การวิเคราะห์ด้วยเทคนิค HPLC ตามข้อกำหนดใน General Chapters: <621> Chromatography, USP 39

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INTRODUCTION

Chromatographic separation techniques

- **Stationary phase**

  a solid or a liquid supported on a solid or a gel
  - packed in a column
  - spread as a layer
  - distributed as a film
  - applied by other techniques
- **Mobile phase**
  - gas
  - liquid
  - supercritical fluid

<621> Chromatography

This chapter contains :

- General procedures
- Definitions
- Calculations of common parameters
- Describes general requirements for system suitability
- Quantitation

GENERAL PROCEDURES

- Paper Chromatography
- Thin-Layer Chromatography
- Column Chromatography
- Gas Chromatography (GC)
- Liquid Chromatography (LC)

Liquid Chromatography (LC)

- High-pressure liquid chromatography
- High-performance liquid chromatography

Stationary phase

The modified silica or polymeric beads are modified by the addition of long-chain hydrocarbons

The specific type of packing indicated by the "L" (L1 – L101)

Reagents/Chrom reagents/Chromatographic columns/Packings

Guard column

Guard column : requirements

(a) the length of the guard column must be NMT 15% of the length of the analytical column

(b) the inner diameter must be the same or smaller than that of the analytical column

(c) the packing material should be the same as the analytical column and contain the same bonded phase

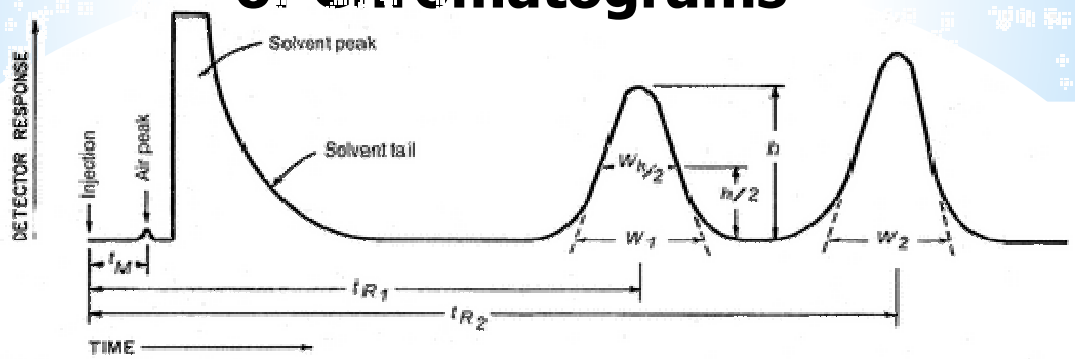
all system suitability requirements must be met with the guard column installed

Mobile phase

solvent or mixture of solvents

(individual monograph)

Definitions and Interpretation of Chromatograms



t_{R1} and t_{R2} are the respective retention times.
h is the height, h/2 is the half-height.
$W_{h/2}$ is the width at half-height, for peak 1.
W_1 and W_2 are the respective widths of peaks 1 and 2 at the baseline.
t_M is the retention time of unretained component.

Retention Factor (k)

capacity factor (k′)

$$k = \frac{\text{amount of substance in stationary phase}}{\text{amount of substance in mobile phase}}$$

$$k = \frac{\text{time spent by substance in stationary phase}}{\text{time spent by substance in mobile phase}}$$

The retention factor of a component may be determined from the chromatogram:

$$k = (t_R - t_M)/t_M$$

Resolution (R_S)

The resolution is the separation of two components in a mixture

$$R_S = 2 \times (t_{R2} - t_{R1})/(W_1 + W_2)$$

Where electronic integrators are used, it may be convenient to determine the resolution, by the equation:

$$R_S = 1.18 \times (t_{R2} - t_{R1})/(W_{1,h/2} + W_{2,h/2})$$

Number of Theoretical Plates (N)

N is a measure of column efficiency

$$N = 16(t_R/W)^2$$

Where electronic integrators are used

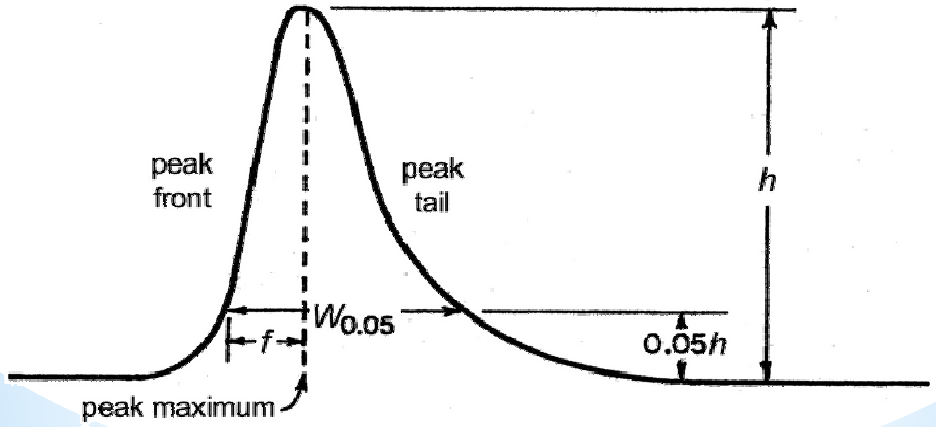
$$N = 5.54\left(\frac{t_R}{W_{h/2}}\right)^2$$

In the event of dispute, only equations based on peak width at baseline are to be used.

Symmetry Factor (A_s)

The tailing factor

$$A_S = W_{0.05}/2f$$



SYSTEM SUITABILITY

System suitability tests are used to verify that the chromatographic system is adequate for the intended analysis.

- Resolution
- Tailing factor
- Number of theoretical plates
- Signal to noise ratio
- %RSD

%RSD

Replicate injections of a standard preparation

- if the requirement %RSD $\leq$ 2.0%
  → data from five replicate injections to calculated

- if the requirement %RSD > 2.0%
  → data from six replicate injections to calculated

%RSD

For Drug substance

The maximum permitted %RSD is calculated for a series of injections of the reference solution:

$$\%RSD = KB\sqrt{n}/t_{90\%,n-1}$$

where K is a constant

B is the upper limit given in the definition of the individual monograph minus 100%

n is the number of replicate injections of the reference solution ($3 \leq n \leq 6$)

$t_{90\%,n-1}$ is the Student's t at the 90% probability level (double sided) with n − 1 degrees of freedom

	Number of Individual Injections			
	3	4	5	6
B (%)	Maximum Permitted RSD			
2.0	0.41	0.59	0.73	0.85
2.5	0.52	0.74	0.92	1.06
3.0	0.62	0.89	1.10	1.27

Factors

- Mobile phase :
  composition, ionic strength, temperature and pH
- Stationary phase :
  type of support, particle size, porosity, and specific surface area
- Reverse-phase and other surface modification of the stationary phases, the extent of chemical modification
- Flow rate, column dimensions, column temperature, and pressure

Adjustments

- **pH of Mobile Phase**

→ within ± 0.2 units of the value or range specified

Exp.

Mobile phase : pH 3.0
permitted change 2.8 – 3.2

Applies to both gradient and isocratic separations

Adjustments

- Concentration of Salts in Buffer

→ within ±10%

Exp.

Mobile phase : 1.0M Potassium phosphate
permitted change 0.9 – 1.1 M

* if the permitted pH variation is met

Applies to both gradient and isocratic separations

Adjustments

- Ratio of Components in Mobile Phase

→ Minor component : ± 30% relative,
Not exceed ± 10% absolute

Adjustments

- Ratio of Components in Mobile Phase

→ Binary mixtures

Exp.1

Specific ratio of 50:50

30% → 50 x 30/100 = 15%

maximum permitted change 10% absolute

"Adjusted within range 40:60 to 60:40"

Adjustments

- Ratio of Components in Mobile Phase

→ Binary mixtures

Exp.2

Specific ratio of 2:98

30% → 2 x 30/100 = 0.6%

"Adjusted within range 1.4:98.6 to 2.6:97.4"

Adjustments

- Ratio of Components in Mobile Phase

→ Ternary mixtures

Exp. Specific ratio of 60:35:5

2nd component

30% → 35 x 30/100 = 10.5%

adjusted only within the range of 25% – 45% absolute

"Adjusted within range 50:45:5 to 70:25:5"

3rd component

30% → 5 x 30/100 = 1.5%

adjusted permit change 3.5% – 6.5% absolute

"Adjusted within range 58.5:35:6.5 to 61.5:35:3.5"

Adjustments

- Wavelength of UV-Visible Detector

→ not permitted

Error ±3 nm

Adjustments

- Stationary Phase

column length : see Particle Size

column inner diameter : see Flow Rate

Adjustments

- Particle Size

Column length(L)/particle size(dp)
L/dp ratio between -25% to +50%

For gradient separations :

~~changes in length~~
~~column inner diameter~~
~~particle size~~

"When particle size is not mentioned in the monograph, → using the largest particle size consigned in the USP definition of the column"

Reagents/Chrom reagents/Chromatographic columns/ Packings

Packings

L1—Octadecyl silane chemically bonded to porous or nonporous silica or ceramic microparticles, 1.5 to 10 µm in diameter, or a monolithic silica rod.

L2—Octadecyl silane chemically bonded to silica gel of a controlled surface porosity that has been bonded to a solid spherical core, 30 to 50 µm in diameter.

L3—Porous silica particles, 1.5 to 10 µm in diameter, or a monolithic silica rod.

L4—Silica gel of controlled surface porosity bonded to a solid spherical core, 30 to 50 µm in diameter.

L5—Alumina of controlled surface porosity bonded to a solid spherical core, 30 to 50 µm in diameter.

L6—Strong cation-exchange packing-sulfonated fluorocarbon polymer coated on a solid spherical core, 30 to 50 µm in diameter.

L7—Octylsilane chemically bonded to totally porous or superficially porous silica particles, 1.5-10 µm in diameter, or a monolithic silica rod.

Packings

L1—Octadecyl silane chemically bonded to porous or nonporous silica or ceramic microparticles, 1.5 to 10 µm in diameter, or a monolithic silica rod.

Exp.1

monograph ⇨ L1 4.6 x 100 mm, 5µm
modified ⇨ L1 4.6 x 150 mm, 5µm

monograph

L(mm)/dp(µm) = 100/5 = 20000

Permitted change = -25% to +50%

-25% = 20000 x -25/100 = -5000

50% = 20000 x 50/100 = 10000

L/dp ratio 15000 - 30000

modified

L(mm)/dp(µm) = 150/5 = 3000(

Permitted

Exp.2

monograph ⇨ L1 4.6 x 150 mm, 5µm
modified ⇨ L1 4.6 x 250 mm, 5µm

monograph

L(mm)/dp(µm) = 150/5 = 30000

Permitted change = -25% to +50%

-25% = 30000 x -25/100 = -7500

50% = 30000 x 50/100 = 15000

L/dp ratio 22500 - 45000

modified

L(mm)/dp(µm) = 250/5 = 50000

Not permitted

Adjustments

- Flow rate

$$F_2 = F_1 \times [(dc_2^2 \times dp_1)/(dc_1^2 \times dp_2)]$$

F_1 , F_2 = flow rates
dc_1 , dc_2 = column diameters
dp_1 , dp_2 = the particle sizes

* the flow rate can be adjusted by ±50% (isocratic only)

Exp.1

monograph ⇨ L1 4.6 x 150 mm, 5µm, flow rate 1.5 ml/min
modified ⇨ L1 2.1 x 75 mm, 2.5µm, flow rate ?

$$F_2 = F_1 \times [(dc_2^2 \times dp_1)/(dc_1^2 \times dp_2)]$$

$$F_2 = 1.5 \times [(2.1^2 \times 5)/(4.6^2 \times 2.5)]$$

$$= 0.6 \text{ ml/min}$$

Exp.2

monograph ⇨ L1 3.9 x 300 mm, flow rate 1.0 ml/min
modified ⇨ L1 4.6 x 150 mm, 5µm, flow rate ?

$$F_2 = F_1 \times [(dc_2^2 \times dp_1)/(dc_1^2 \times dp_2)]$$

$$F_2 = 1.0 \times [(4.6^2 \times 10)/(3.9^2 \times 5)]$$

$$= 2.8 \text{ ml/min}$$

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EXAMPLES: Adjustments in column length, internal diameter, particle size, and flow rate can be used in combination to give equivalent conditions (same *N*), but with differences in pressure and run time. The table below lists some of the more popular column configurations to give equivalent efficiency (*N*), by adjusting these variables.

Length (*L*, mm)	Column Diameter (*dc*, mm)	Particle Size (*dp*, µm)	Relative Values				
			L/*dp*	*F*	*N*	Pressure	Run Time
250	4.6	10	25,000	0.5	0.8	0.2	3.3
150	4.6	5	30,000	1.0	1.0	1.0	1.0
150	2.1	5	30,000	0.2	1.0	1.0	1.0
100	4.6	3.5	28,600	1.4	1.0	1.9	0.5
100	2.1	3.5	28,600	0.3	1.0	1.9	0.5
75	4.6	2.5	30,000	2.0	1.0	4.0	0.3
75	2.1	2.5	30,000	0.4	1.0	4.0	0.3
50	4.6	1.7	29,400	2.9	1.0	8.5	0.1
50	2.1	1.7	29,400	0.6	1.0	8.5	0.1

For example, if a monograph specifies a 150-mm × 4.6-mm; 5-µm column operated at 1.5 mL/min, the same separation may be expected with a 75-mm × 2.1-mm; 2.5-µm column operated at 1.5 mL/min × 0.4 = 0.6 mL/min, along with a pressure increase of about four times and a reduction in run time to about 30% of the original.

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Adjustments

- Injection volume

can be adjusted with accepted precision, linearity, and detection limits

Applies to both gradient and isocratic separations

Adjustments

- Column Temperature

can be adjusted by as much as ± 10°

Applies to both gradient and isocratic separations

QUANTITATION

- External Standard Method
- Internal Standard Method
- Normalization Procedure
- Calibration Procedure

External Standard Method

Comparing the response obtained with the sample solution to the response obtained with a standard solution

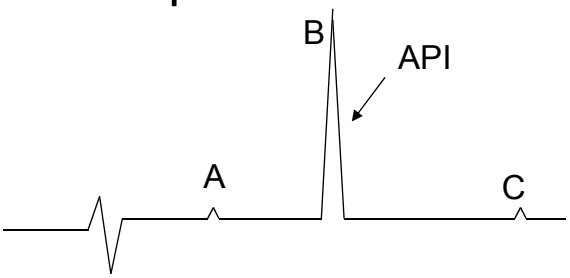
Internal Standard Method

Equal amounts of the internal standard are introduced into the sample solution and a standard solution

Comparing the ratios of peak response and the internal standard in the sample solution with the ratios of peak response and the internal standard in the standard solution

Normalization Procedure

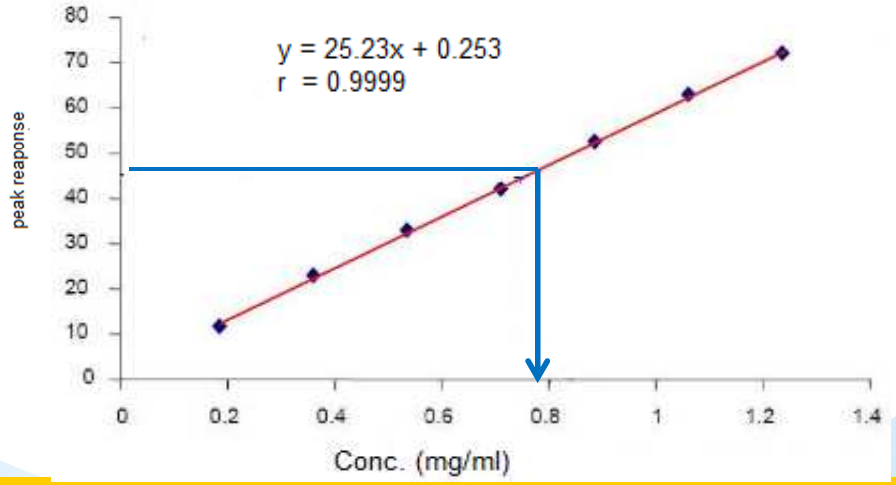
Determining the area as a percentage of the total area of all the peaks



Excluding :
solvents, reagents, mobile phase, the sample matrix and those at or below the limit at which they can be disregarded

Calibration Procedure

Calculated from the measured signal of the analyte and its position on the calibration curve

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