# CONTENTS

# Introduction of high sensitivity analysis using nano-flow LC/MS/MS

## — Evaluation of Method for Determination of Statin Drug and Metabolites in Plasma Using Nano-flow LC/MS/MS —

# Background

## *Needs for High Sensitivity Analysis*

- Reduction of volume of collected blood
  Too much volume may affect calculation of PK parameters.
  For animal care.

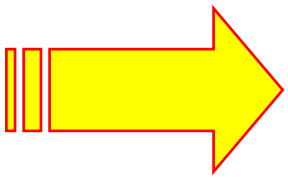
- Microdose clinical trials
  The volume for microdosing in human is defined to be less than 1/100$^{th}$ of the therapeutic dose, predicted from animals and in vitro models, and also a maximum of 100 µg.

- Incurred Sample Reanalysis (ISR)
  ISR is conducted to evaluate incurred sample reproducibility and accuracy.

# Features of nano-flow LC/MS/MS Analysis

- Lower sample diffusion achieved by use of thin LC capillary tubes (eg. ID 20 µm) and low flow rate.

- Spraying ultrafine charged droplets leads to rapid desolvation, as a result, efficiency of ionization is increased.



**High sensitivity analysis is expected**

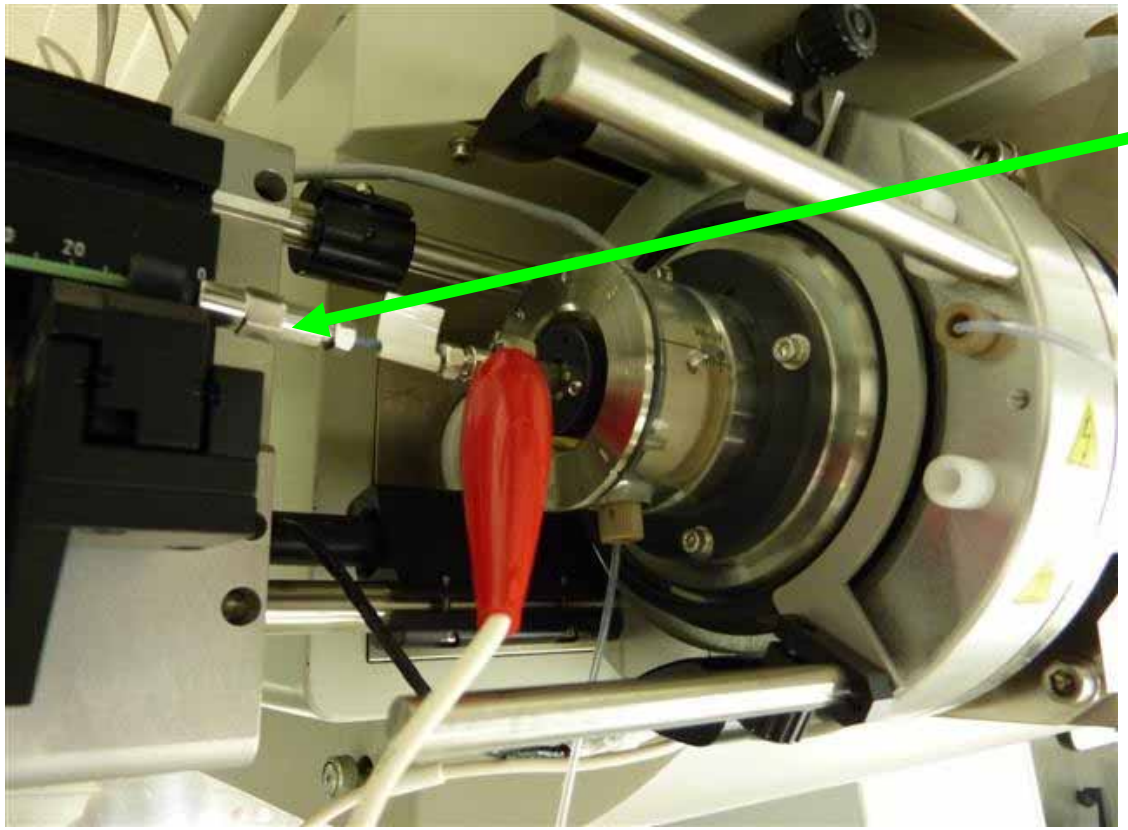
# Nano-flow LC-MS/MS system

**Nano-flow LC : Paradigm MS2 (Michrom)**
**MS/MS : 4000 Q TRAP(AB/MDS Sciex)**

# Nano-flow LC/MS/MS System (interface)

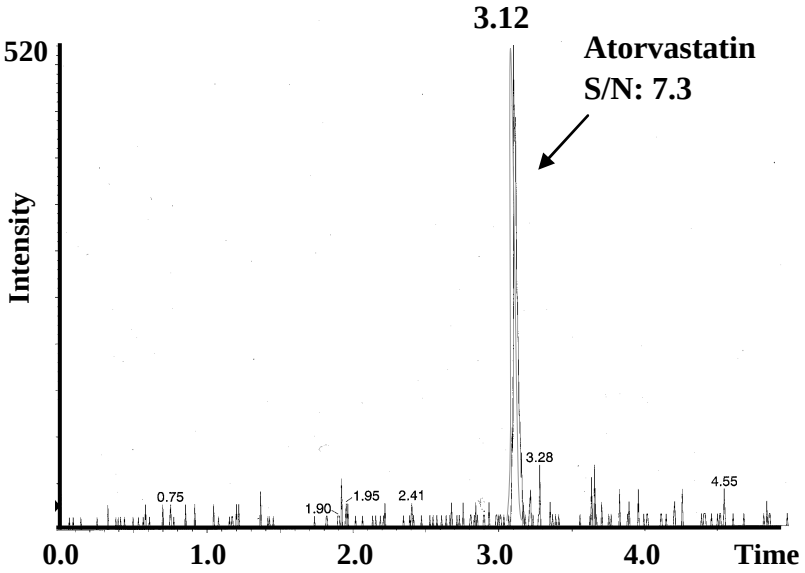
## H4 Nanospray Ion Source (Developed by AMR, Inc.)

**→Spray is constantly injected into MS/MS**



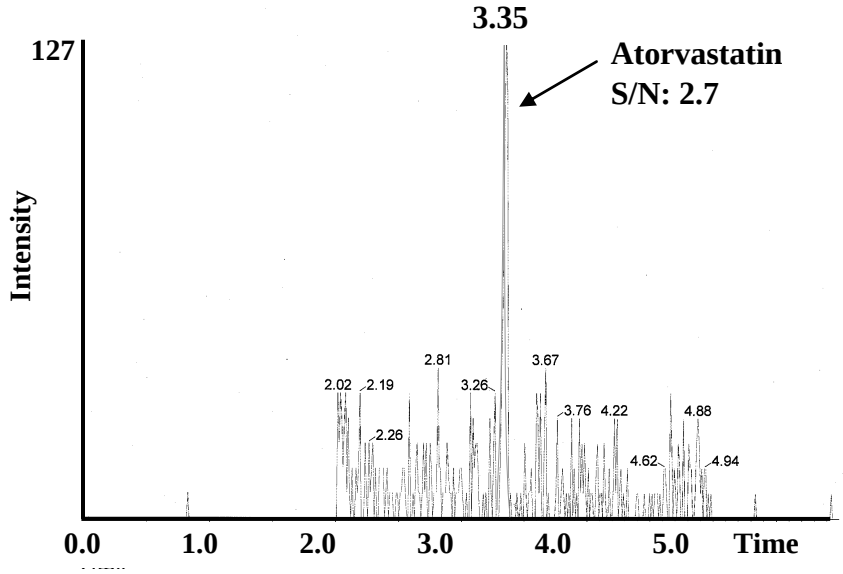
# Comparison to Semi-micro System

25 fg on column




4000QTRAP

50 fg on column




API5000

2.7-fold higher signal-to-noise ratio with ½ injection volume.

# Quantitative Analysis

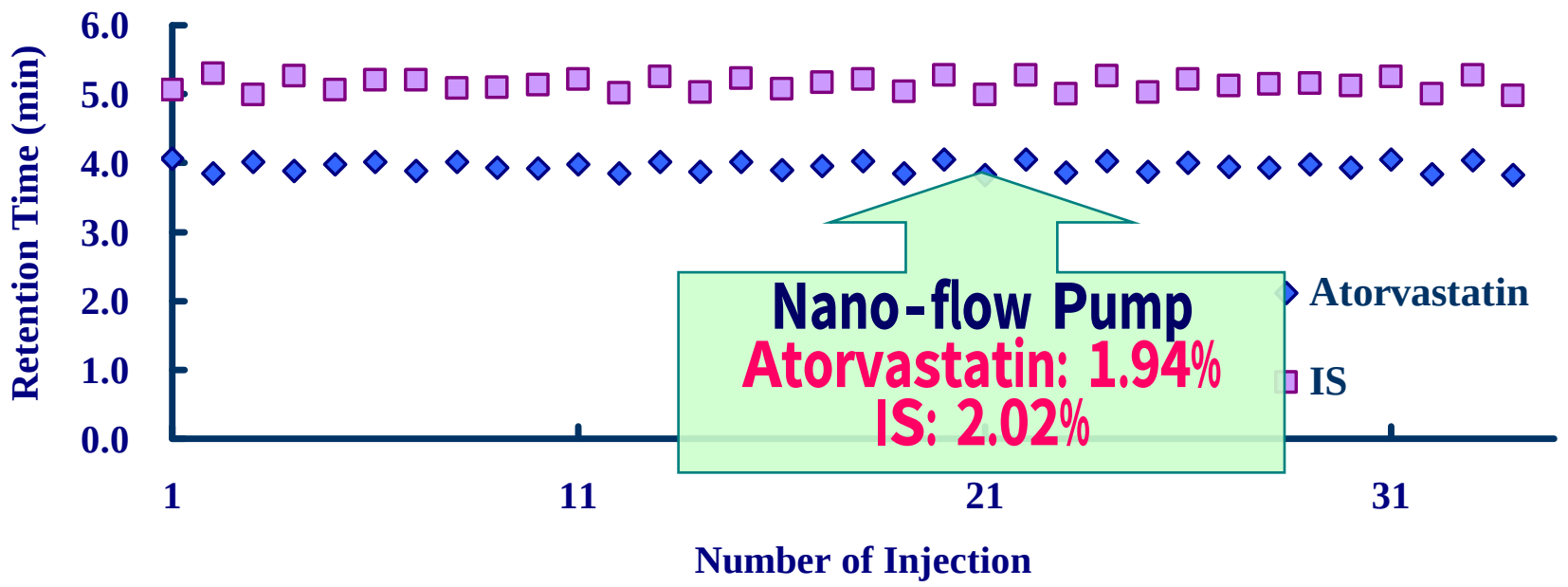
| Item | Conditions | Result |
|---|---|---|
| Calibration curve | — | 1.00 to 200 ng/mL |
| Selectivity | 6 individuals | No interfering peaks were observed on the chromatograms of analyte in rat plasma. |
| Matrix effect | LQC | 101.0 % (%Accuracy)<br>10.9 % (%CV) |
| Accuracy | LLQC | 111.0% |
| | LQC, MQC and HQC | 87.5 to 101.5 % |



Accepted

# Performance Test

## Variation in Retention Time



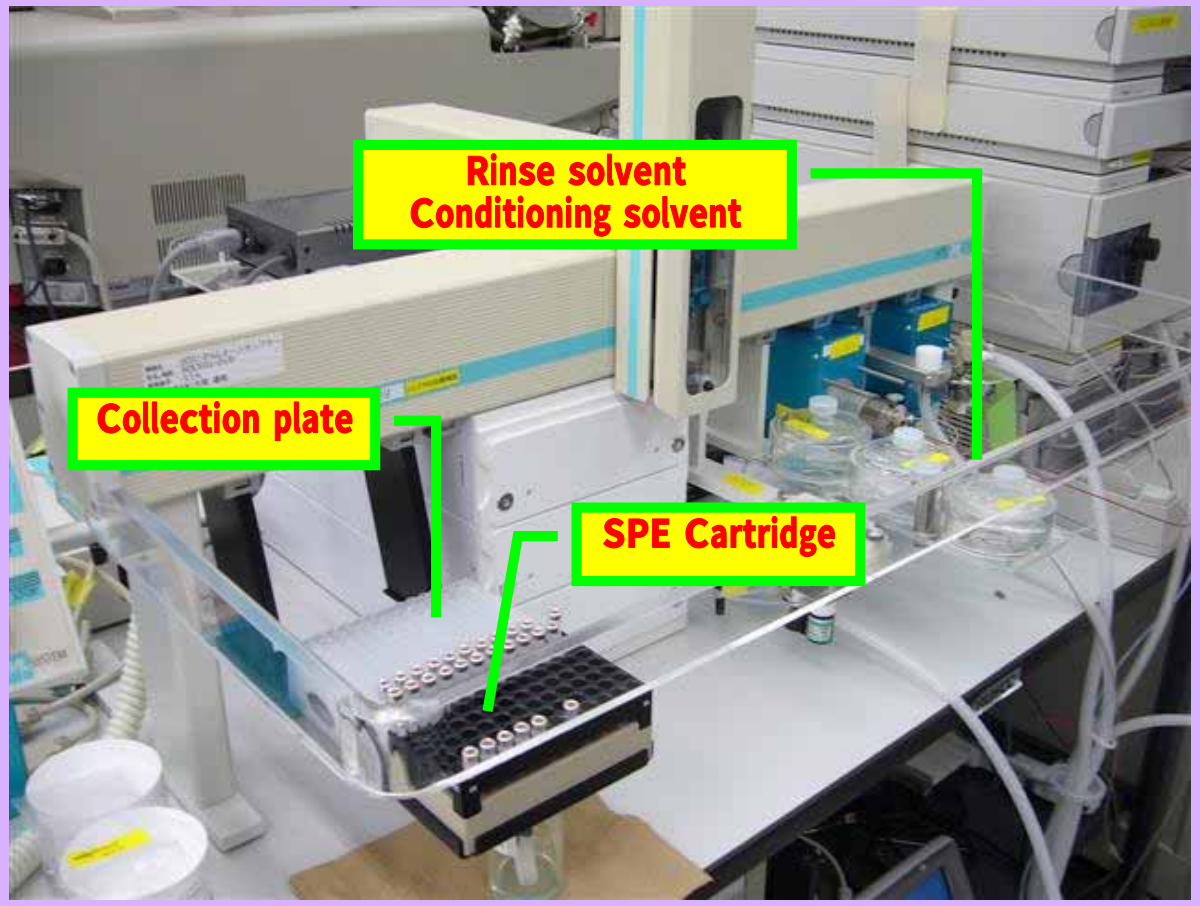
# Conclusion

- Signal-to-noise ratio obtained with nano-flow LC system was 2.7-fold higher that with semi-micro system.
- CV(%) of retention time of analyte was within 2%, as a result, nano flow pump was successfully controlled to provide constant flow.
- As a result of quantitative analysis, acceptable data were obtained in following parameters; Calibration curve, Selectivity, Matrix effects, and Accuracy and precision.

# Pragmatic approach to online SPE/LC/MS using CTC PAL
## – Method Development for Determination of Atorvastatin and Its Metabolites in human Plasma Using ITSP System–

# ITSP System

ITSP can automatize the process of conditioning, sample loading, elution and injection using PAL.



# Expected Benefit

1. **Efficient sample processing**
2. **Reduction of re-analysis caused by human error**
3. **Saving reagent consumption**
4. **Improvement of reproducibility in SPE and analysis**

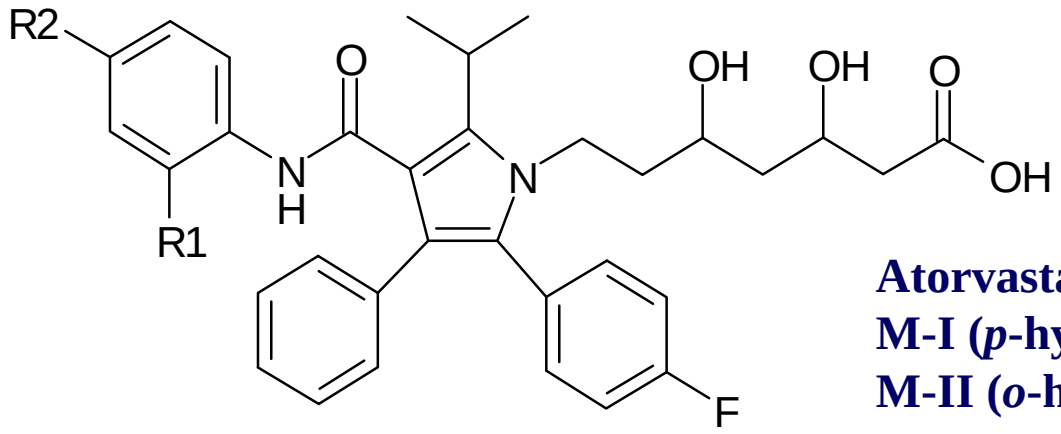
**etc**

ITSP system could have a high degree of availability for processing of huge number of samples.
(e.g. clinical examination, clinical research・・・ )

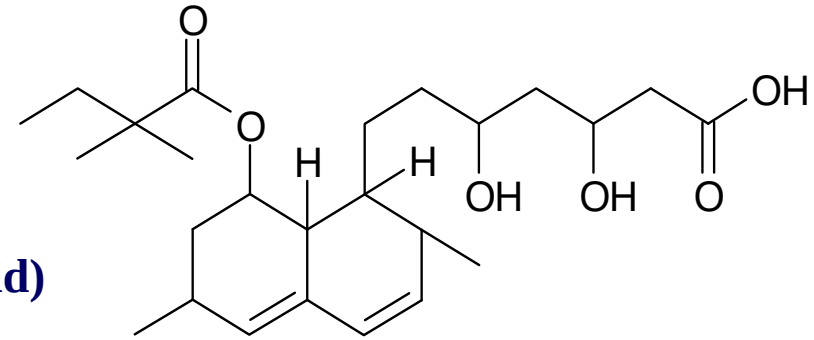
# Evaluation Item

- **Calibration curve**
  **(Range: 0.250 to 100 ng/mL in human plasma)**
- **Accuracy and precision**
  **(4 levels; LLQC, LQC, MQC, and HQC)**
- **Recovery (3 analytes and IS)**
- **System Continuity (20 peak areas of IS)**
- **Contamination (Solvent and eluate)**

# Structures of Atorvastatin and Its Metabolites



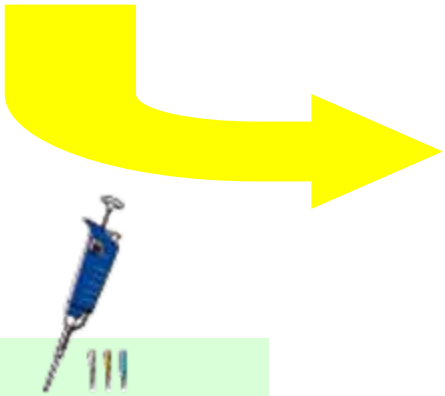

**Atorvastatin** / **R1:H, R2:H**
**M-I (*p*-hydroxy atorvastatin)** / **R1:H, R2:OH**
**M-II (*o*-hydroxy atorvastatin)** / **R1:OH, R2:H**




**IS (Simvastatin hydroxy acid)**

# Pretreatment Procedure

**Plasma sample**





**POINT**
Dilution with buffer.

## ITSP

**STEP 1** SPE cartridge conditioning

**STEP 2** SPE cartridge conditioning

**STEP 3** Load onto SPE cartridge

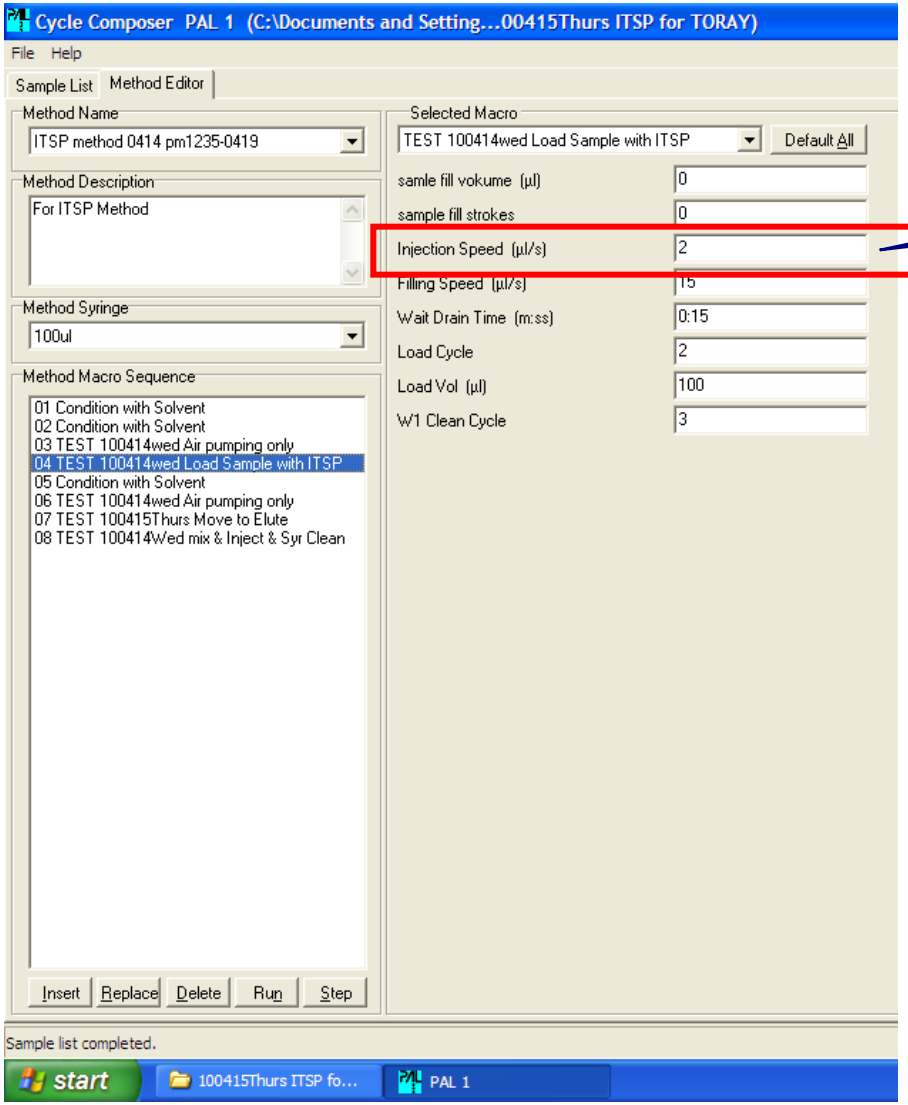
**STEP 4** Wash

**STEP 5** Elute

**STEP 6** Dilution

**STEP 7** Injection

# Pretreatment Procedure




**POINT**

**Injection Speed [µl/s]**

Detailed movement for each part of each step can be controlled by ITSP because many parameters can be set.

Sample could be slowly infiltrated into the solid phase cartridge and, as a result, recovery is expected to be constant.

# Evaluation Item

- **Calibration curve**
  **(Range: 0.250 to 100 ng/mL in human plasma)**
- **Accuracy**
  **(4 levels; LLQC, LQC, MQC, and HQC)**
- **Recovery (3 analytes and IS)**
- **System Continuity (20 peak areas of IS)**
- **Contamination (Solvent and eluate)**

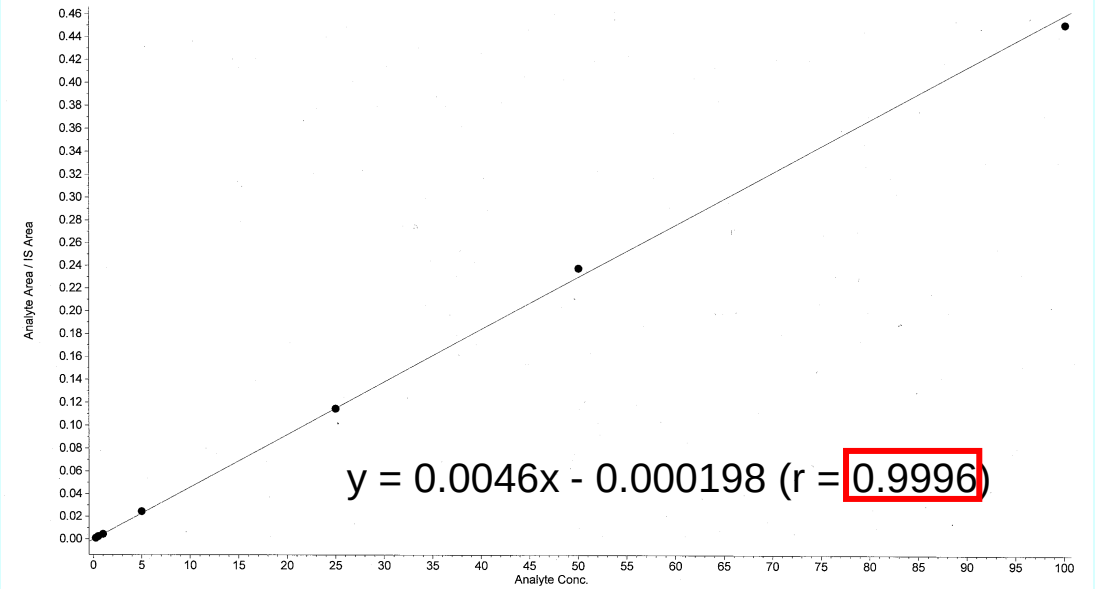
# Calibration Curve of Atorvastatin

(1/*x* weighting)

| Atorvastatin concentration (ng/mL) | | | | | | |
|---|---|---|---|---|---|---|
| 0.250 | 0.500 | 1.00 | 5.00 | 25.0 | 50.0 | 100 |
| 0.215 | 0.538 | 0.984 | 5.36 | 24.9 | 51.6 | 98.1 |
| *86.0* | *107.6* | *98.4* | *107.2* | *99.6* | *103.2* | *98.1* |

Upper value: Observed concentration (ng/mL)

Lower value: %Nominal




**Acceptance criteria**

%Nominal: 85.0 to 115.0% (80.0 to 120.0% at the LLOQ)

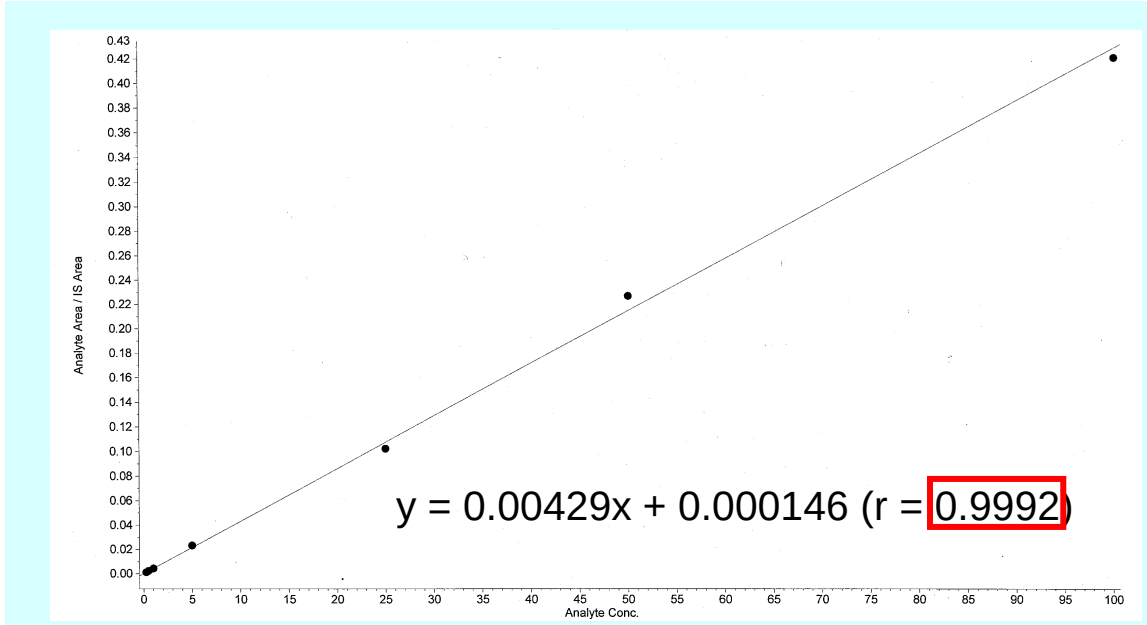
# Calibration Curve of M-I

(1/*x* weighting)

| M-I concentration (ng/mL) | | | | | | |
|---|---|---|---|---|---|---|
| 0.250 | 0.500 | 1.00 | 5.00 | 25.0 | 50.0 | 100 |
| 0.236 | 0.505 | 0.983 | 5.38 | 23.8 | 52.8 | 98.1 |
| *94.4* | *101.0* | *98.3* | *107.6* | *95.2* | *105.6* | *98.1* |

Upper value: Observed concentration (ng/mL)

Lower value: %Nominal



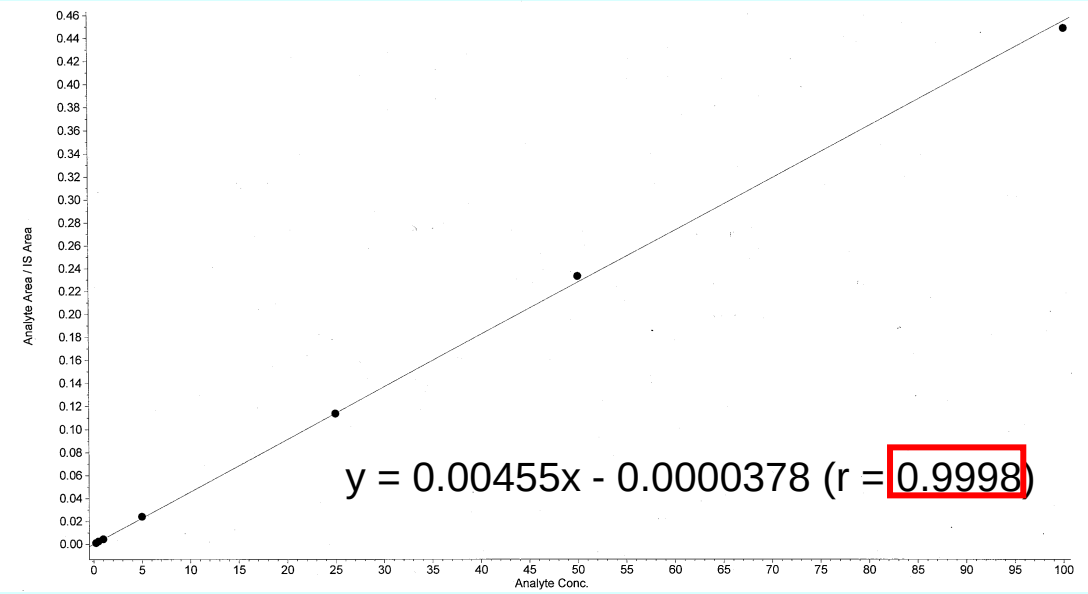
# Calibration Curve of M-II

(1/*x* weighting)

| M-II concentration (ng/mL) | | | | | | |
|---|---|---|---|---|---|---|
| 0.250 | 0.500 | 1.00 | 5.00 | 25.0 | 50.0 | 100 |
| 0.223 | 0.539 | 0.964 | 5.30 | 25.0 | 51.2 | 98.6 |
| *89.2* | *107.8* | *96.4* | *106.0* | *100.0* | *102.4* | *98.6* |

Upper value: Observed concentration (ng/mL)

Lower value: %Nominal



# Evaluation Item

- Calibration curve
  (Range: 0.250 to 100 ng/mL in human plasma)
- **Accuracy**
  **(4 levels; LLQC, LQC, MQC, and HQC)**
- Recovery (3 analytes and IS)
- System Continuity (20 peak areas of IS)
- Contamination (Solvent and eluate)

# Assay Reproducibility

## Quantitative results

| Compound name | Atorvastatin | | | | M-I | | | | M-II | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| QC sample | LLQC 0.250 | LQC 0.500 | MQC 5.00 | ULQC 80.0 | LLQC 0.250 | LQC 0.500 | MQC 5.00 | ULQC 80.0 | LLQC 0.250 | LQC 0.500 | MQC 5.00 | ULQC 80.0 |
| Found Concentration (ng/mL) | 0.301 | 0.524 | 4.68 | 71.9 | 0.215 | 0.468 | 4.47 | 69.5 | 0.235 | 0.561 | 4.90 | 72.5 |
| | 0.263 | 0.384 | 5.61 | 77.7 | 0.211 | 0.520 | 5.24 | 69.9 | 0.213 | 0.464 | 5.34 | 77.1 |
| | 0.287 | 0.495 | 4.66 | 70.7 | 0.247 | 0.448 | 4.29 | 68.2 | 0.202 | 0.466 | 4.54 | 72.1 |
| Mean | 0.284 | 0.468 | 4.98 | 73.4 | 0.224 | 0.479 | 4.67 | 69.2 | 0.217 | 0.497 | 4.93 | 73.9 |
| SD | 0.019 | 0.074 | 0.54 | 3.7 | 0.020 | 0.037 | 0.50 | 0.9 | 0.017 | 0.055 | 0.40 | 2.8 |
| %Nominal | 113.5 | 93.5 | 99.7 | 91.8 | 89.7 | 95.7 | 93.3 | 86.5 | 86.7 | 99.4 | 98.5 | 92.4 |

$$\%\text{Nominal} = \frac{X}{C_{nom}} \times 100$$

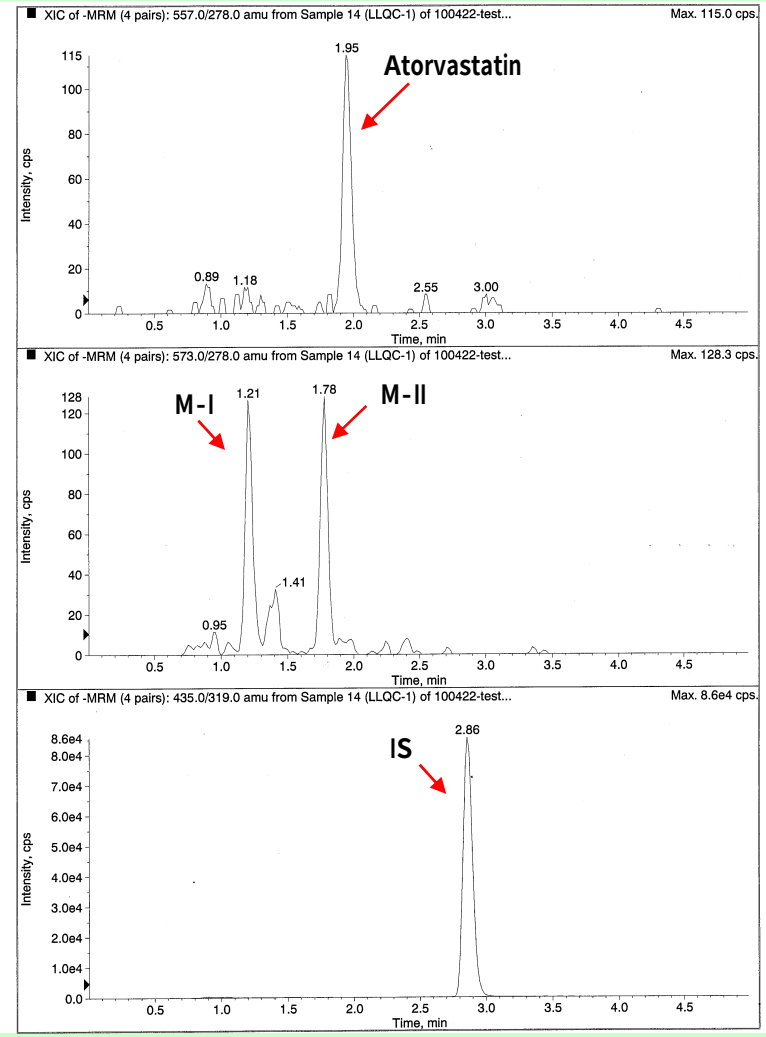
$C_{nom}$ : Nominal concentration (ng/mL)

X: Mean found concentrations (n=3)

**Acceptance criteria**

%Nominal: 85.0 to 115.0% (80.0 to 120.0% for LLQC)

# Representative Chromatograms




**LLQC sample: 0.250 ng/mL**

## Analytical condition

**Analytical column：**
CAPCELLPAK C18 AQ 5 μm
2.0 mm I.D. × 150 mm (Shiseido Co, Ltd)

**Mobile Phase：**
A：$H_2O$-$CH_3CN$-HCOOH (950:50:1, v/v/v)
B：$CH_3CN$-$H_2O$-HCOOH (950:50:1, v/v/v)

**Gradient Condition：**
A/B=35/65 (0.5 min Hold) - (3.5 min)
A/B=10/90 (1.5 min Hold)

**Flow rate：** 0.4 mL/min

**Column oven temperature：** 40 °C

# Evaluation Item

- Calibration curve
  (Range: 0.250 to 100 ng/mL in human plasma)
- Accuracy
  (4 levels; LLQC, LQC, MQC, and HQC)
- **Recovery (3 analytes and IS)**
- System Continuity (20 peak areas of IS)
- Contamination (Solvent and eluate)

# Recovery

| Sample | %Recovery | | | |
|---|---|---|---|---|
| | Atorvastatin | M-I | M-II | IS |
| LQC | 72.7 | 98.6 | 89.3 | 67.8 |
| MQC | 75.6 | 91.3 | 82.5 | — |
| HQC | 73.0 | 78.5 | 71.6 | — |
| Overall mean | 73.8 | 89.5 | 81.1 | 67.8 |

%Recovery was calculated based on the peak area of each QC sample for analyte or IS recovery to the peak area of the reference QC sample.

%Recovery of analytes was more than 70%.

# Evaluation Item

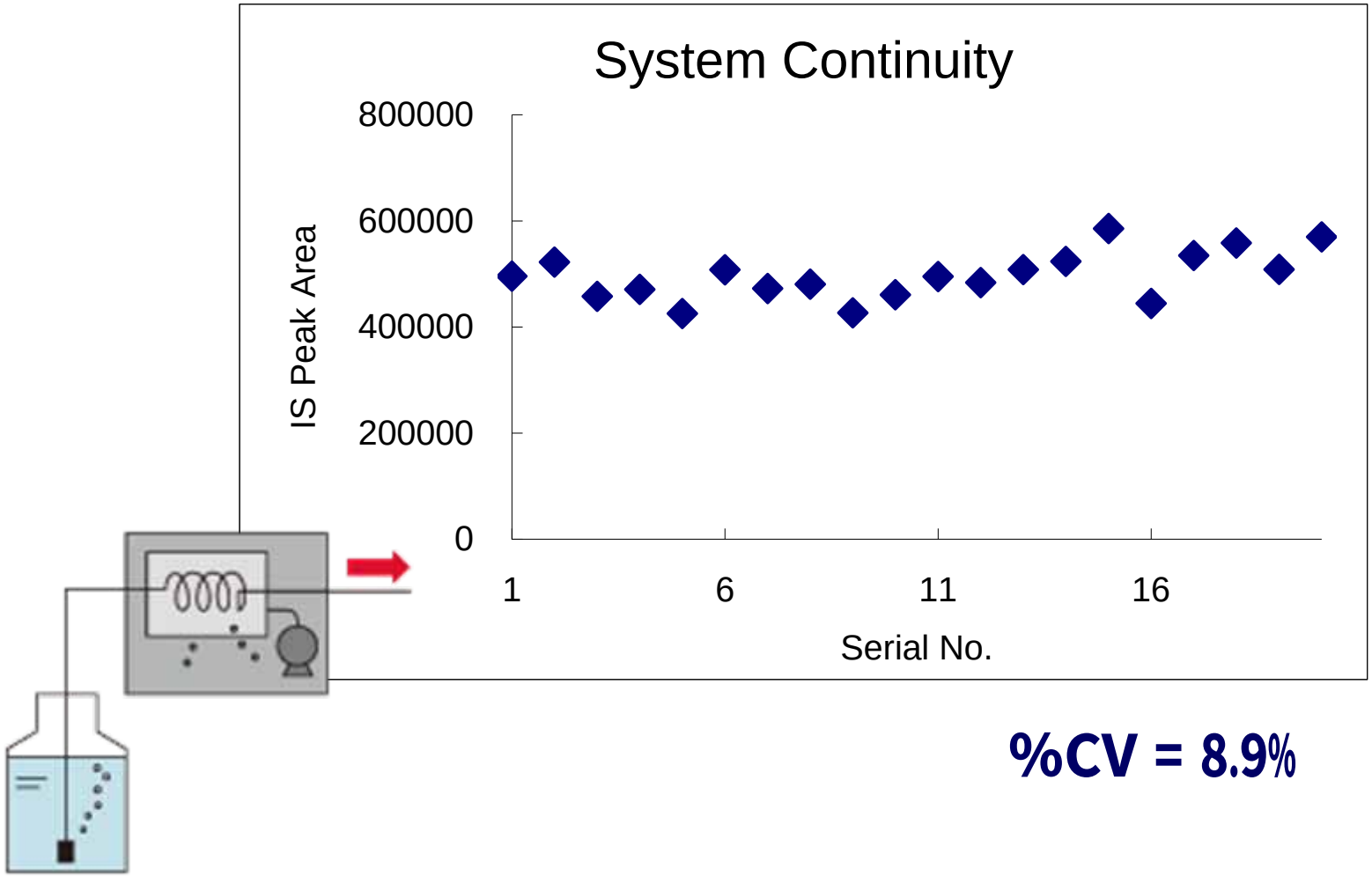
- Calibration curve
  (Range: 0.250 to 100 ng/mL in human plasma)
- Accuracy
  (4 levels; LLQC, LQC, MQC, and HQC)
- Recovery (3 analytes and IS)
- **System Continuity (20 peak areas of IS)**
- Contamination (Solvent and eluate)

# System Continuity

System continuity was evaluated based on %CV calculated from the twenty IS (internal standard) peak areas.

| Serial No. | IS peak area | Serial No. | IS peak area |
|---|---|---|---|
| 1 | 495622 | 11 | 495066 |
| 2 | 522284 | 12 | 483692 |
| 3 | 457797 | 13 | 508182 |
| 4 | 470868 | 14 | 523555 |
| 5 | 425415 | 15 | 585504 |
| 6 | 507861 | 16 | 444373 |
| 7 | 472654 | 17 | 534831 |
| 8 | 480629 | 18 | 558283 |
| 9 | 426602 | 19 | 508397 |
| 10 | 460637 | 20 | 569788 |
| | Mean | 496602 | |
| | SD | 44407 | |
| | %CV | 8.9 | |

# System Continuity




**%CV = 8.9%**

# Evaluation Item

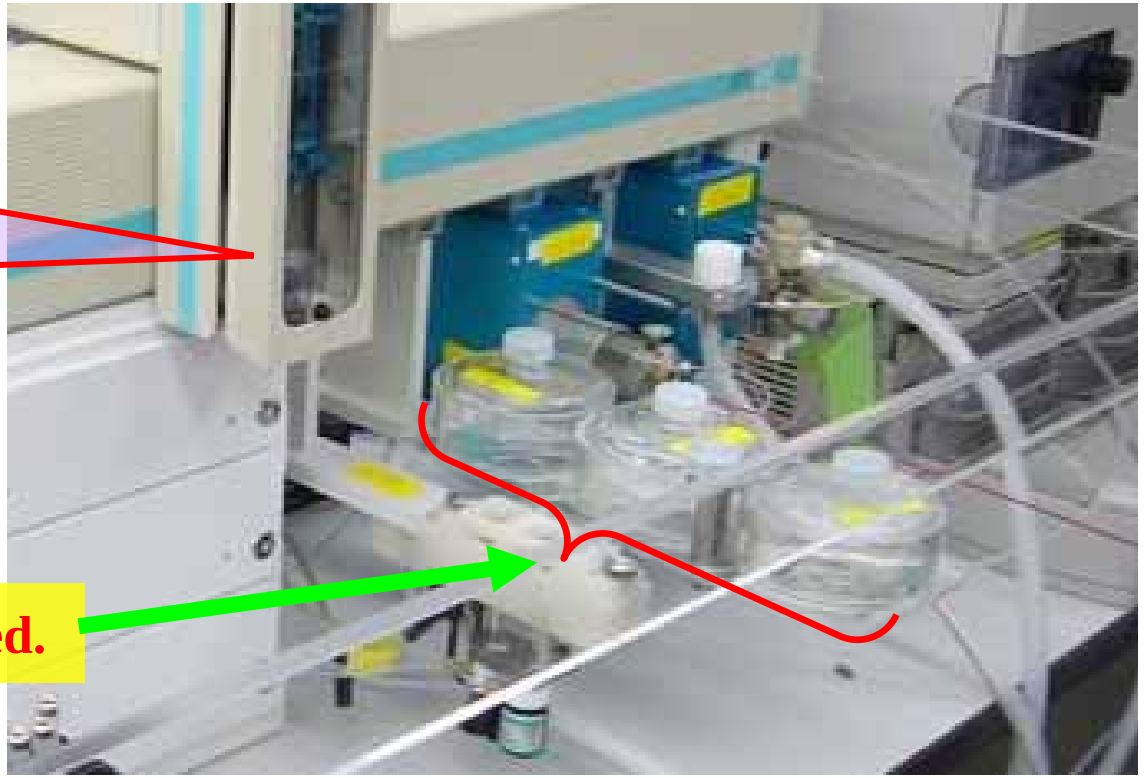
- Calibration curve
  (Range: 0.250 to 100 ng/mL in human plasma)
- Accuracy
  (4 levels; LLQC, LQC, MQC, and HQC)
- Recovery (3 analytes and IS)
- System Continuity (20 peak areas of IS)
- **Contamination (Solvent and eluate)**

# Contamination

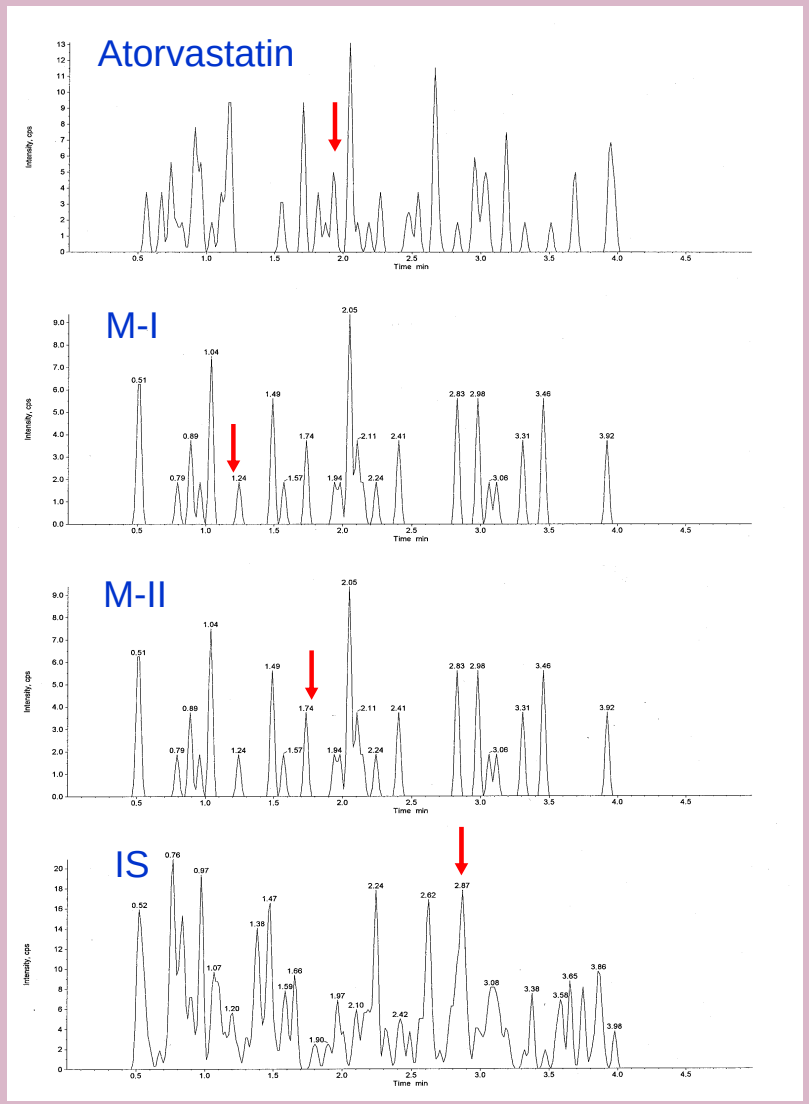
After the determination of all analytical samples was completed, a solvent sample was pretreated with the same method as the analytical samples.

Analytical samples were pretreated for all steps using a syringe without human intervention.

**Contamination may be suspected.**



# Contamination




No peaks were detected at the retention times of the analytes and internal standard on the chromatograms of the solvent sample.

No contamination

# Overlap Injection

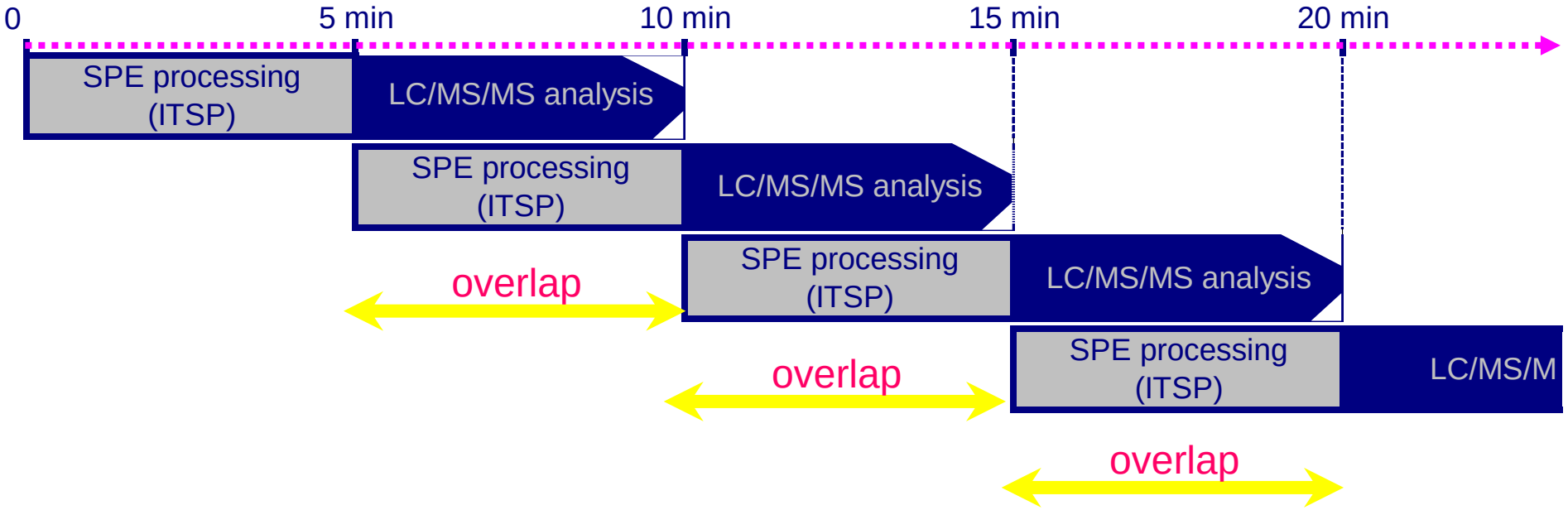
An analytical sample is pretreated by ITSP as soon as the previous sample has been injected into LC/MS/MS.

Time for analysis and pretreatment of the next sample is overlapped.

5 min/sample

# Overlap Injection

## Workflow



# Conclusion

| Item | Conditions | Result |
|---|---|---|
| Calibration curve | — | 0.250 to 100 ng/mL |
| Accuracy | LLQC | 86.7 to 113.5% |
| | LQC, MQC and HQC | 86.5 to 99.7% |
| Recovery test | Atorvastatin | 72.7 to 75.6% |
| | M-I | 78.5 to 98.6% |
| | M-II | 71.6 to 89.3% |
| | IS | 67.8% |
| System continuity | IS peak area (n = 20) | 8.9% (%CV) |
| Cotamination | — | Not detected |

This method can be useful for quantitative assay on Atorvastatin and its metabolites in human plasma.

*Thank you for your attention.*

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