【S-7】

Full validation of high-throughput bioanalytical method for the new drug in plasma by LC-MS/MS and its applicability to toxicokinetic analysis

Sang Beom Han

College of Pharmacy, Chung-Ang University

Modern drug discovery requires rapid pharmacokinetic evaluation of chemically diverse compounds for early candidate selection. This demands the development of analytical methods that offer high-throughput of samples. Naturally, liquid chromatography/tandem mass spectrometry (LC-MS/MS) is choice of the analytical method because of its superior sensitivity and selectivity. As a result of the short analysis time(typically 3-5 min) by LC-MS/MS, sample preparation has become the rate- determining step in the whole analytical cycle. Consequently tremendous efforts are being made to speed up and automate this step.

In a typical automated 96-well SPE(solid-phase extraction) procedure, plasma samples are transferred to the 96-well SPE plate, internal standard and aqueous buffer solutions are added and then vacuum is applied using the robotic liquid handling system. It takes only 20-90 min to process 96 samples by automated SPE and the analyst is physically occupied for only approximately 10 min.

Recently, the ultra-high flow rate liquid chromatography(turbulent-flow chromatography) has sparked a huge interest for rapid and direct quantitation of drugs in plasma. There is no sample preparation except for sample aliquotting, internal standard addition and centrifugation. This type of analysis is achieved by using a small diameter column with a large particle size(30-50㎛) and a high flow rate, typically between 3-5 ㎖/min.

Silica-based monolithic HPLC columns contain a novel chromatographic support in which the traditional particulate packing has been replaced with a single, continuous network (monolith) of porous silica. The main advantage of such a network is decreased backpressure due to macropores (2 μm) throughout the network. This allows high flow rates, and hence fast analyses that are unattainable with traditional particulate columns.

The reduction of particle diameter in HPLC results in increased column efficiency. The

use of small particles (<2 um), however, requires pressures beyond the traditional 6,000 psi of conventional pumping devices. Instrumental development in recent years has resulted in pumping devices capable of handling the requirements of columns packed with small particles.

The staggered parallel HPLC system consists of four fully independent binary HPLC pumps, a modified autosampler, and a series of switching and selector valves all controlled by a single computer program. The system improves sample throughput without sacrificing chromatographic separation or data quality. Sample throughput can be increased nearly four-fold without requiring significant changes in current analytical procedures.

The process of Bioanalytical Method Validation is required by the FDA to assess and verify the performance of a chromatographic method prior to its application in sample analysis. The validation should address the selectivity, linearity, accuracy, precision and stability of the method. This presentation will provide an overview of the work required to accomplish a full validation and show how a chromatographic method is suitable for toxicokinetic sample analysis. A liquid chromatography/tandem mass spectrometry (LC-MS/MS) method developed to quantitate drug levels in dog plasma will be used as an example of the process.

목차

1. **LC-MS/MS introduction**
2. High-throughput system
   - Sample preparation
   - *Analytical system*
3. Bioanalytical method validation
4. Case report of TK study

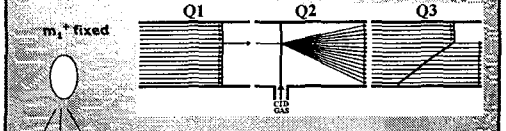
Challenges in High-Throughput Analysis (LC-MS/MS)

- Speed : Analyse more samples/unit time
  - increase in sample throughput
  - ✓ Extraction : automation, on-line extraction
  - ✓ LC separation : fast LC
  - ✓ Equilibration : high flow
- Sensitivity : LOD and LOQ to meet regulatory requirements
  - ✓ Compatible mobile phase : *less salts & more organic*
  - ✓ Matrix suppression : eliminate it
- Selectivity : increased specificity (MS/MS) provides increased productivity
  - ✓ Co-eluting suppression : chromatographic resolution
  - ✓ Matrix effect : minimize interfering components

Triple Quad Basic Scan Modes

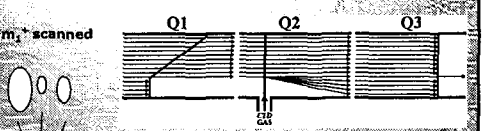
- **Q1 full scan**
- **Q3 full scan**
- **Selected ion monitoring (SIM)**
- **Product ion scan**
- **Precursor ion scan**
- **Constant neutral loss scan**
- **Multiple reaction monitoring (MRM)**

MS/MS Product Ion Scan



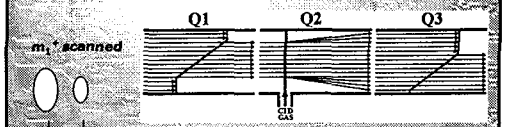
- After identification, the precursor ion is sent into the collision cell and fragmented by CID
- Q1 is fixed, Q3 sweeps a given mass range
- Used for structural elucidation
- First step to developing quantitative method

MS/MS Precursor Ion Scan



- Q1 sweeps a given mass range, Q3 is fixed
- Used to determine the "origin" of particular product ion(s) created in the collision cell
- Frequently used for drug metabolite identification

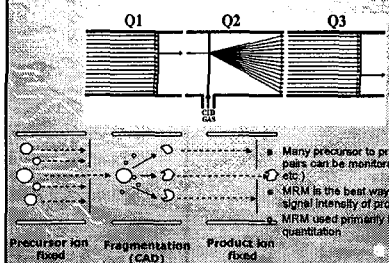
Constant Neutral Loss Scan



- Q1 & Q3 both scan a given mass range but with a constant difference between ranges scanned
- Spectrum indicates which ions lose a neutral species equal to Q1 - Q3 difference
- Complement to Precursor Ion Scan

7

Multiple Reaction Monitoring (MRM)



- Many precursor to product ion pairs can be monitored (A-B, A'-B', etc.)
- MRM is the best way to maximize signal intensity of product ions
- MRM used primarily for quantitation

8

목차

1. LC-MS/MS introduction
2. **High-throughput system**
   - **Sample preparation**
   - **Analytical system**
3. Bioanalytical method validation
4. Case report of TK study

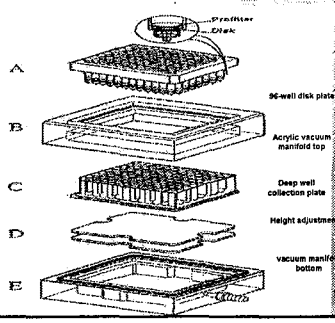
Common Sample preparation limitations

- Liquid/liquid extraction is:
  - Lengthy and complicated
  - Expensive (cost of solvent, solvent elimination...)
  - Less selective due to a solubility Differentiation versus Functional Group Differentiation
  - Less flexible due to the limited number of possible "pairs of phase"
- Protein precipitation is:
  - Less selective by not removing interferences
  - Not easy to automate

SPE might help !

10

96-Well Solid Phase Extraction Plate - vacuum manifold system



11

Comparison in consumable cost between disk cartridge and

96 well plate for every 100 samples[a]

Disk cartridge		96-well plate	
Cartridge	$154	Plate	$209
Receiving Tube	$41	Receiving Plate	$4.7
Injection vials	$60		
Pipette Tips	$10		
Total	$265	Total	$213

[a] Based on invoices incurred from January to August 1997.

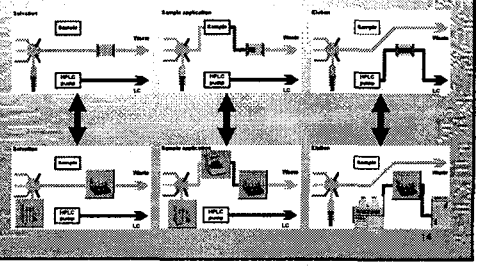
12

Sample Preparation

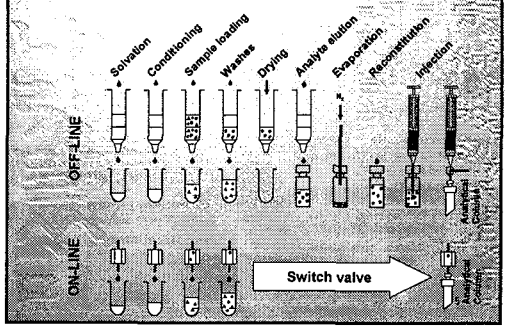
- Off line SPE extraction:
  not automatic
  Multiple steps
  Time consuming
- Risk of loss of product
  during the elution process
  degradation during the evaporation process
- Dangerous sample handling

on line Solid Phase

On-line SPE의 원리



Advantages of on line SPE



Laminar vs. Turbulent Flow

Reynolds Number

$$Re = \frac{\text{Interial Forces}}{\text{Viscous Forces}}$$

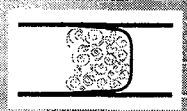
$$Re = \frac{\mu D}{\eta}$$

where :
μ = linear velocity
D = Dia. of pipe
η = kinematic viscosity

Laminar

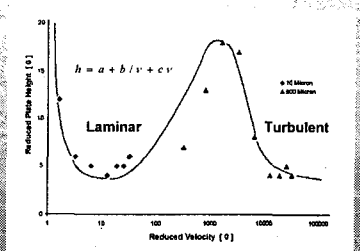
Re ≤ 2000

Turbulent



Re ≥ 3000

Van Deemter Curve:
Laminar vs. Turbulent Flow Chromatography

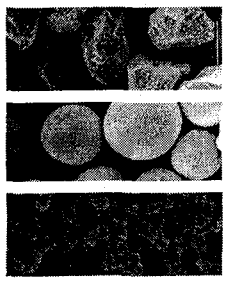


Plasma sample analysis

- Plasma sample
- Precipitate proteins
- Centrifuge
- Transfer organic phase
- Evaporate
- Dissolve mobile phase
- Centrifuge
- Inject onto column
- detection

- Plasma sample
- Centrifuge
- Inject plasma
- detection

Chromatography - The Stationary Phases



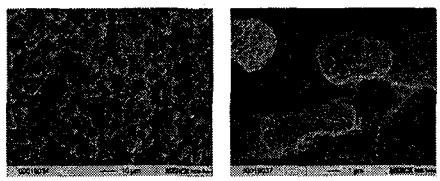
irregular

spherical

monolithic

Defined bimodal pore structure

SEM of a cross section of a monolithic silica rod

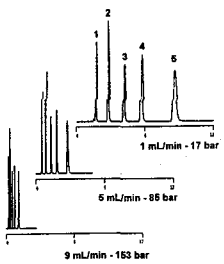


Macropores: 2 µm

Mesopores: 13 nm

Total porosity 92% (65 - 75% for conventional particulate columns)

Speed and Quality in Practice

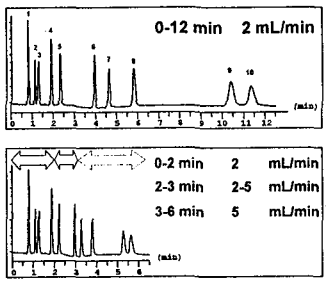


Monolithic column RP-18
(100 mm x 4.6 mm)

ß-blockers

Mobile	Acetonitrile/ 0.1% TFA in water
Phase	(20/80; v/v)
Flow rate	1-9 ml/min
Detection	222 nm
Sample	1. Atenolol 2. Pindolol 3. Metoprolol 4. Celiprolol 5. Bisoprolol

Applying Special Techniques



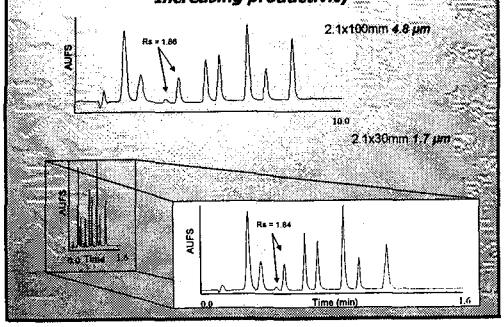
Flow Gradient

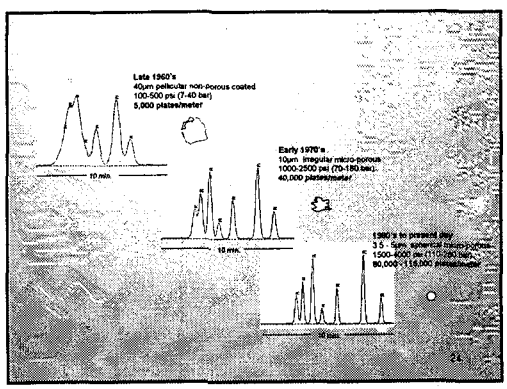
Separation of 10 β-blocking drugs

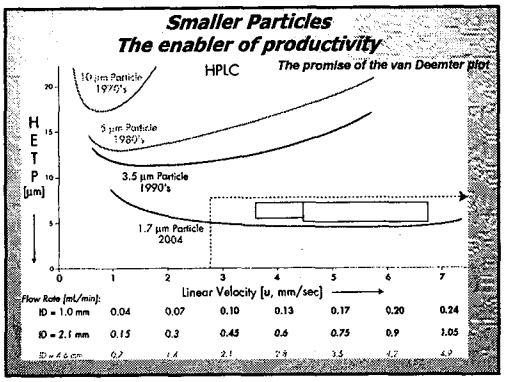
Monolithic column
RP-18
(100 X 4.6 mm)

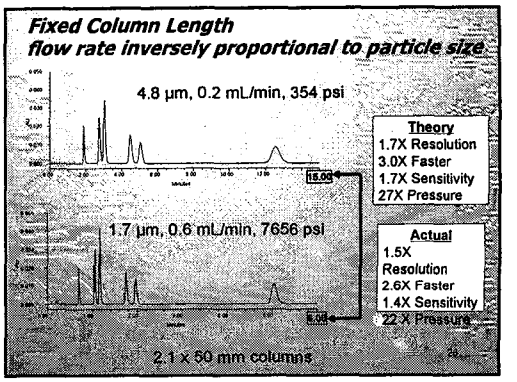
Particle Size Evolution

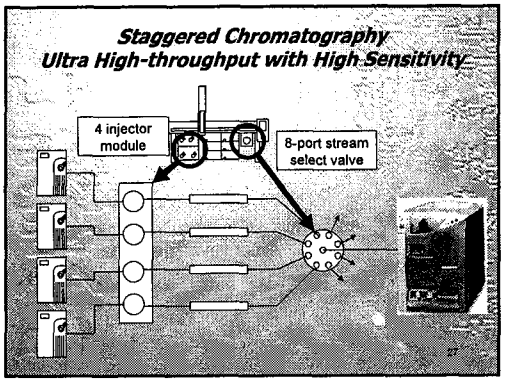
Increasing productivity

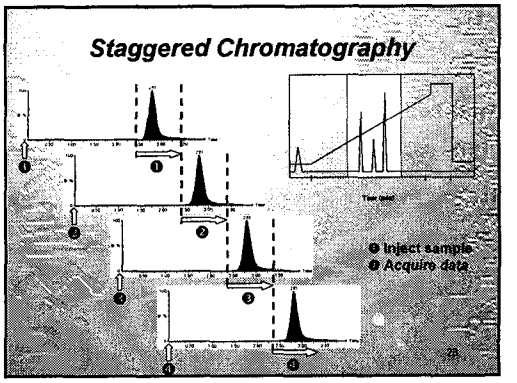


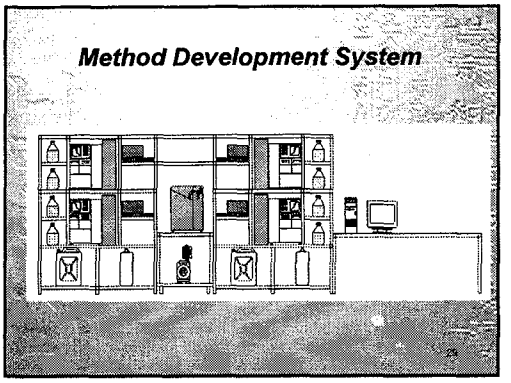


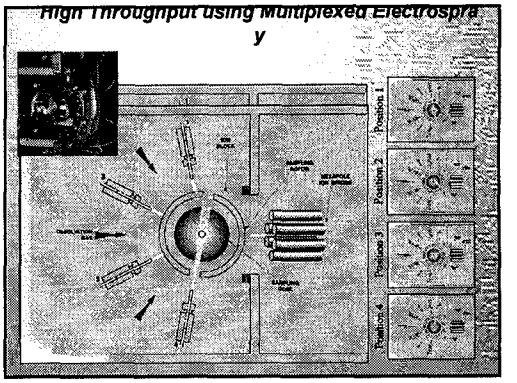

Smaller Particles
The enabler of productivity
HPLC
The promise of the van Deemter plot
10 µm Particle 1970's
5 µm Particle 1980's
3.5 µm Particle 1990's
1.7 µm Particle 2004
HETP [µm]
Linear Velocity [u, mm/sec]
Flow Rate [mL/min]:
ID = 1.0 mm 0.04 0.07 0.10 0.13 0.17 0.20 0.24
ID = 2.1 mm 0.15 0.3 0.45 0.6 0.75 0.9 1.05

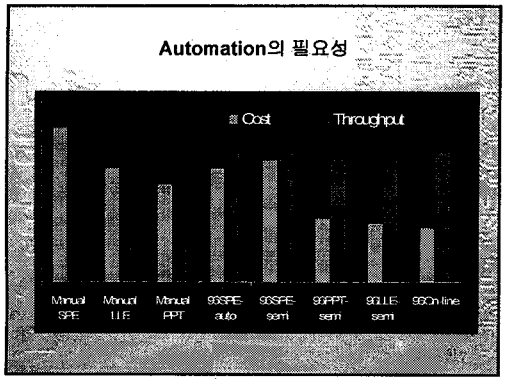

Fixed Column Length
flow rate inversely proportional to particle size
4.8 µm, 0.2 mL/min, 354 psi
1.7 µm, 0.6 mL/min, 7656 psi
2.1 x 50 mm columns
Theory
1.7X Resolution
3.0X Faster
1.7X Sensitivity
27X Pressure
Actual
1.5X Resolution
2.6X Faster
1.4X Sensitivity
22 X Pressure

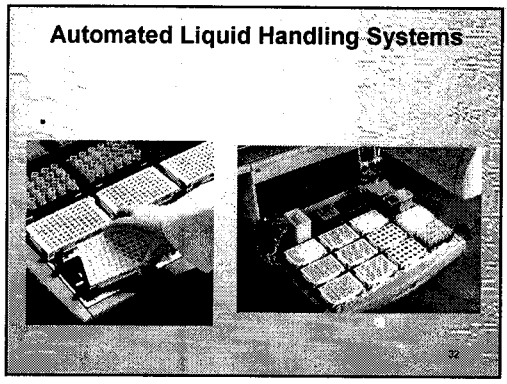

Staggered Chromatography
Ultra High-throughput with High Sensitivity
4 injector module
8-port stream select valve

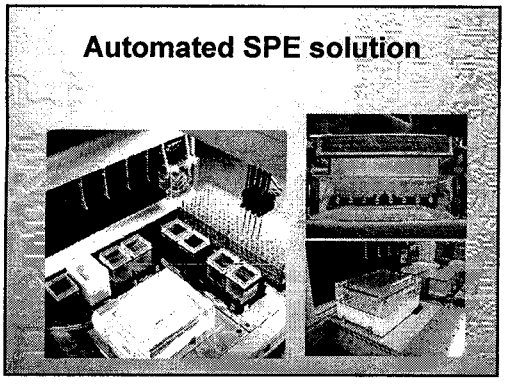

Staggered Chromatography
Inject sample
Acquire data

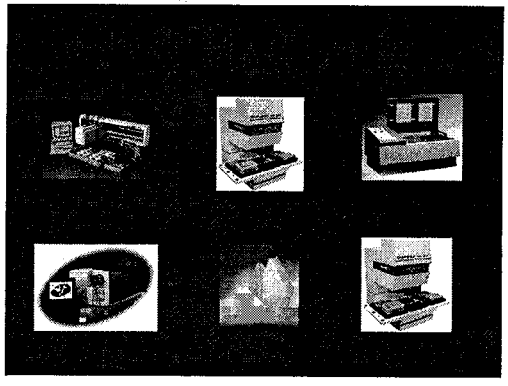

Method Development System

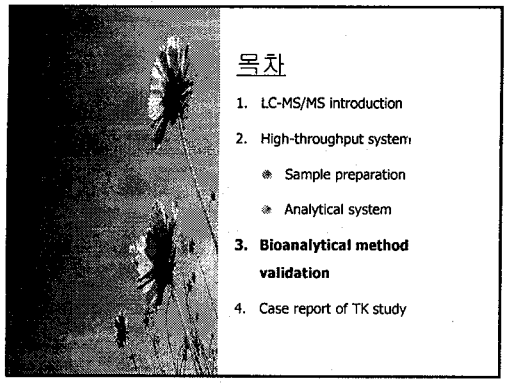

High Throughput using Multiplexed Electrospra y
Position 1
Position 2
Position 3
Position 4

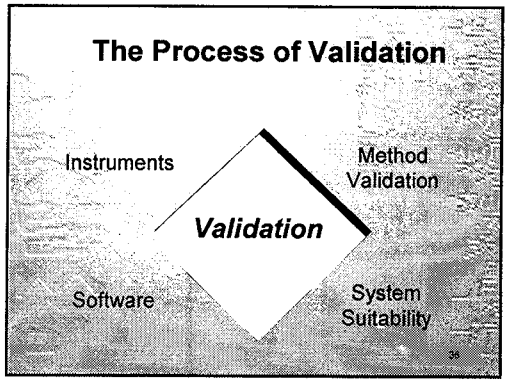

Automation의 필요성
Cost
Throughput
Manual SPE
Manual L.L.E
Manual PPT
96SPE-auto
96SPE-semi
96PPT-semi
96L.L.E-semi
96On-line


Automated Liquid Handling Systems


Automated SPE solution




목차
1. LC-MS/MS introduction
2. High-throughput system
Sample preparation
Analytical system
3. Bioanalytical method validation
4. Case report of TK study


The Process of Validation
Instruments
Method Validation
Validation
Software
System Suitability

Why Method Validation is Important?

- The purpose of analytical measurement is to get consistent, reliable and accurate data.
  - Incorrect measurement results can lead to tremendous costs.
- Equal importance for those working in a regulated and in an accredited environment.
  - U.S. FDA, EMEA, EPA, AOAC, ISO
  - 각국의 규제 및 규정들

37

What is the purpose of Analytical Method Validation?

- Identification of Sources and Quantitation of Potential errors
- Determination if Method is Acceptable for Intended Use
- Establish Proof that a Method Can be Used for Decision Making
- Satisfy FDA or EPA or ISO... Requirements

38

What are the Benefits of Analytical Method Validation?

- Regulatory Compliance
- Assurance that Test data from Methods are Reliable
- Establishment that Test Data are Reproducible, Accurate, Specificity....

39

Validation is an Old Concept But There are Many Problems

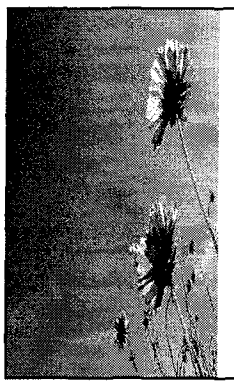
- Lack of documented procedures and documented validation results
- Sampling or Sample preparation step contribute to overall error
- Accessories and materials used for equipment qualification are not qualified
- Limits for Operational Qualification
- Lack of software validation and computer system validation
- Qualification and validation are done at just one particular point in time
- Adaptation of acceptance criteria for qualification of new system

40

Basic guideline of bioanalytical method validation

1. Selectivity/Specificity
2. Linearity
3. Accuracy and Precision
4. Detection limit (LOD)/ Quantitation limit (LOQ)
5. Dilutions
6. QC Samples
7. Stability:
   1) Freeze-thaw stability
   2) Short-term stability
   3) Long-term stability
   4) Stock solution stability
   5) Autosampler stability
8. System Suitability

41



목차

1. LC-MS/MS introduction
2. High-throughput system
   - Sample preparation
   - Analytical system
3. Bioanalytical method validation
4. **Case report of TK study**

Case Report

비글견 혈장 중 LC-MS/MS를 이용한 XXXX-XX의

특성동태 분석을 위한 분석법 검증시험

43

Sample Preparation of XXXX-XX

70 μL Plasma

↓

Add 50 μL of IS (XX000A 50 ng/mL)
Add 10 μL of 1N NaOH,1 mL of TBME
Vortex mix for 30 sec
Centrifuge at 13,000 rpm for 5 min

↓

Upper layer

↓

Evaporated at 40 °C under N_2
Reconstituted of 200 μL of mobile phase
Centrifuge at 13,000 rpm for 5 min

↓

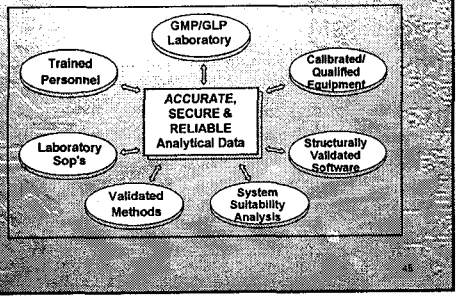
LC-MS/MS

LC-MS/MS analytical conditions

Instrument : API4000, Perkin-Elmer LC
CTC Pal autosampler
Column : Waters XTerra C18 column
(2.1 X 150 mm, 5.0 μm)
Column oven temperature : 40 °C
Mobile phase : 0.5 % trifluoroacetic acid /
Methanol = 20 / 80
Flow rate : 0.2 mL/min
Autosampler temperature : 10°C
Inject volume : 10 μL

44

Requirement for Data integrity



48