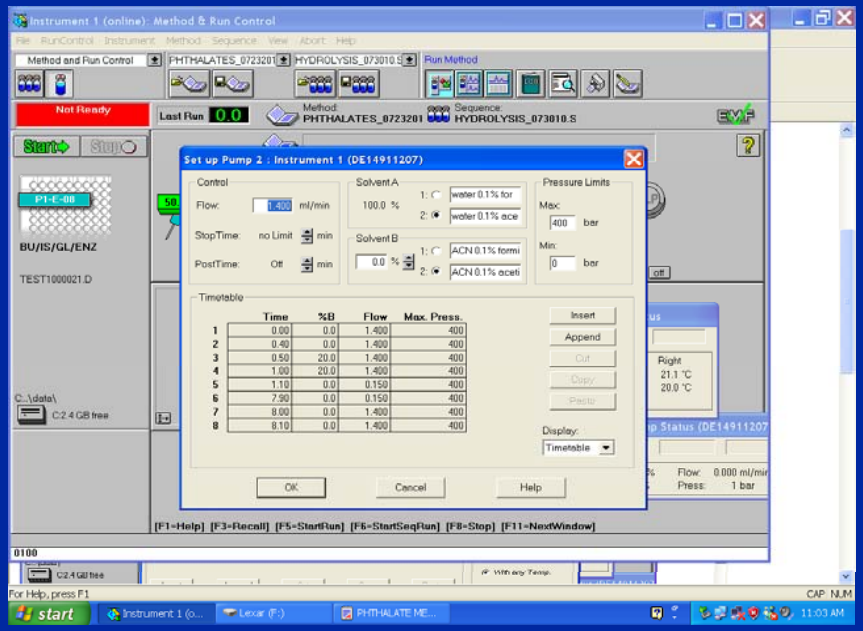
Compound	Transition monitored	Cone /Collision Energy	Retention Time (min)
MEP	192.7→76.9	22/22	~3.43
MBP	220.9→76.9	22/18	~4.22
IS MEP	197.0→78.9	22/19	~3.43
IS MBP	224.7→78.9	25/20	~4.22

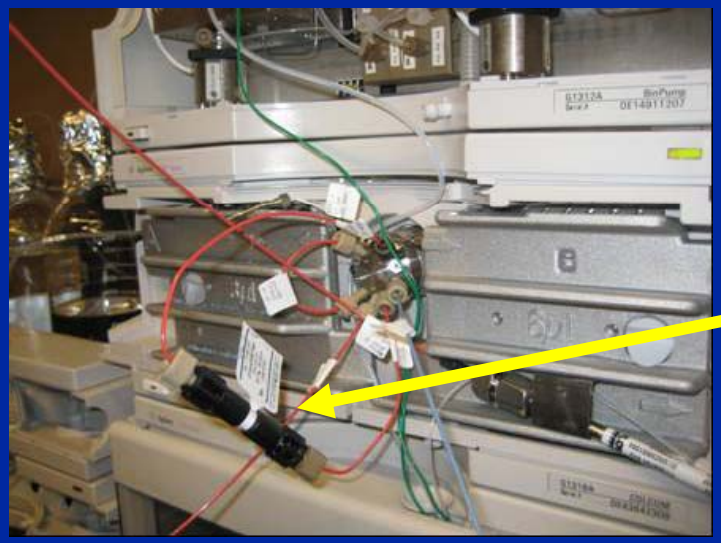
Selected Reaction Monitoring (MRM)



ON-LINE SPE METHOD

- On-line SPE for pre-concentration and purification
  - Column: Chromolith Flash RP-18e column (4.5x25mm, Merck KGaA, Germany)
  - Mobile Phase:
    - A (0.1% Acetic Acid in water);
    - B (0.1% Acetic Acid in Acetonitrile)





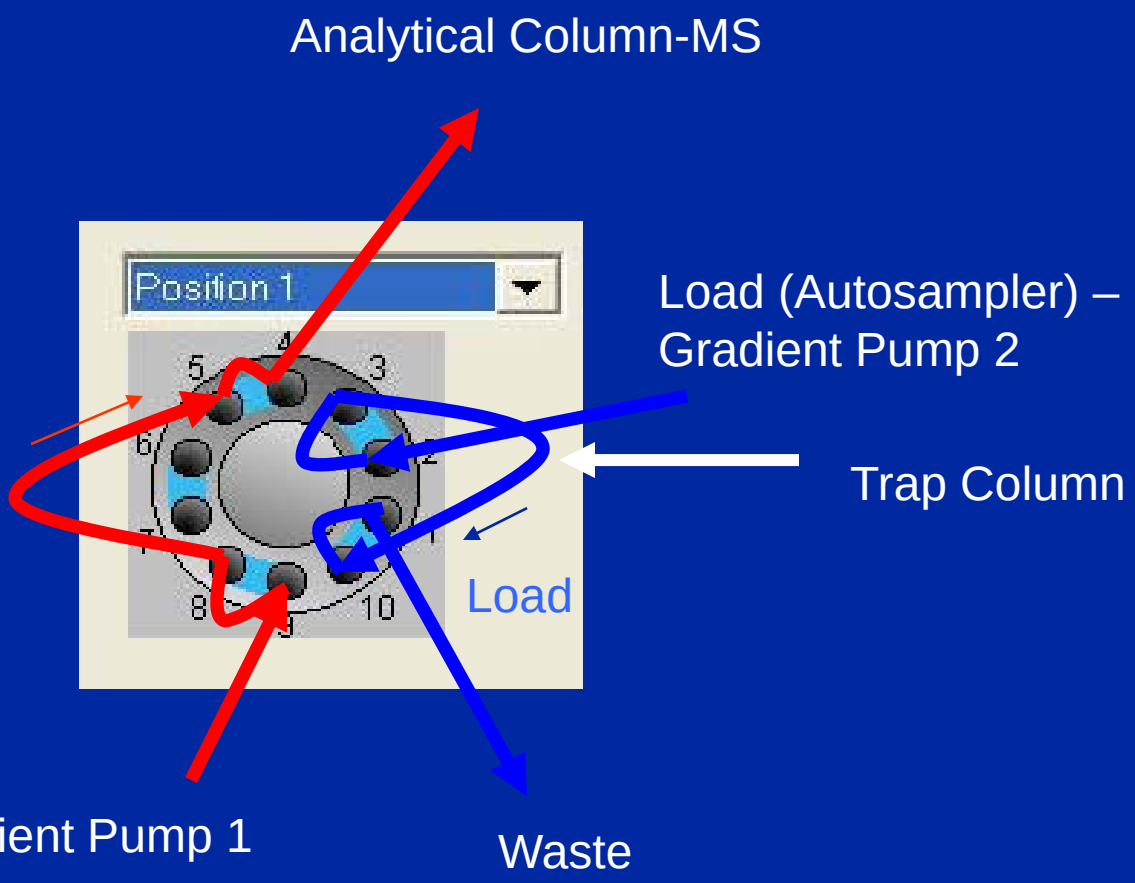
SPE Column

SWITCHING VALVE POSITION 1

Inject (50 -100uL)

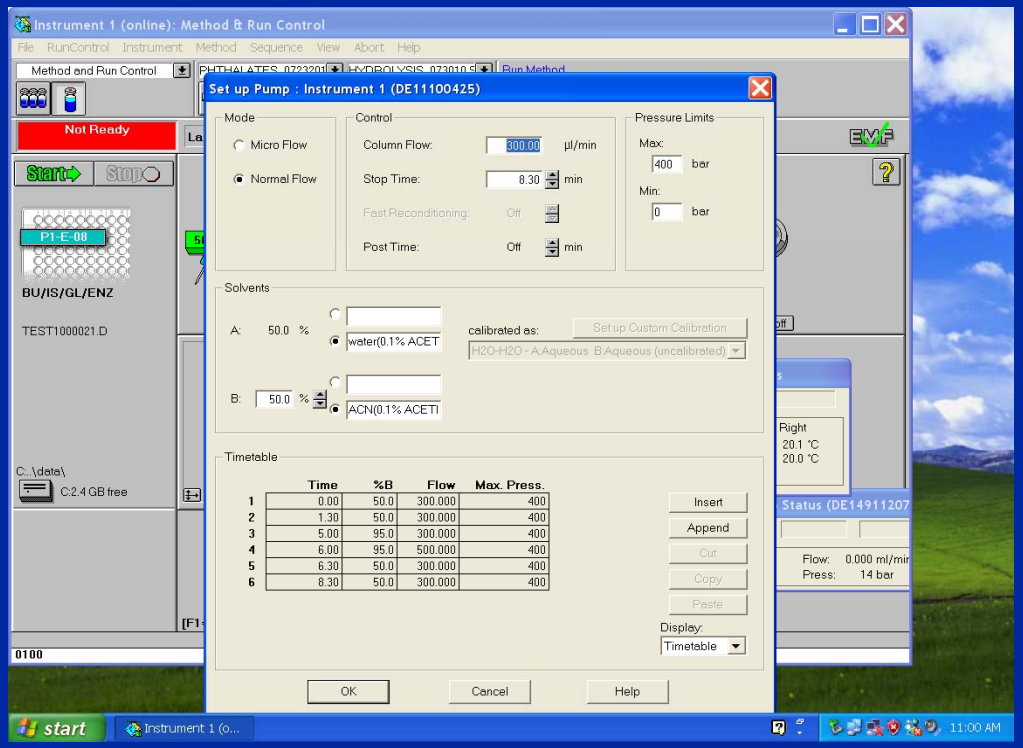
Load on Trap Column while Analytical column is Equilibrating

1 Minute in Position 1



HPLC ANALYTICAL METHOD

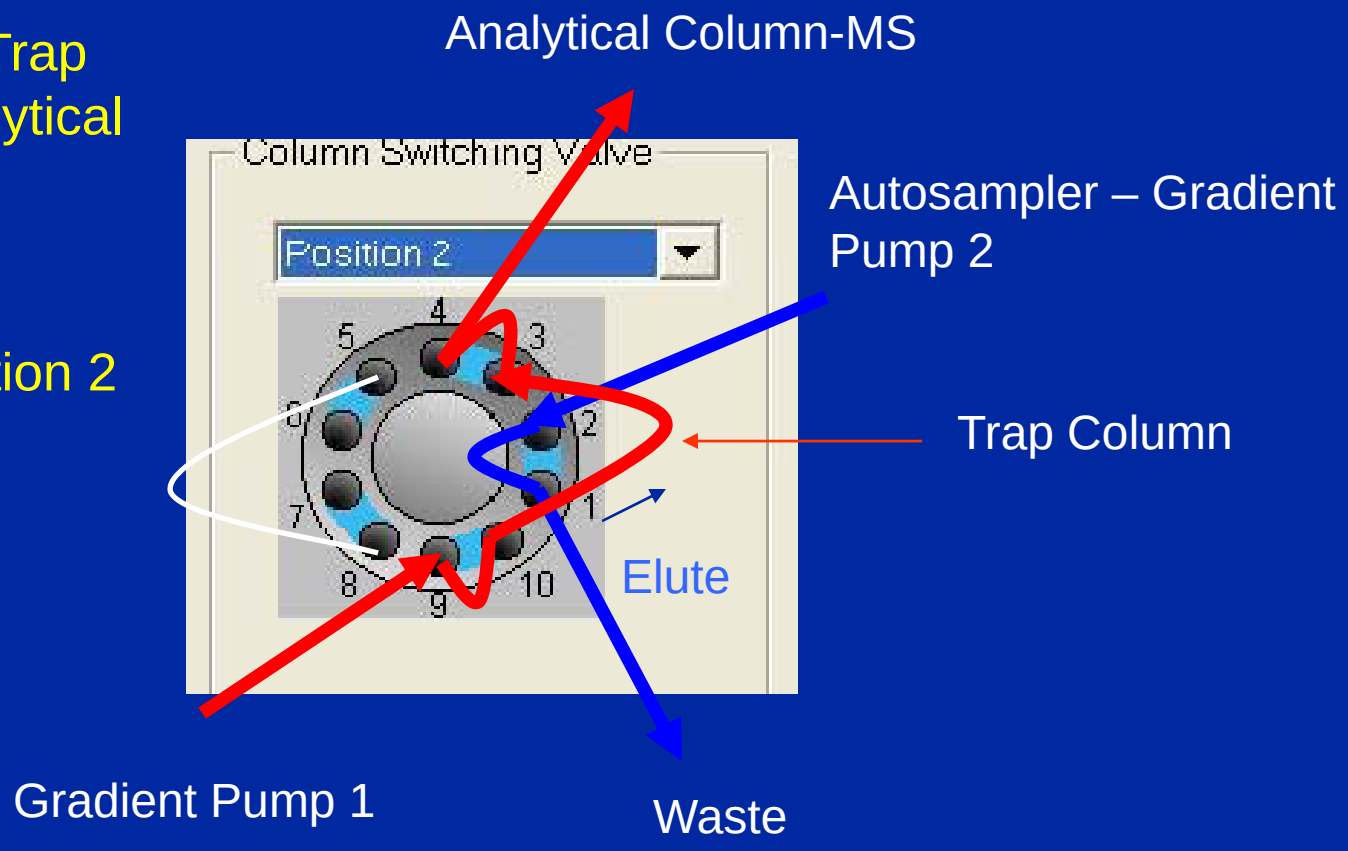
- Column
  - Eclipse Plus, Phenyl Hexyl 2.1x150mm, 5um (Agilent)
  - Mobile Phase:
    - A (0.1% Acetic Acid in water);
    - B (0.1% Acetic Acid in Acetonitrile)



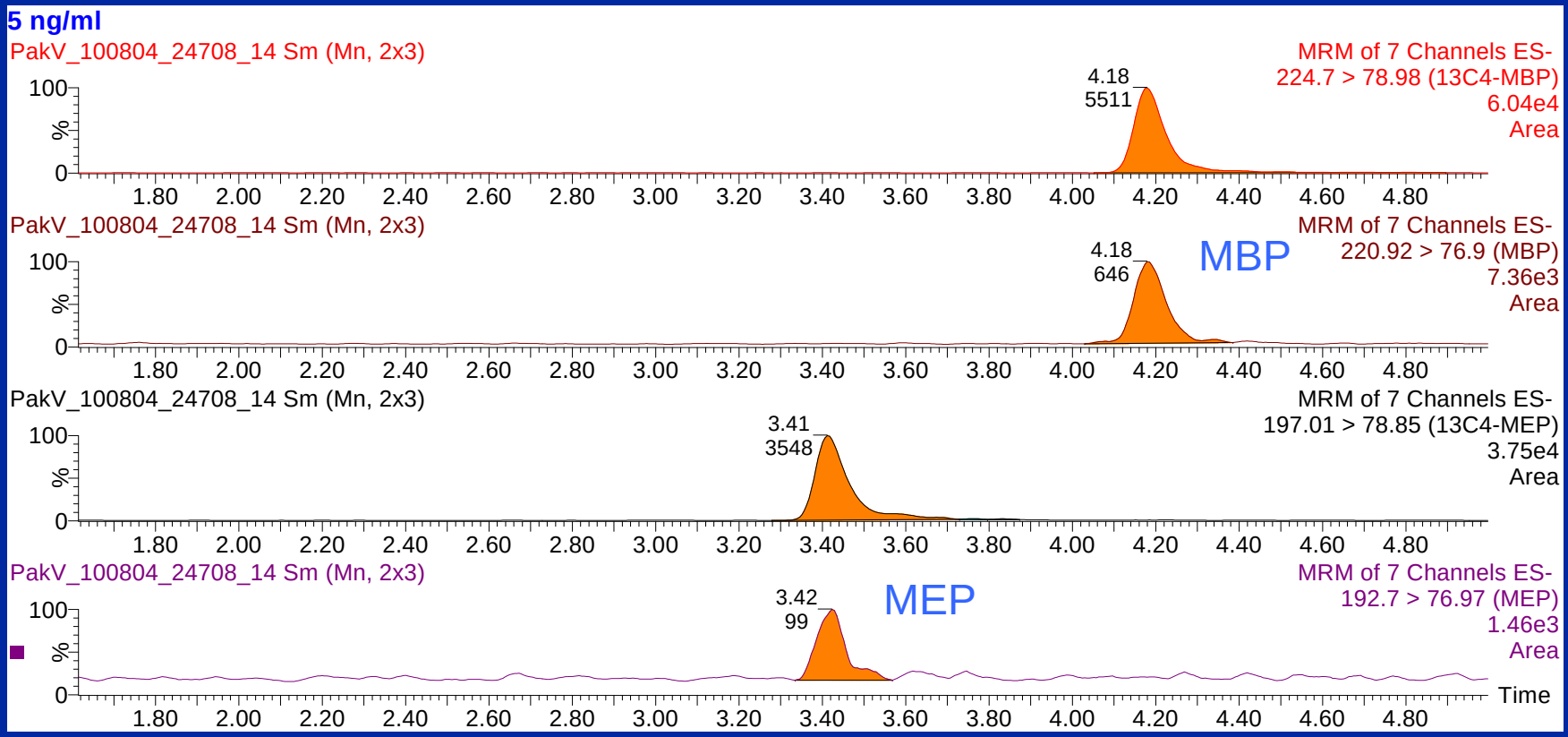
Switching Valve POSITION 2

Back Flush from Trap Column onto Analytical Column to Mass spectrometer

7 minutes in Position 2

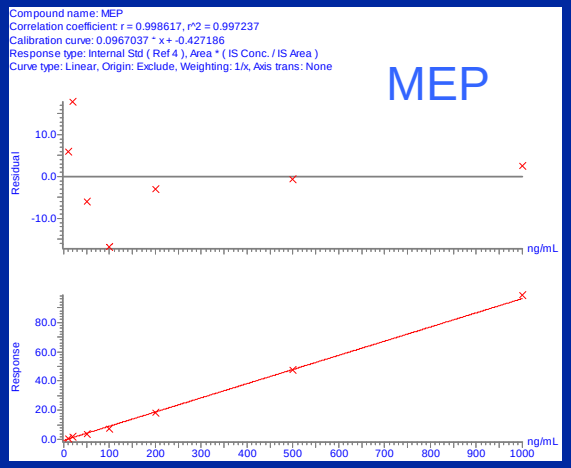


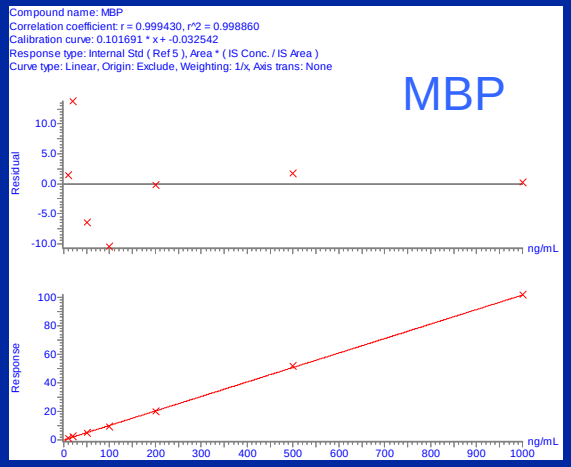
5ng/mL Calibration Solution



MEP/MBP Calibration Curves

- 5ng/mL - 1000ng/mL
- Injection volume: 50uL
- Some Amounts of MEP were Detected in Blank Urine (~30 – 100 ng/mL)
- Some Amounts of MBP wereDetected in Blank Urine (~5 – 40 ng/mL)





Case Study : Summary

- **On-line SPE Method Implemented**
- 122 Urine Samples Analyzed in 3 Runs
- Wide Range of Concentrations Detected Confirming Human Exposure to Phthalates
  - MEP Concentration range: 5 – 2600 ng/mL
  - MBP Concentration range: <5 – 200 ng/mL
- **This Method Could be Used for Other Studies which Require Sample Pre-concentration and Purification**

Conclusions

- **Bioanalysis is an Integral Part of PK/TK/PD Characterization of New Compounds from Discovery Through Various Stages of Drug Development Leading to the Market**
- **LC-MS Triple Quadrupoles are Instruments of Choice for Quantification**
- **Method Development is Challenging and Exciting Part of the Drug Development Process Since Unique Compounds are Used and New Targets Investigated**
- **The Choice of Instrumentation and its Characteristics is Important, but it is Essential to Understand Chemical Properties of the Compounds to be Successful!**

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