METHOD DEVELOPMENT ON *HPLC*

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Introduction

- High Performance Liquid Chromatograph (HPLC) instruments were originated from the fundamental work of Howard and Martin.
- In classical column liquid chromatography, the mobile liquid phase flows slowly through the column by means of gravity therefore gives low column efficiency and long separation time.
- In HPLC, small diameter columns with support particle size in the region of 3-10 μm are used and the eluents is pumped through the column at a constant flow rate.
- HPLC is thus a method of separation in which the stationary phase is contained in a column one end of which is attained to a source of pressurized liquid eluents (mobile phase).

ADVANTAGES OF HPLC:

- High sensitivity.
- High performance.
- Rapid process and hence time saving.
- Continuous monitoring of the column effluent.
- Automation of analytical procedure and data handling.
- Both aqueous and non-aqueous samples can be analyzed with little or no sample pretreatment.
- Variety of solvents and column packing materials are available, which provide a high degree of selectivity for specific analysis.
- Separation components can be easily collected and isolated from the mobile phase for further analysis or characterization.

•It provides a method for determination of multiple components in a single analysis

•Analysis of sample components is not limited by their thermal stability or volatility.

•It is flexible and thus can be used for liquids as well as for solids.

•The solvents used for mobile phase preparation are easily available and economical.

• It has high efficiency of separation.

•Column stability.

•Different varieties of detectors and columns are available.

•Calculation is based on area as well as peak height.

- Faster and better resolution.
- Can be used for both analytical and preparative purposes.
- Can be used for qualitative as well as quantitative estimation.
- Ability to simultaneously analyze a broad spectrum of both closely related and widely different compounds.
- Batch to batch reproducibility and accuracy of results.

Normal-Phase Chromatography

• In normal-phase chromatography (NPC) the stationary phase is more polar than the mobile phase, the opposite of RPC.

• Usually, the mobile phase is a mixture of organic solvents without added water (e.g., isopropanol plus hexane) and the column packing is either an inorganic adsorbent (silica or occasionally alumina) or a polar bonded phase (cyano, diol, or amino) on a silica support.

• Regardless of the mobile or stationary phase used, sample retention in NPC increases as the polarity of the mobile phase decreases (the opposite of RPC).

• NPC has been used for separating both neutral and ionic (or ionizable) compounds, but neutral samples predominate.

• NPC for ionic samples can involve the use of water in the mobile phase, and the retention process is then somewhat complex.

• When ionic samples are separated by NPC, it is usually advisable to add triethylamine to the mobile phase for basic compounds and acetic or formic acid for acidic compounds.

• Neutral samples are often separated equally well by either RPC or NPC, the main difference being a reversal of elution order for the two HPLC methods.

• In NPC, less polar (hydrophobic) compounds elute first, while more polar (hydrophilic) compounds leave the column last.

• Usually, RPC separation should be tried first.

• More often, the need for a change from RPC to NPC will become apparent after initial RPC experiments show either inadequate retention or poor selectivity for different solvents and/or columns.

Reasons to use Normal-Phase Chromatography

1. The sample is unretained by RPC (too hydrophilic).
2. The sample is too strongly retained by RPC (too hydrophobic).
3. RPC separation is unable to achieve adequate band spacing ($\alpha \approx 1$).
4. The sample contains positional isomers, stereoisomers, or diastereomers.
5. Recovery of significant amounts of organic-soluble sample components is desired (preparative HPLC, Chapter 13).
6. The sample is dissolved in a non-polar solvent (causing direct-injection problems if using a RPC column).

Comparision of selectivity of NPC and RPC

Compounds having different functional groups
  Larger α values for silica vs. C_{18}; more similar α values for polar-bonded-phase NPC columns vs. C_{18} in RPC.

Homologs or compounds differing in carbon number
  Larger α values for RPC vs. NPC

Isomers
  Much larger α values for silica vs. C_{18}; larger α values for polar-bonded-phase columns vs. C_{18}.

The advantages and disadvantages of NPC

Advantages	Disadvantages
1. Very large changes in separation selectivity are possible by changing either the mobile phase or column packing (especially for inorganic packings such as silica). 2. Columns are quite stable when using non-aqueous mobile phases. 3. Many organic compounds are more soluble in normal-phase solvents (a special advantage in preparative HPLC). 4. Pressure drop is lower due to lower-viscosity solvents. 5. Useful for samples which may decompose in aqueous solutions.	1. Ionic samples are more easily separated by RPC. 2. Controlling solvent strength can be less predictable and more tedious than in RPC. 3. Column plate numbers in NPC are sometimes lower than in RPC. 4. Lower-boiling solvents are more prone to evaporation and bubble formation, especially at higher room temperatures (less convenient). 5. For unmodified silica: a. Retention can be variable because of water uptake by the column packing. b. Gradient elution may not be practical because of solvent demixing and water uptake by silica columns. 6. Higher cost of purchase and disposal of organic solvents.

RETENTION IN NORMAL-PHASE CHROMATOGRAPHY

General Aspects

• While retention in RPC is believed to resemble a partition process retention in NPC, on the other hand, appears to occur by an adsorption process.

Sample and Solvent Localization.

• Polar sample and solvent molecules are strongly attracted to polar groups *(adsorption sites)* on the surface of the column packing.

• The adsorption sites are silanols (-SiOH) in the case of silica.

• For cyano, amino, or diol columns commonly used in NPC, the bonded-phase ligands and/or silanols can be the adsorption sites.

• Polar sample molecules consist of one or more polar functional groups attached to a hydrocarbon residue such as hexane or benzene.

• The non-polar hydrocarbon will be attracted to adsorption sites only weakly, in contrast to the attached polar groups.

• In the case of the mono-substituted compounds X-benzene and Y-benzene, the polar group X or Y is attracted to an adsorbent site A.

• When group X or Y is very polar, this attraction will be quite strong, and group X or Y becomes attached or *localized* onto an adsorbent site.

• When two or more polar groups are present in the same molecule, it may not be possible for each of these groups to localize at the same time.

• Localization of very polar solvent molecules is also possible, with the important practical consequence of enhanced selectivity control.

• Polar sample and solvent molecules interact strongly with the stationary phase in NPC.

• As a result, NPC usually allows more control over selectivity than RPC, by changing either the strong solvent (B) or the column.

• The strong interaction of solvents and solutes with NPC stationary phases requires a somewhat different approach to solvent classification and method development.

Mobile-Phase Effects

• The mobile phase for NPC is chosen in the same general way as for RPC.

• A weak (non-polar) solvent A and a strong (polar) solvent B are first selected and then blended to obtain a mobile phase of intermediate polarity that will provide $0.5 < k < 20$ for the sample.

• During the adjustment of % B for adequate retention, changes in selectivity with % B should be noted so that the resolution of the critical band pair can be optimized (solvent-strength selectivity).

• If separation is still inadequate, a different strong solvent can be selected for additional changes in selectivity.

• If further improvement in separation is required, mixtures of the latter strong solvents can be explored.

Solvent Strength.

• The strength of different solvents or solvent mixtures for NPC can be represented by a parameter ε^0 which can be measured experimentally.

Values of ε^0 for some commonly used HPLC solvents are

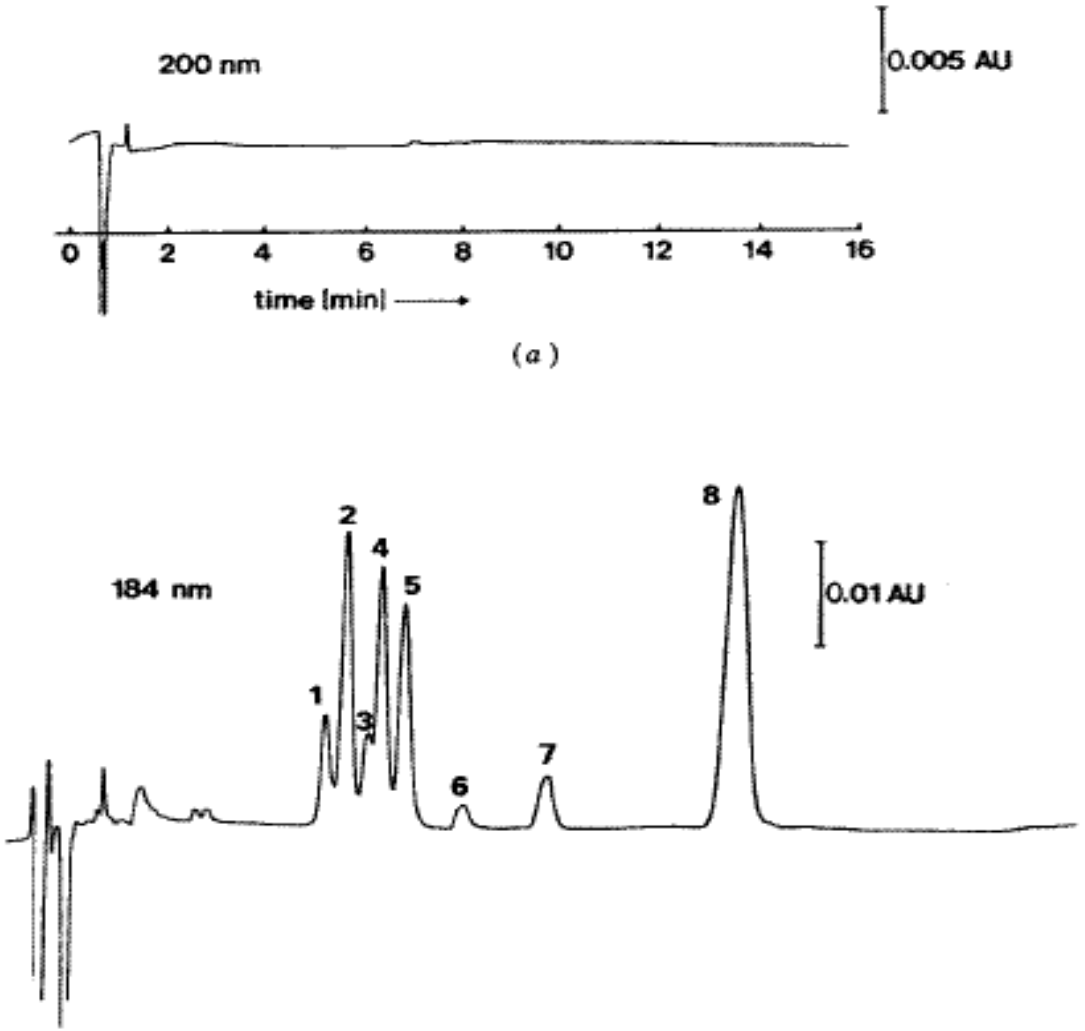
Solvent	ε°	Localization	Basic?	UV[b]
Hexane, heptane, octane	0.00	No	[c]	201
1,1,2-Triflurotrichloroethane (Freon FC-113)	0.02	No	[c]	235
Chloroform	0.26	No	[c]	247
1- or 2-Chloropropane	0.28	No	[c]	225
Methylene chloride	0.30	No	[c]	234
2-Propyl ether	0.32	Minor	[c]	217
1,2-Dichloroethane	0.34	No	[c]	234
Ethyl ether	0.38	Yes	Yes	219
MTBE[d]	0.48	Yes	Yes	225
Ethyl acetate	0.48	Yes	No	256
Dioxane	0.51	Yes	Yes	215
Acetonitrile	0.52	Yes	No	192
THF	0.53	Yes	Yes	230
1- or 2-Propanol	0.60	Yes	[e]	214
Methanol	0.70	Yes	[e]	210

a Silica used as absorbent. ***b*** Minimum UV wavelength; assumes that maximum baseline absorbance (100% B) is 0.5 AU. ***c*** Solvent basicity is irrelevant for non-localizing solvents. ***d*** Methyl t-butyl ether. ***e*** Different selectivity due to presence of proton donor group.

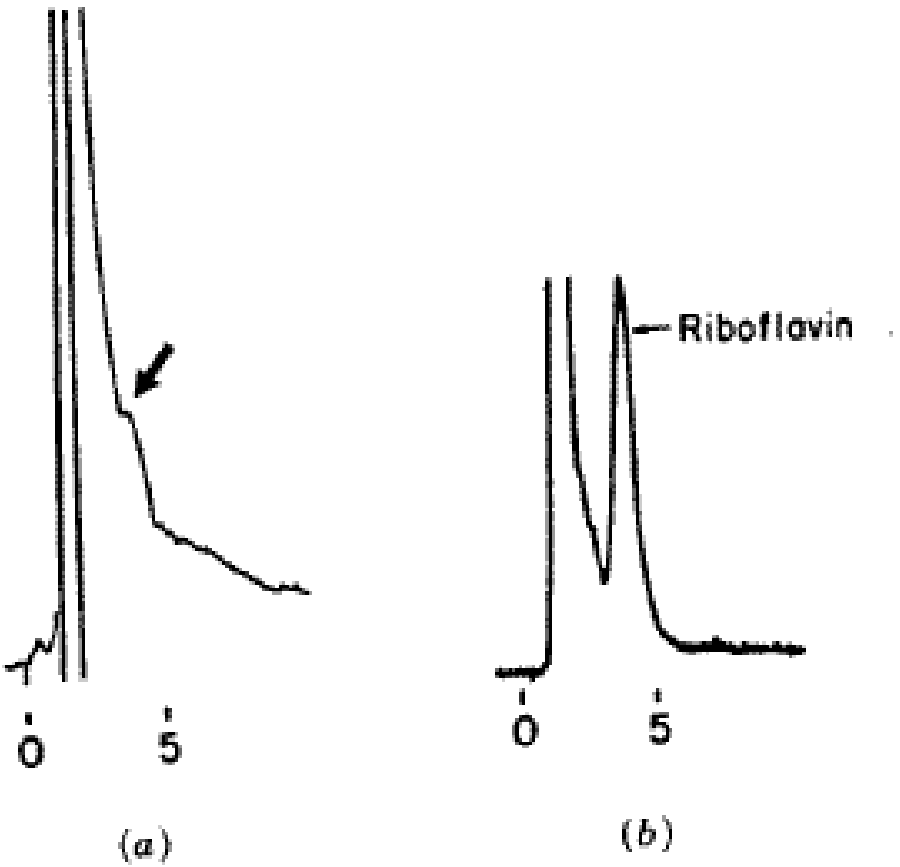
In most cases HPLC method development is carried out with UV detection using either a variable-wavelength (spectrophotometer) or a diode array detector. Alternative detectors are selected primarily when:

- Samples have little or no UV absorbance.
- Analyte concentration are too low for UV detection.
- Sample interference are important.
- Qualitative structural information is required.

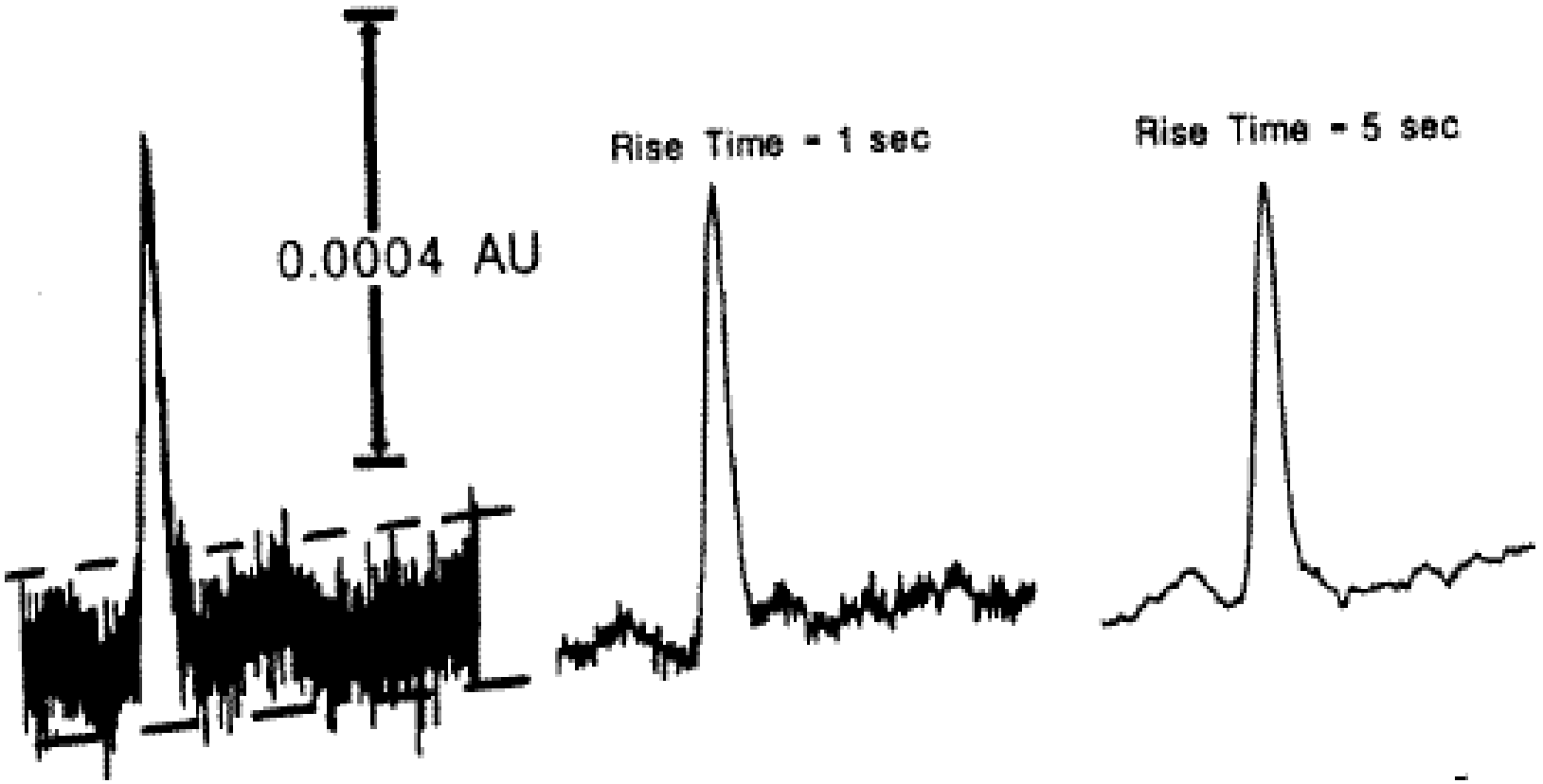
Detector type and operation affect the relative response of sample components and potential interferences in interrelated ways: sensitivity, selectivity and base line noise. In the same separation with different detector settings base line noise is much reduced, with an increase in S/N ratio.



HPLC of aged sample of n-butanol at 200 and 184 nm



Detection of Riboflavin a) by UV at 365 and b) using a fluorescence detector at exitation wavelength 365 and emission wavelength 530 nm.



Effect of signal to noise ratio on separation of the compound

Steps in HPLC method Development

- Information on samples define separation goals
- Need for special HPLC procedure sample preparation etc.
- Choose column and detector conditions
- Choose LC Method, Preliminary run, estimate best separation conditions
- Optimize separation conditions
- Check for problems or requirement of special procedure
- Quantitative calibration or Recover purified material or Qualitative method
- **Validate Method for release to routine laboratory**

On the basis of the applications, there are two types of HPLC.

Analytical HPLC**:**

Preparative HPLC:

HPLC can be operated by two types:

Isocratic:

Gradient system:

TERMINOLOGIES USED

RETENTION TIME: The degree of retention of a particular compound in a mixture is expressed as its retention time (RT). It is defined as the time that elapses from the moment of sample is introduced to the point of maximum concentration of the eluted peak.

Retention time is affected by:

1. Change in temperature
2. Change in column and
3. Change in flow rate

ANALYSIS: Pharmaceutical analysis is defined as the detection or measurement of an analyte in a pharmaceutical formulation. In pharmaceutical analysis the analyte concentration is fixed within the known manufacturing limits.

Number of theoretical plates: The number of theoretical plates (n) is expressed by the following mathematical expression,

$$n = 16 (Tr. / W)^2 = 5.54 (Tr / W_{1/2})^2$$

Where, W = Peak with

$W_{1/2}$ = Peak width at half height of the peak.

T_r = Retention time of the peak.

Height equivalent to a theoretical plate**:**

The thickness of the layer of the column in which one " Partition " is considered to occur is called the height equivalent to a theoretical plate (HETP) and is given by the equation,

The HETP can be given by the expression,

$$HETP = \frac{\text{Length of column}}{n}$$

Column Capacity ratio:

$$K = \frac{T_r - T_0}{T_0}$$

Where,

T_r = Retention time of retained component

T_0 = Retention time of unretained component

- It is preferred than the retention time as it is remain constant and is independent of flow rate.
- If K value is too high with an aqueous solvent, the problem may be solved by adding appropriate organic modifier until adequate K values are obtained.
- If the K value is too low and no organic solvent is present then either the pH or the ionic strength or composition of eluent may be changed.
- If changes in the mobile phase does not effect the separation then increase in column length or change in packing type may be necessary.

Resolution:

$$Rs = 2[T_2-T_1]/[W_1+W_2]$$

The value of Rs should always be greater than 1. R_s = 2.0 or greater is a desirable

$$\alpha = \frac{K_2}{K_1}$$

Where, K_1 = Capacity factor of the other component .

K_2 = Capacity factor of longer retained component.

When $K_2 = K_1$ i.e. $\alpha = 1$, two bands are not resolved. Therefore, α should be sufficiently greater than 1 to get better-resolved peaks. This can be obtained by optimizing capacity factor of one component. When the two bands are partially overlapped and $T_R >> t_0$ either α or N must be increased. Unless only a small increase in Rs is required (<30%), however, it is usually better to attempt an increase in α for this situation. When the two bands are badly overlapped with $t_R >> t_0$, an increase in α is normally required.

Controlling Sample Retention by Changing Solvent Strength

HPLC Method	How Solvent Strength Is Usually Varried
Reversed Phase	Water (A) plus organic solvent (B) (e.g., water-acetonitrile); increase in %B decreases k.
Normal Phase	Nonpolar organic solvent (A) plus polar organic solvent (B) (e.g., hexane-propanol); increase in % B decreases k.
Ion Pair	Same as reverse phase.
Ion Exchange	Buffered aqueous solution plus added salt (e.g.; 5 mM sodium acetate plus 50 mM NaCl); increase in ionic strength (NaCl concentration) decreases k.

Separation Goals in HPLC Method Development

Goal*	Comments
Resolution	Precise and rugged quantitative analysis requires that R_s be greater than 1.5.
Separation time	<5-10 minutes is desirable for routine procedures.
Quantitation	<2% (1SD) for assay; 5% for less-demanding analyses; <15% for trace analyses.
Pressure	<150 bar is desirable, <200 is usually essential (new column assumed).
Peak height	Narrow peaks are desirable for large signal/noise ratio.
Solvent composition	Minimum mobile phase use per run is desirable.

*Roughly in order of decreasing importance but may vary with analysis requirements

Important Information Concerning Sample composition and Properties:

1. Number of compound present
2. Chemical structure of compounds
3. Molecular weights of compounds
4. PKa values of compounds
5. UV spectra of compounds
6. Concentration range of compounds in sample of interest
7. Sample solubility

Stationary phase.

Mobile phase:

Degassing may be carried out in several ways:

1. Filtration.
2. By warming the solvent under reduced pressure.
3. By stirring it vigorously with a magnetic stirrer.
4. By bubbling helium gas through the solvent reservoir.
5. By sonication.

Criteria for selecting mobile phase: -

The choice of mobile phase depends upon the separation.

It should be of desired polarity.

It should be economical.

It should not be toxic.

It should be easily available.

Its eluting power should be proper.

Special grade of solvents (known as HPLC grade) are available for HPLC.

Column/Solvent selection Guide:

MW<2000

	RP	Adsorption	Partition
Non polar/non ionic	**RP-2/8/18**	**Si**	**amino,CN,Diol**
	ACN:Water	Heptane,	ACN,MeOH
	MeOH:Aq	ACN,$CHCl_3$	CH_2Cl_2
		CH_2Cl_2	

Soluble in Organic Solvent

	RP(pH control)	RP(ion pair)	Partition
Polar/ionic	**RP-2/8/18**	**RP-2/8/18**	**amino,CN,Diol**
	ACN/MeOH	ACN/MeOH	THF/H_2O
	+Aq. Buffer	+Aq. Buffer	Buffer,
		+ion pairing Ag.	MeOH

MW<2000 Soluble in Water

Non ionic	RP **RP-2/8/18** ACN:Water MeOH:Water	Partition **amino,CN,Diol** ACN,MeOH/Water
Acids or bases	**RP (pH control)** **RP-2/8/18** ACN/MeOH +Aq. Buffer	**RP (ion pair)** **RP-2/8/18** ACN/MeOH +Aq. Buffer +ion pairing Agent.
ionic-Amphoteric	RP(pH control) **RP-2/8/18** ACN/MeOH +Aq. Buffer +ion pairing Agent	Ion exchange columns **Ax/Cx** Aq. Buffer

The separation in HPLC can be achieved by**:**

a) Adsorption

b) Partition

c) Ion-exchange

d) Size Exclusion.

OR

by a combination of above methods.

TYPES OF COLUMN:

The following types of chromatographic columns are commercially available.

C-8, C-18, phenyl, amino, cyano, diol, silica material.

Symmetry column: These columns give us a high standard of reproducibility for confidence in long-term compliance of HPLC method, with unmatched peak symmetry for maximum sensitivity and accurate quantitation. These columns provide

•Unmatched column-to-column reproducibility.

•Superior peak shapes for all compound classes' especially basic compounds without limiting the choice of operating pH.

•They allow decreasing the limit of detection and quantitation.

•These columns are very useful because they cover all key properties of the silica, bonded phase and final chromatography.

Styragel Column: These are very useful for characterization of organic polymers with different ranges of molecular weights.

•Low to medium molecular weight samples.

•Medium to high molecular weight samples

•Ultra high molecular weight samples.

•These columns are packed with styrene divinylbenzene particles and provide high resolution and efficiency whether used alone or in a column bank.

•These are also useful at high temperature analysis.

APPARATUS FOR HPLC:

There are four basic components of a modern HPLC device

1. A solvent delivery system, including pump.
2. A means of introducing the sample i.e. sample injection system.
3. A chromatographic column with pre column.
4. Detector and recording system.

STRATEGY FOR DEVELOPING AN ANALYTICAL PROCEDURE

The first step of developing chromatographic analysis is to define the problem and state of the purpose of analysis.

In order to define the problem, the following questions should be asked,

•Is the analysis is going to be used routinely for a large number of samples? Then ease of analysis and simplicity is of great importance.

•Is a qualitative or quantitative analysis required?

•Is it necessary to separate all the constituents in the sample or a small group?

•Are the constituents similar in structure or widely diverse?

•Are the constituents present in similar concentration or is one constituent present in a large amount and other in trace amount?

•If quantitative analysis is requested, what levels of accuracy and precision are required?

•A precision of ±1 or 2% for major components of a sample.

•Can the sample be easily prepared for analysis?

•For how many different sample matrices should the method be designed?

•Are the components present that may interfere with the analysis of constituents of interest?

•What HPLC equipment and operator skills are present in the laboratory that will use the final method?

•Can the column be thermostated, and is an HPLC system for gradient elusion available?

•Will the method be run on equipment of different design and manufacturer?

•What HPLC experience and academic training do the operators have?

The next step is a literature search to find out if these compounds have been separated using other chromatographic techniques.

For example, the condition used in TLC or open column chromatography can be adapted for HPLC.

This serves as a starting point and saves valuable time.

Sample pre treatment and detection

Samples come in various forms:

1. Solution ready for injection
2. Solutions that require dilution, buffering, addition of an internal standard, or other volumetric manipulation
3. Solids that must first be dissolved or extracted
4. Samples that require sample pretreatment to remove interferences and/or protect the column or equipment from damage.

Setting up a method:

1. Assess polarity of the compound.
2. Carry out a U.V. scan of the analyte and an internal standard (if using).
3. Optimize chromatographic condition to separate drug, impurities starting materials and I.S.
4. Record chromatographic conditions.
5. Assess reproducibility and construct a calibration curve.
6. Assess precision of the assay method.
7. Calculate LOD and LOQ.
8. Assess the dynamic range.

A total analysis involves the following steps,

1. Sample collection and preparation

Each sample requires a different approach with respect to the sample collection and preparation procedure. Chemical sample may not need sample pre-treatment, as the % of impurity is less. Biological samples require sample filtration because large number of components of different nature is present. Sample filtration can be performed with membrane type filters. Deproteination of biological samples is also necessary. Several methods of protein removal can be used:

1.Ultrafiltration

2.Precipitation

3.Denaturation by heating

2. Chromatography

The majority of analysis can now be carried out using Reverse Phase Column Chromatography (RPLC). So RPLC is the method of choice unless the desired separation can not be achieved. C_{18} as a bonded phase on 3 to 10 μm silica particle containing columns called C_{18} column is used in separation of more than 80 % of all RPLC.

3. Conditions for high resolution

The combination of appropriate stationary and mobile phases and mode of elution. Although most separations are carried out at ambient temperatures, some separation will be improved at elevated temperature.

After the selection of mobile phase and stationary phase, the optimal flow rate and elution mode is determined.

If the sample to be analysed contains very complex or a mixture of compounds of diverse structure & retention behavior, then either a ternary mixture of solvents can be used isocraticaly or gradient elution.

In some cased buffer solution can be used in different pH, ionic strength. The buffer cations that can be used include Na^+, K^+, $NH4^+$ & anion PO_4^{3-}, CH_3COO^-, and citrate. The stability of buffer and ability to maintain the desired pH are very important consideration in choosing a buffering agent.

When the need of adding organic modifier to an aqueous diluent, the first considerations are the solubility of the solutes and the compatibility of the solvent with water. Methanol and acetonitrile are the widely chosen organic modifier. Ethanol, dimethyl sulfoxide, dimethyl formamide, tetrahydrofuran, dioxane are also used.

For separation of ionic or ionizable compounds ion association technique or ion exchange chromatography is used.

Mode of elution and flow rate:

•Whenever possible, Isocratic elution should be used because it eliminates turnaround time on the column and thus shortens overall analysis time.

• Retention reproducibility is more predictable with isocratic elution because equilibration of the column after gradient elution is necessary.

•But when adequate resolution can not be achieved within a reasonable length of time then only gradient elution is advisable.

•Gradient elution can be stepwise or continuous.

•Flow rate doesn't affect the retention order or K values.

•But increases in flow rate will decrease in retention time and peak width.

• When retention is adequate and a faster analysis or sharper peaks are desired, flow rate may be increased.

OPTIMISATION OF AN ANALYSIS

•In solving a general elution problem compromise may be necessary among the goals of optimisation of analysis time, resolution, detection sensitivity.

•Some times optimisation of one of these parameters is made at expense of one or more of the other parameters. For example To optimize the retention time many operating parameters should be considered.

Composition of eluent: Depending on the types of compounds involved, the problem of inadequate resolution can be tackled by changing the pH of the eluent or the amount or type of organic modifier in the solution. If mobile phase changes can not provide adequate resolution of peak longer columns (or double or multiple columns) or slightly different types of packing (C_2, C_8 or phenyl) can be tried.

Elution mode: If isocratic elution does not provide the desired resolution, the most obvious way to improve the resolution is either by isocratic elution with mixed solvent or by gradient elution.

However, if the capacity factors of the compounds to be eluted are equally affected by the modifier, then gradient elution may solve the problem.

Flow rate: Flow rate doesn’t affect the retention order or K (capacity factor) values. But increases in flow rate will decrease in retention time and peak width. When retention is adequate and a faster analysis or sharper peaks are desired, flow rate may be increased.

UV detection

General considerations

• The light source is typically a deuterium lamp, which provides acceptable light intensity from 190 to 400 nm.

• When measurements at visible wavelength 400-700 nm are required, a high-energy tungsten-halide lamp is often used.

• Most of the measurements are carried below 400 nm.

•Light from the lamp passes through a UV transmitting flow cell connected to the column and impringes on a diode that measures the light intensity I.

• Light from the lamp also directed a reference diode for measurement of the original light intensity I_0.

• The detector electronics then converts the signal from the two diodes into absorbance A.

$$A = \log (I_0/I)$$

Analyte concentration *c* in the flow cell

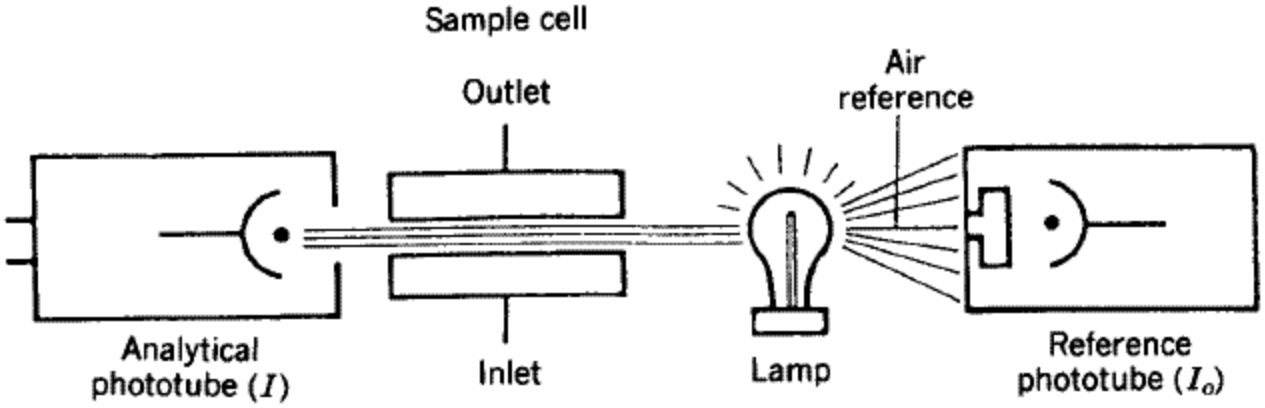
$$A = abc$$

• Ageneral goal in selecting experimental conditions that affect detection is to maximize the signal S of sample components of interest.

• In Variable wavelength detectors the wavelength selection is achieved with a diffraction grating.

• Light from the lamp enters the grating assembly through an entrance slit and focused on the grating by mirror A.

•The orientation of the grating can be varied so as to direct monochromatic light of a selected wavelength on to a second mirror B and from there to the exit slit.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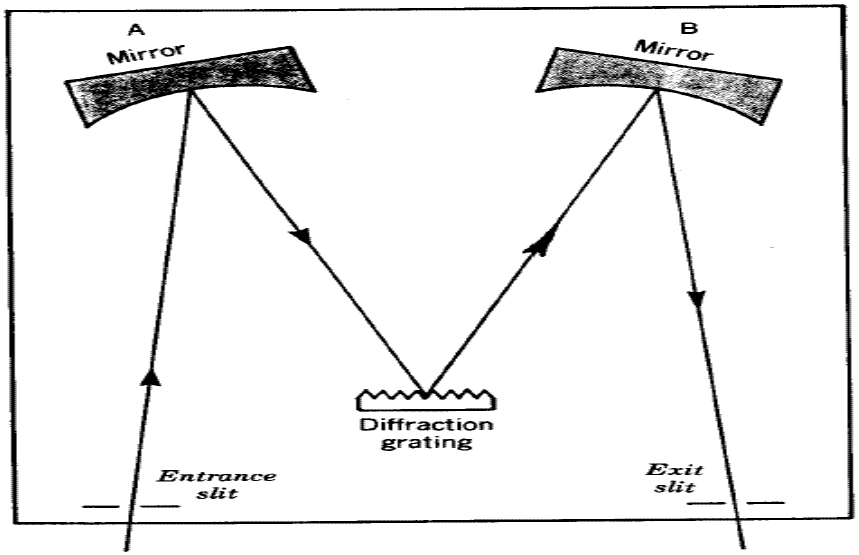
A
Mirror
B
Mirror
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Entrance
slit
Exit
slit

For variable-wavelength detectors: the grating is placed between the lamp and the flow cell.

For DAD: the grating is placed after the flow cell, so that light of different wavelengths can be measured simultaneously with an array of sensing diodes.

Choice of wavelength:

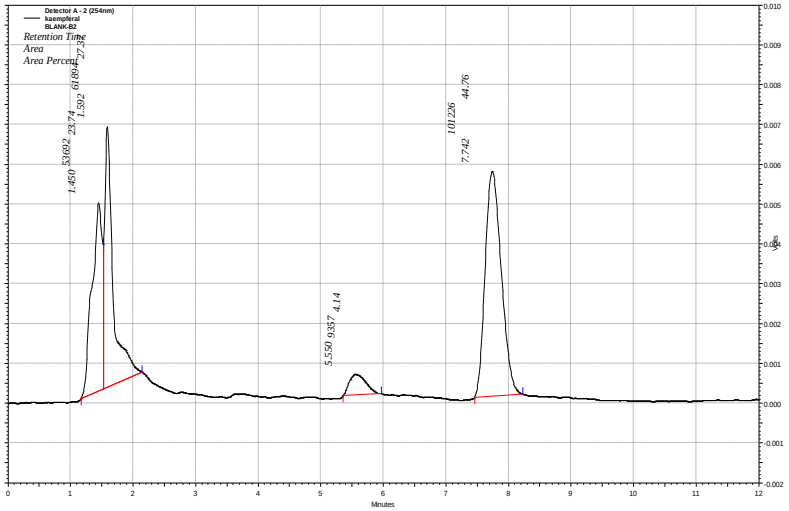
• This requires a knowledge of of the UV spectra of individual sample components (Picrolive).

• If analytical standards are available check UV spectra before method development.

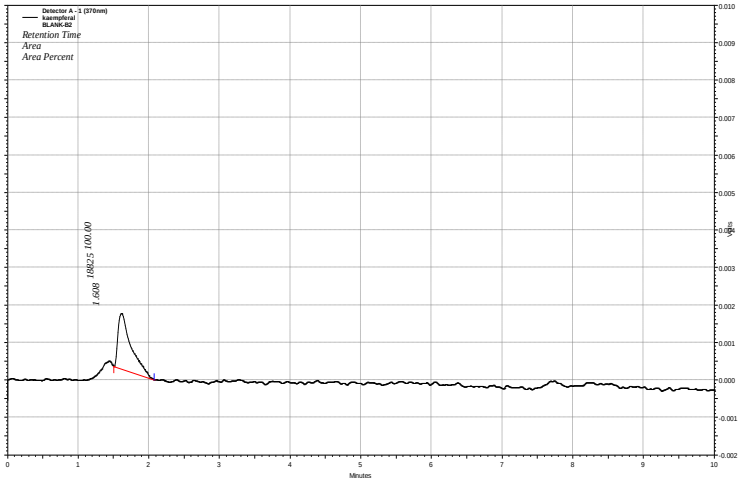
•A DAD permits the acquisition of UV spectra for all sample components during method development.

Sample absorbance as a function of molecular structure:

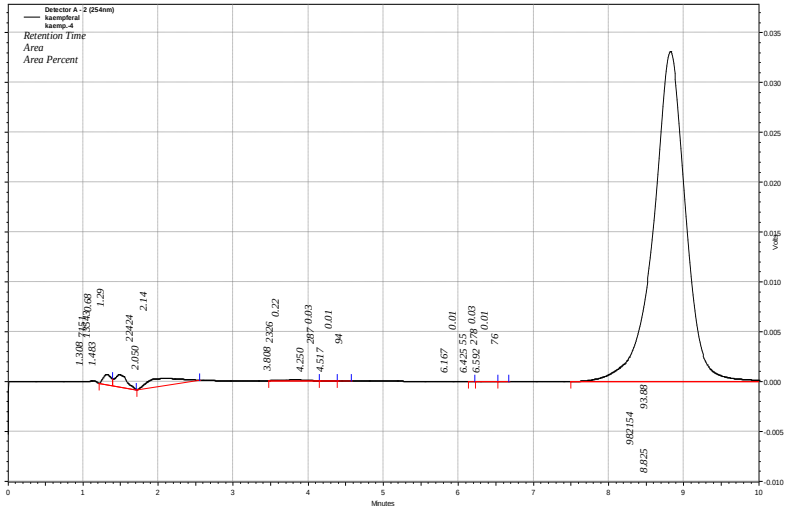
- The wavelength chosen for UV detection must provide acceptable absorbance by the various analytes in the sample combine with the acceptable light transmittance by the mobile phase.
- It is also important to select a wavelength at which sample interferences have minimal absorption. (Kaempferol).
- The detector signal is proportional to the molar absorptivity of the compound of interest.
- The adequate sensitivity for the analysis of major sample components molar absorptivity must be greater than 10 at some wavelength above 185 nm.
- For trace analysis the molar absorptivity of the trace compound must be more than 1000.
- With molar absorptivity less than 100 it is not possible to analyze.



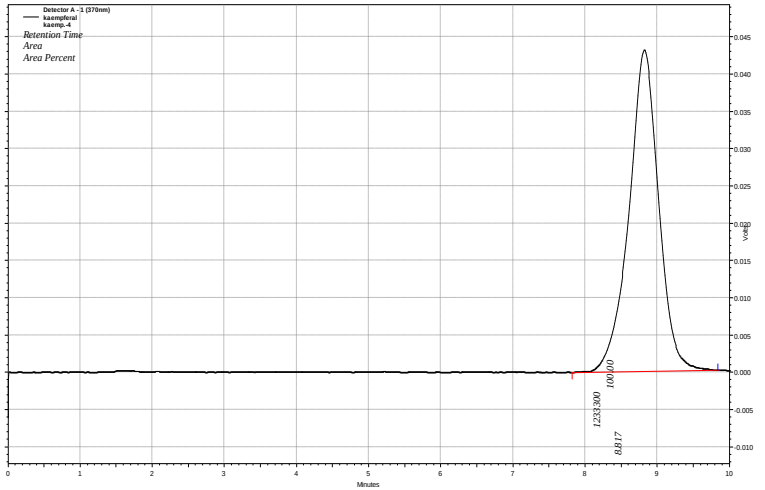
Blank Blood at 254 nm



Blank Blood at 370 nm



Kaempferol at 254 nm



Kaempferol at 370 nm

Organic compounds not suitable for UV detectors:

• Saturated hydrocarbons and their amino and nitrile derivatives.

• Saturated hydrocarbons with ether, hydroxy, chloro, COOH, COOR groups have molar absorptivity less than 100 and may required UV detection at low UV values (185 – 210 nm).

• When wavelength is less than 210 the sample interferences generally absorb strongly and the choice of mobile phase solvents and additives is some what restricted.

• Aromatic compounds have molar absorptivity more than 1000 and detected at UV values more than 210 nm).

TABLE 3.1 Representative Molar Absorptivity Values for Some Common Functional Groups

Compound Type	Chromophore	Wavelength (nm)	Molar Absorptivity
Acetylide	$-C\equiv C-$	175–180	6,000
Aldehyde	$-CHO$	210	1,500
		280–300	11–18
Amine	$-NH_2$	195	—
Azido	$C=N$	190	5,000
Azo	$-N=N$	285–400	3–25
Bisulfide	$-S-S$	194	5,500
		255	400
Bromide	$-Br$	280	300
Carboxyl	$-COOH$	200–210	50–70
Ester	$-COOR$	205	50
Ether	$-O-$	185	1,000
Iodide	$-I-$	260	400
Ketone	$C=O$	195	1,000
		270–285	15–30
Nitrate	$-ONO_2$	270	12
Nitrile	$-C\equiv N$	160	—
Nitrite	$-ONO$	220–230	1,000–2,000
		300–400	10

Nitro	$-NO_2$	210	Strong
Nitrose	$-N=O$	302	100
Oxime	$-NOH$	190	5,000
Sulfone	$-SO_2$	180	—
Sulfoxide	$S=O$	210	1,500
Thioether	$-S-O$	194	4,600
		215	1,600
Thioketone	$C=S$	205	Strong
Thiol	$-SH$	195	1,400
Unsaturation	$-(C=C)_3-$	260	35,000
Conjugated	$-(C=C)_4-$	300	52,000
	$-(C=C)_5-$	330	118,000
Aliphatic	$-C=C-$	190	8,000
	$-(C=C)_2-$	210–230	21,000
Alicyclic	$-(C=C)_2-$	230–260	3,000–8,000
Miscellaneous	$C=C-C\equiv C$	291	6,500
compounds	$C=C-C=N$	220	23,000
	$C=C=C=O$	210–250	10,000–20,000
		300–350	Weak
	$C=C-NO_2$	229	9,500
Benzene	C_6H_6	184	46,700
		202	6,900
		255	170
Diphenyl	$C_{12}H_{10}$	246	20,000

Mobile-Phase absorbance as a function of composition:

- The mobile phase must transmit sufficiently.
- Baseline noise increases significantly when A > 0.7 for mobile phase.
- Mobile phase A must be < 0.5 for detection.
- If Mobile phase A is >1.0 the detector became unusable.

TABLE 3.3 Useful Solvent Mixtures with Low Background Absorbances (< 0.5 AU) at ≥ 200 nm

Aqueous mobile-phase mixtures
0–26% methanol–water
0–28% isopropanol
0–20% THF
0–100% acetonitrile–water
ACN–water with additives
0.2% acetic acid
0.4% trifluoroacetic acid
250 m*M* NaCl
> 25 m*M* potassium (or sodium) phosphate (pH < 5)
25 m*M* sodium (or potassium) phosphate (pH 6.8)

• Normal-phase chromatography uses solvents that are generally more strongly absorbing – Detection at higher wavelength is usually required.

TABLE 3.4 UV Absorbance of Normal-Phase Solvents as a Function of Wavelength

	Absorbance (A) at Wavelength (nm) Indicated						
Solvent	200	210	220	230	240	250	260
Ethyl acetate	>1.0	>1.0	>1.0	>1.0	>1.0	>1.0	0.10
Ethyl ether	>1.0	>1.0	0.46	0.27	0.18	0.10	0.05
Hexane	0.54	0.20	0.07	0.03	0.02	0.01	0.00
Methylene chloride	>1.0	>1.0	>1.0	1.4	0.09	0.00	0.00
Methyl-*t*-butyl ether	>1.0	0.69	0.54	0.45	0.26	0.11	0.05
n-Propanol	>1.0	0.65	0.35	0.15	0.07	0.03	0.01
i-Propanol	>1.0	0.44	0.20	0.11	0.05	0.03	0.02
Tetrahydrofuran	>1.0	>1.0	0.70	0.50	0.30	0.16	0.09

Signal, Noise and assay precision:

• Precise results are of prime importance when carrying out quantitative analysis by HPLC.

• Signal refers to the baseline-corrected absorbance of analytical peak and noise refer to the width of the baseline.

• Baseline noise usually has two components:

Short term (high frequency) from stray light and the detector electronics, it is more important for a rise time of 0.1s.

Long time from temperature fluctuations, pump noiseand/or a dirty column, it is more important for a rise time of 0.5s.

• If assay precision is affected by noise (small S/N values), the imprecision (CV) should increase for small peak absorbance values or smaller concentration of analyte.

• At lower concentrations CV increases as the concentration of of analyte decreases.

Maximizing S/N for better assay precision:

- Better precision is obtained by increasing S/N ratio.
- A maximum signal can be obtained by choosing a wavelength that gives maximum absorbance.
- Signal can be increase by

    Increase in the analyte concentration. (C_0) *

    Increase in the inject sample volume (Vs) *

    Increase in column efficiency (N)

    Decrease in the column volume (Vm)

    Decrease in analyte retention (k)

* Too large sample volume leads to peak broadening with loss of resolution.

• Noise increases as light intensity falls off.

• An increase in high-frequency noise can be expected as the detector lamp age.

• When the lamp energy dropped to 15% 0f its original value a doubling of noise was noted.

• The precision of a method can vary with time for the same detector or when different detectors are used.

• Pump pulsation is also responsible to long term noise.

• Baseline noise of this type is characterized by a regular rise and fall of the baseline.

• Pulse dampers can markedly reduce the effect of pump pulsation.

• When on line blending is used to prepare the mobile phase and the absorbances of the two or more solvents being mixed are different, the mobile phase may show oscillation in absorbance.

• The result is a repeating up and down movement of the baseline.

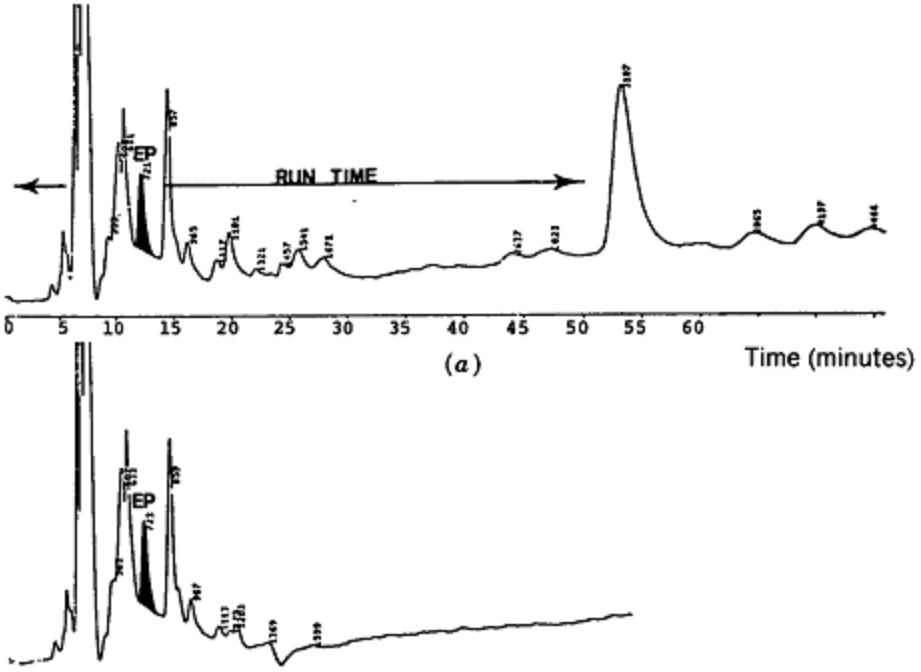
• This absorbance related noise can be eliminated by adding a nonretained, UV absorbing compound to one of the two solvents being mixed on-line to equalize their absorbances.

• Another kind of long term noise results from contamination of the column by prior sample injections.

• Such samples may contain compounds that leave the column at a later time; over time, several such late eluters (from different prior samples) may overlap to produce an irregular baseline.

Noise due to late eluters can be reduced in several ways:

• Sample cleanup prior to HPLC, (also enhance detection by removing sample interferences and/or concentrating the analyte)
• Column cleanup with a strong solvent,
• Use of a guard column, gradient elution
• Column switching



Effect of late eluters on long-term baseline noise. Isocratic reversed-phase analysis of plasma extract for drug EP. (*a*) Separation without column switching; (*b*) separation with column switching.

TABLE 3.6 Systematic Approach for Maximizing UV Detection Sensitivity (S/N')[a]

1. Select wavelength for maximum ε (S).
2. Inject largest possible sample volume (S).
3. Concentrate sample for increase in mass injected (S).
4. Reduce k to minimum possible (but no baseline upset or interference peaks) (S).
5. Consider alternative (non-UV) detector if Eq. 3.4 indicates UV detection unlikely to be acceptable (S).
6. Increase detector time constant (N').
7. Ensure that aged lamp is replaced with newer lamp (S, N').
8. Use pulse damper to eliminate pump noise if necessary (N').
9. Match UV absorbance of A and B solvents if on-line mixing is used (N').
10. Minimize late eluters with sample cleanup, gradient elution, or column switching (N').

[a] (S) and (N') for each operation indicates an effect on signal or noise, respectively.

Diode Array UV Detectors:

- **DAD allow simultaneous collection of chromatograms at different wavelengths during a single run.**
- **UV spectrum of each separated peak is also obtained as an important tool for selecting an optimum wavelength for the final HPLC method.**
- **By examining the UV spectrum for a peak from beginning to end, peak purity can be evaluated.**
- **Another way to test peak purity is to separate the sample by an alternative method, e.g. reversed-phase HPLC followed by normal-phase HPLC.**
- **The use of a DAD is also important for peak tracking or the matching of peaks that contain the same compound between different experimental runs during method development.**

CDRI compound No. 93/478 (I)

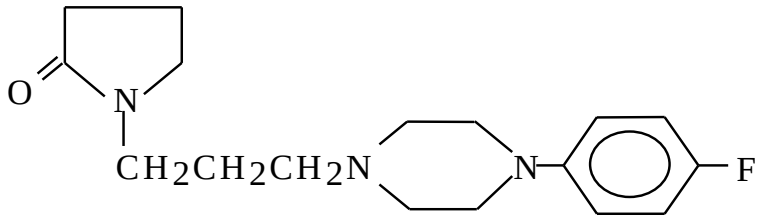


Figure. 2

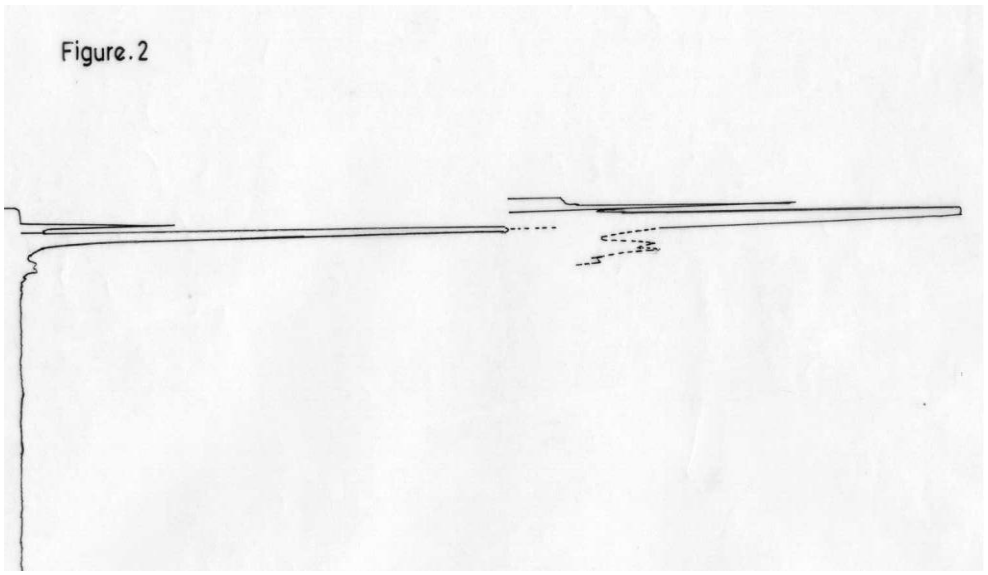


Figure. 3

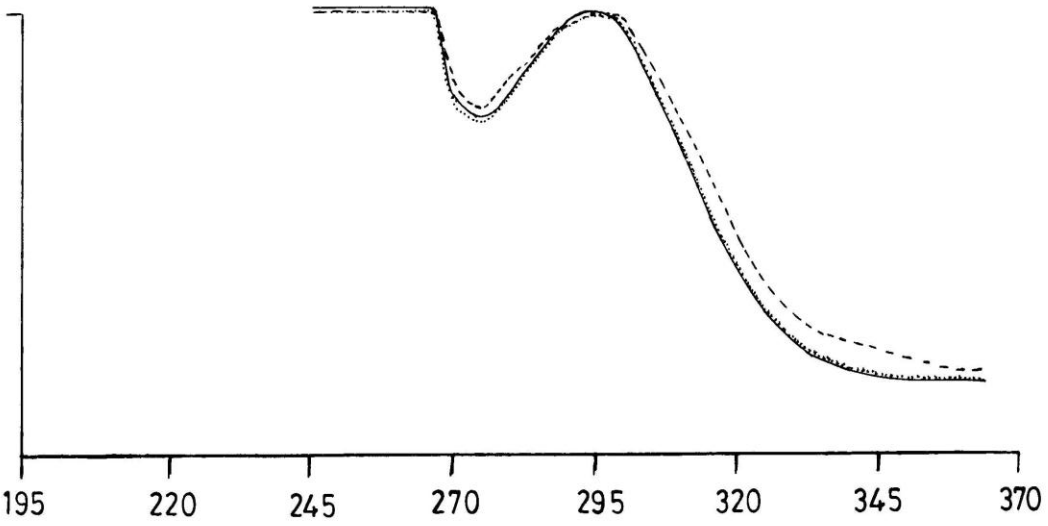


Figure . 4

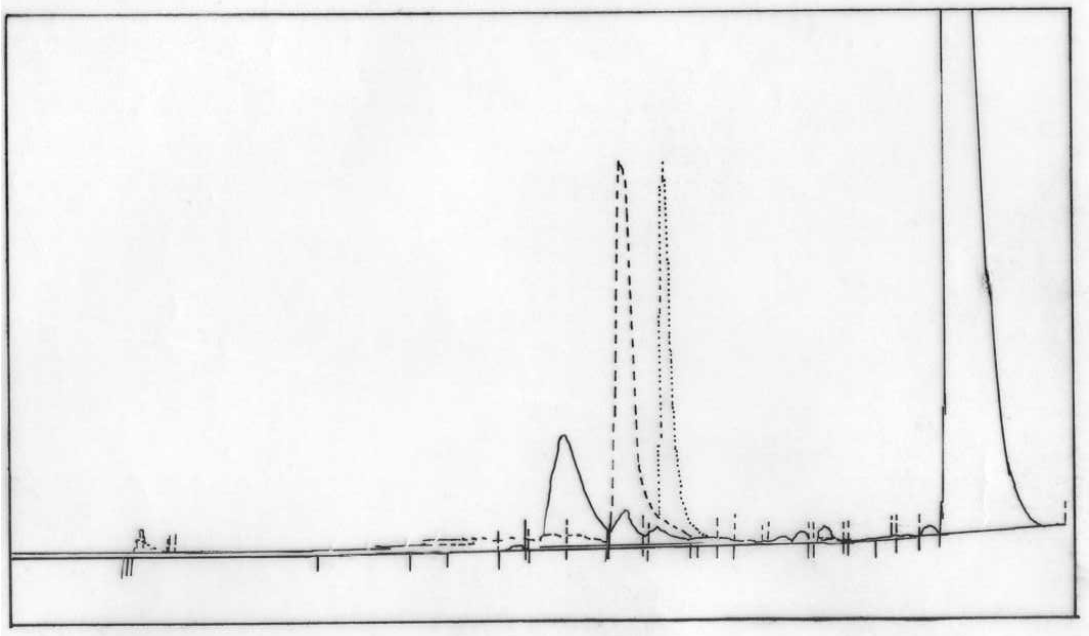
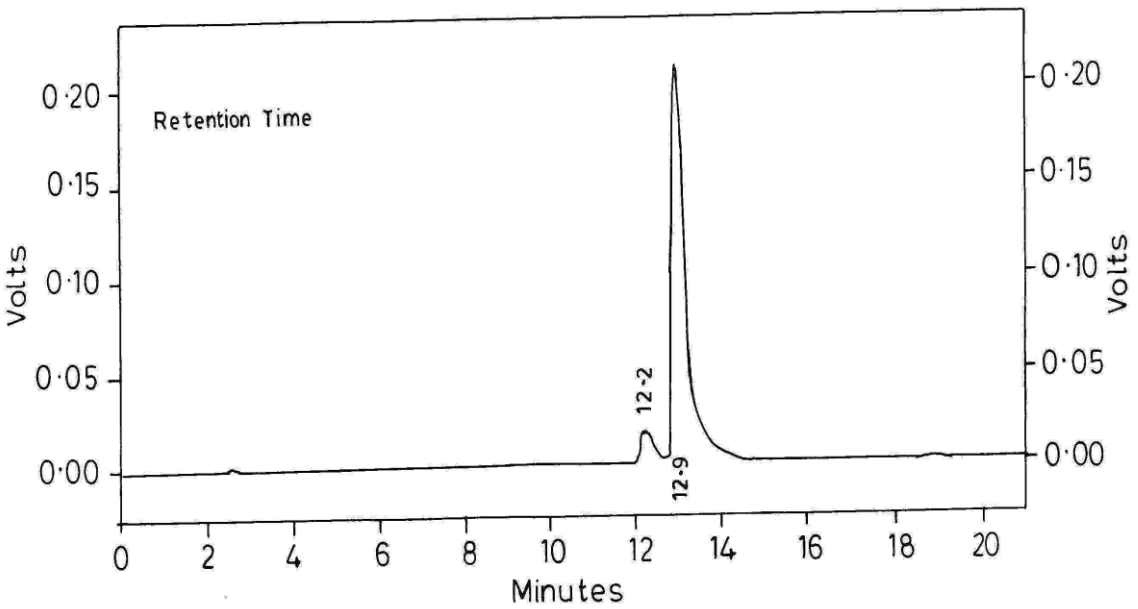
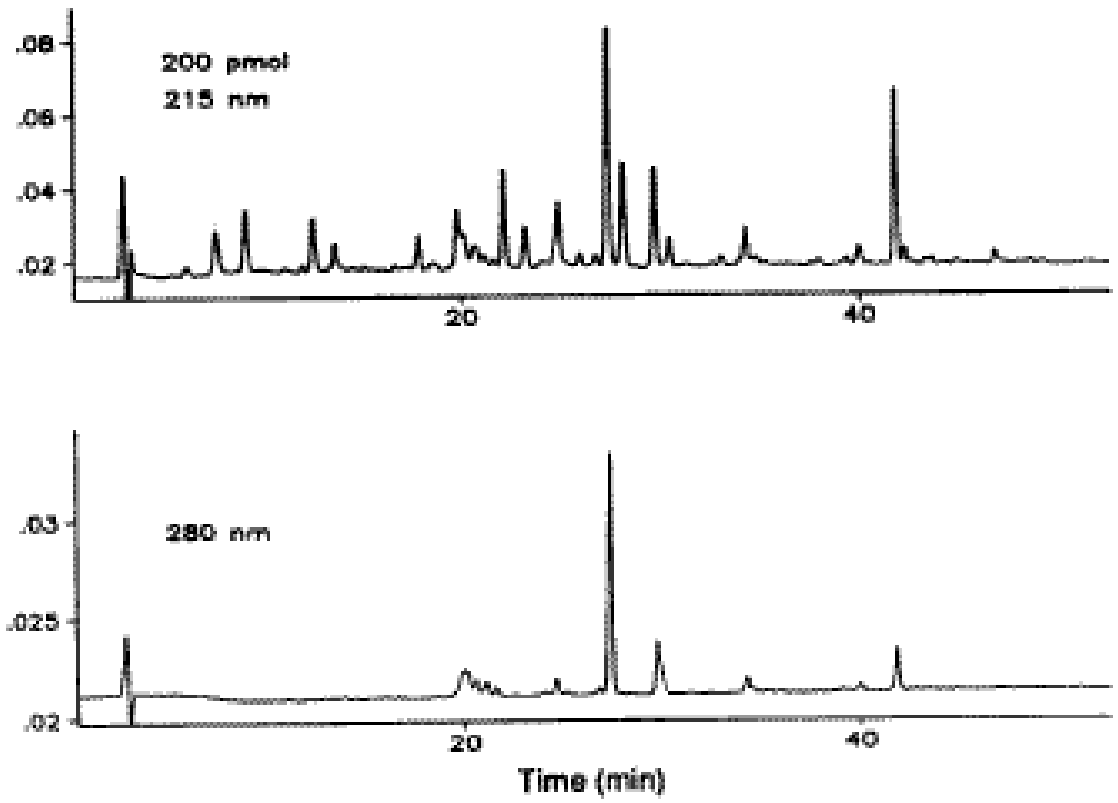


Figure. 5


Retention Time
0·20
0·15
0·10
0·05
0·00
Volts
12·2
12·9
0
2
4
6
8
10
12
14
16
18
20
Minutes
— D: \AKD \93478 \93478j~1, Detector A-2



Display of a single chromatogram at different wavelength

Fluorescence Detection:

• Detection based on analyte fluorescence (FL) are sensitive and selective.

• These are ideal for trace analysis and complex sample matrices.

• FL detection is typically three orders of magnitude more sensitive than UV.

• HPLC procedures with FL detection are used routinely for assays in the low ng/ml. range, and concentrations of low pg/mL often can be measured.

• The linearity range for these detectors is potentially similar to that of UV detectors

Other HPLC detectors

- Several times UV detectors may prove unsuitable because the analyte(s) of interest have no UV absorption.
- The analyte(s) has only a small absorptivity ($\varepsilon < 100$).
- The mobile phase may not allow low UV detection.
- The analyte concentration is relatively low.

Universal Detection:

The universal detectors give a response for almost all sample components, including those with poor UV sensitivity.

Universal detectors are used primarily in two applications:

1. For samples with very low values of e,
2. To provide a more representative analysis for unknown samples by means of area normalization.

Universal detectors can also be used with mobile phases that absorb strongly in the UV region.

The Refractive index (RI) detector:

• Refractive index is a physical property of all compounds.

• Any compound can be detected at moderate levels.

• The mobile phase components, including solvents and additives, will also show significant RI response.

• Gradient elusion using RI detectors is impractical.

• Other factors such as a need for stringent temperature control, the effects of dissolved gases in mobile phase, and lack of sensitivity for trace analysis, limit the use of the RI detector for many routine applications.

VALIDATION OF AN ANALYICAL METHOD

Validation of an analytical method is the process by which it is established, by laboratory studies, that the performance characteristics of the method meet the requirements for the indented analytical application.

Why validation?

- To demonstrate the performance and reliability of a method.
- To generate confidence on the results.

Validation parameters:

- Analytical reference standard.
- Selectivity.
- Limit of detection. (LOD)
- Limit of quantification. (LOQ)
- Range and linearity.
- Precision.
- Accuracy.
- Stability.

Analytical reference standard:

Standard should be of known form.

Standard should be of known purity.

Should be monitored to ensure no decomposition or contaminaton.

Limit of detection: The lowest amount of analyte, which can be detected but not quantified. (S/N = 3)

Limit of quantification: The lowest amount of analyte, which can be measured with acceptable precision and accuracy.

Precision: The closeness of the replicate determination by inter-day and intra day variation.

$$\% \text{ RSD} = \text{SD}*100/\text{Mean}$$

ANOVA and Confounded Precision

$$\% \text{ RSD} = (\text{Mean Square Variance})1/2 *100/\text{Mean}$$

Accuracy: Closeness of the determined concentration to the true value.

$$\% \text{ DFA} = \frac{(\text{Mean Calculated Concentration} - \text{Concentration Taken})*100}{\text{Concentration taken}}$$

Difference between Pharmaceutical analysis and bio-analysis:

- Concentration
- Concentration range
- Matrix effects
- Cellular material or debris
- Macromolecules
- Metabolites or precursors
- Concomitant drug therapy
- Species differences.

VALIDATION OF A BIO-ANALYICAL METHOD

- Validate the method for each matrix.
- Preparation of spiked samples.
- Analyte stability.
- Standard solution stability.
- Stability in the bio-analytical matrix.
- Recovery.
- Linearity of detector response.
- Range and linearity of the assay.
- Precision and accuracy of the experiments.
- Selectivity.

Validation Parameters as per USP guide lines

Analytical performance parameter	Quantitation of major components and active ingredients	Quantitation of impurities and degradation products	Limit test of impurities and degradation products	Determination of performance characteristics
Accuracy	Yes	Yes	$	$
Precision	Yes	Yes	No	Yes
Specificity	Yes	Yes	Yes	$
Limit of detection	No	No	Yes	$
Limit of quantitation	No	Yes	No	$
Linearity	Yes	Yes	No	$
Range	Yes	Yes	$	$
Ruggedness	Yes	Yes	Yes	Yes
$ May be	**required,**	**depending on**	**the nature of**	**the specific test**

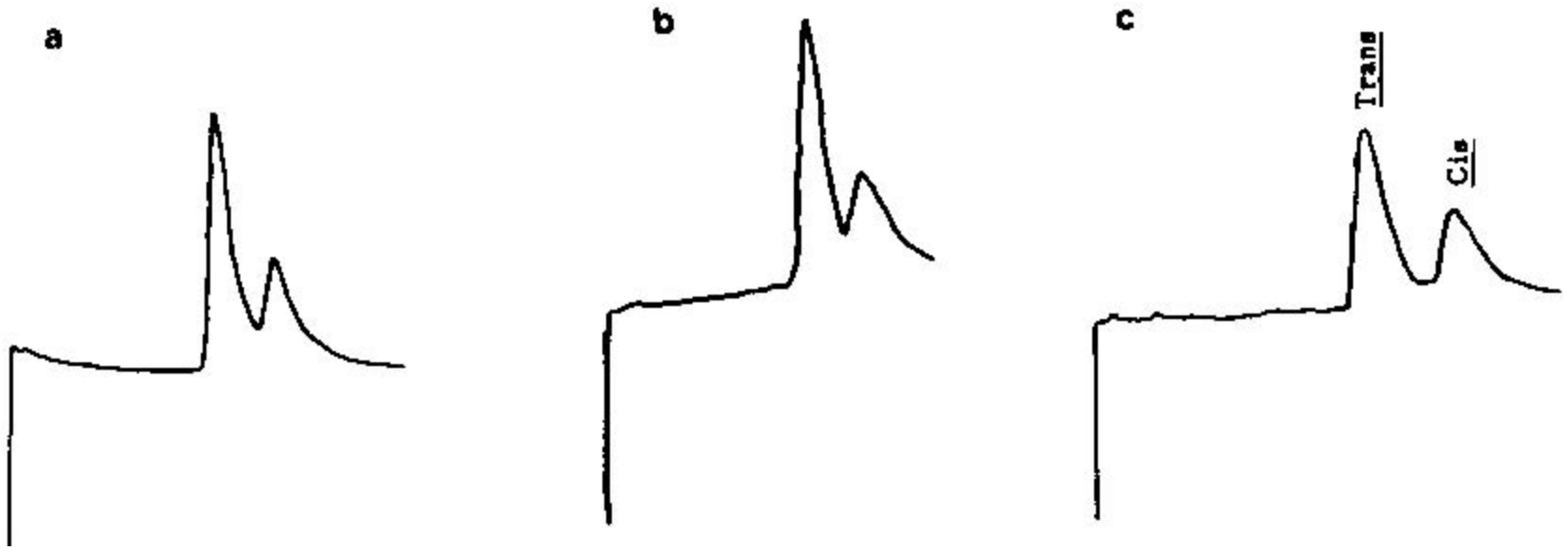
Validation Parameters as per ICH guide lines

Type of analytical procedure	Identification	Impurities testing Quantitative	Impurities testing limit test	Assay
Accuracy	No	Yes	No	Yes
Precision				
a. Repeatability	No	Yes	No	Yes
b. Intermediate precision	No	Yes	No	Yes
Specificity	Yes	Yes	Yes	Yes
Limit of detection	No	No	Yes	No
Limit of quantitation	No	Yes	No	No
Linearity	No	Yes	No	Yes
Range	No	Yes	No	Yes

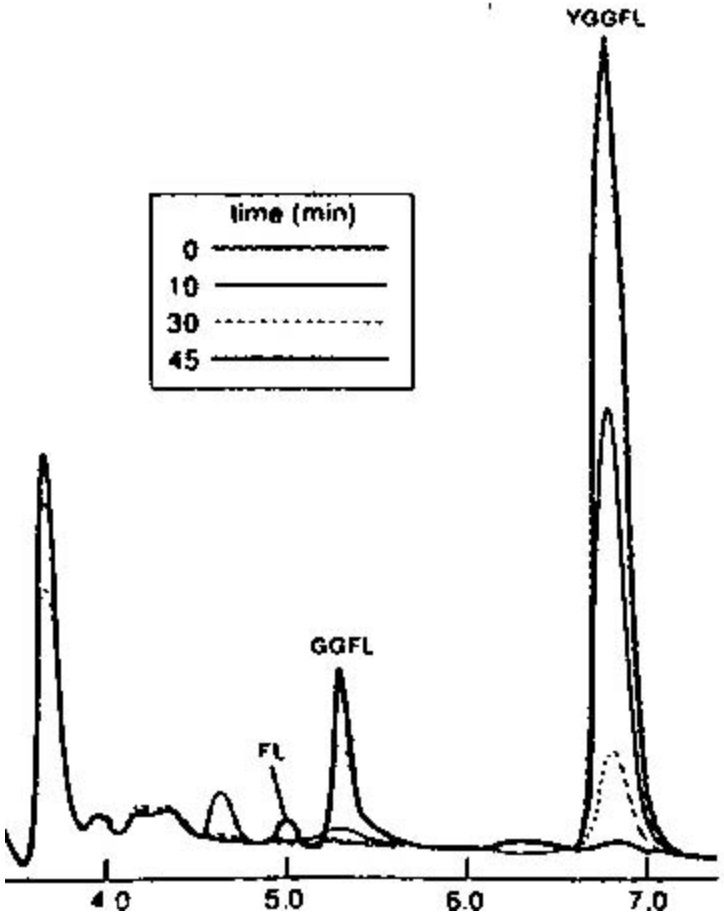
System suitability parameters for recommendations (USP)

Parameter	Recommendation
Retention factor (k)	The peak should be well resolved from other peaks and the void volume, generally k should be > 2.0.
Repeatability	A relative standard deviation (RSD) of 1% or less for five or analysis is desirable.
Relative retention	This parameter is nonessential when the resolution is stated.
Resolution (R_s)	R_s should be > 2.0 between the peak of interest and the closest eluted potential interferent (impurity, excipients, degradation product or internal standard).
Tailing factor (T)	T should be 2 or less.
Theoretical plate Number (N)	Generally, the method should provide more than 2000 plates.

Effect of pH on the separation of centchroman



Enzymatic degradation of Leucine enkephaline



Separation of arteether isomers

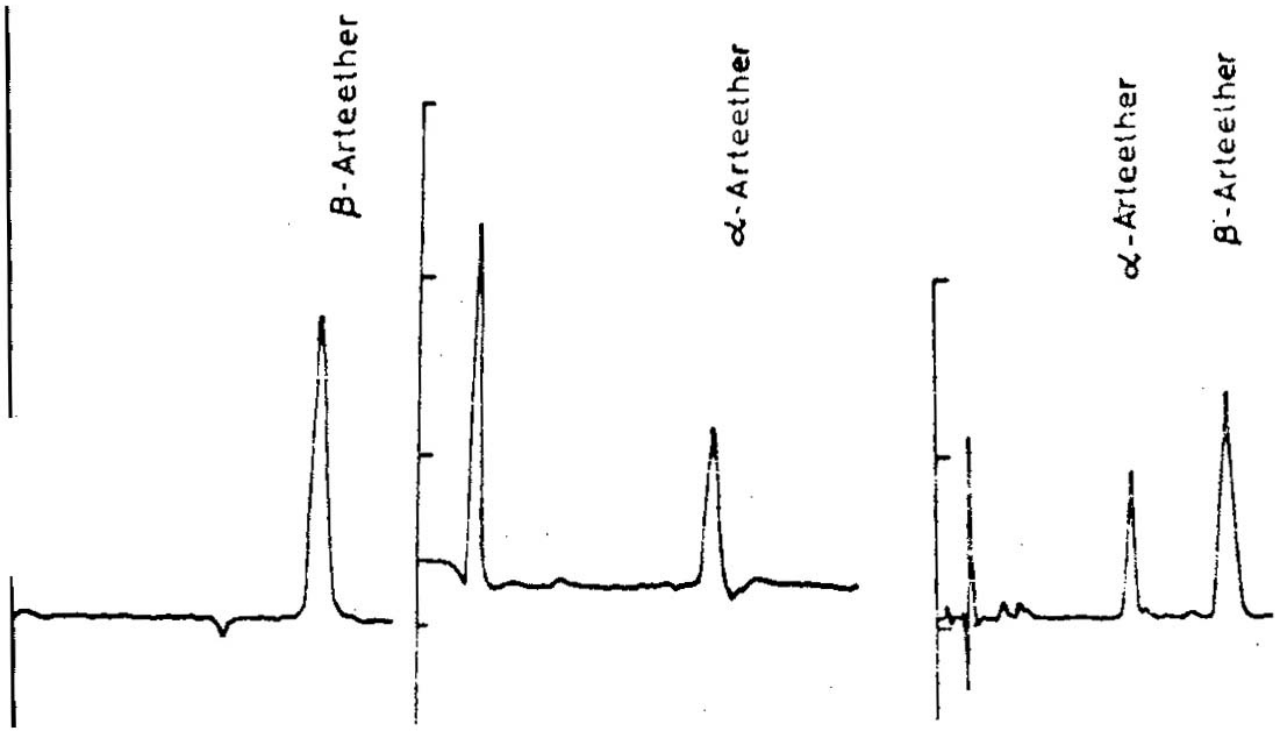
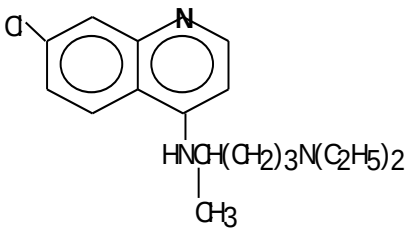
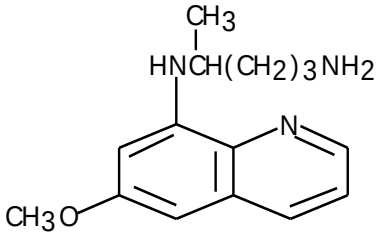


Figure-1

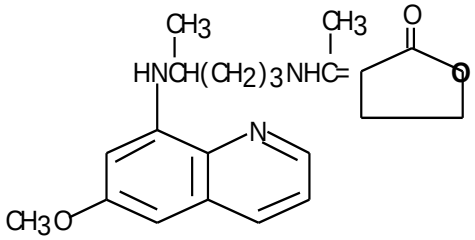
Structure of Chloroquine, Primaquine and Bulaquin



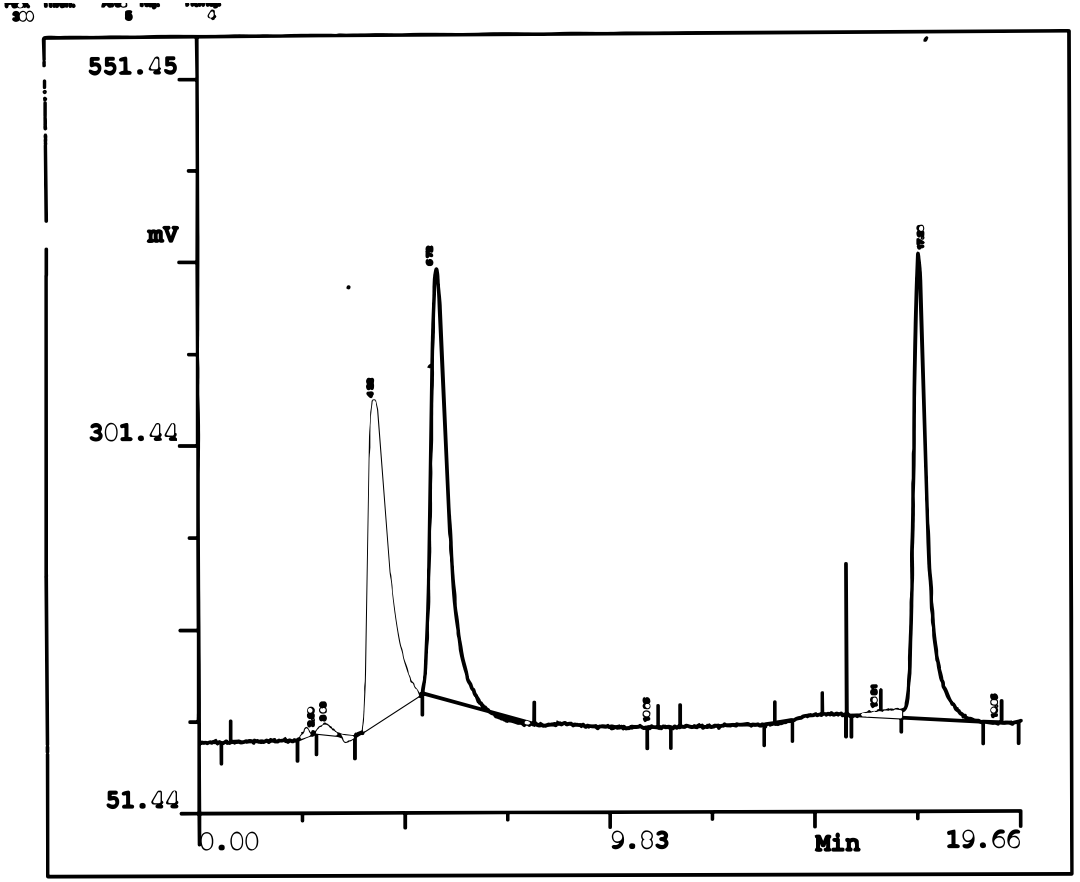
Chloroquine

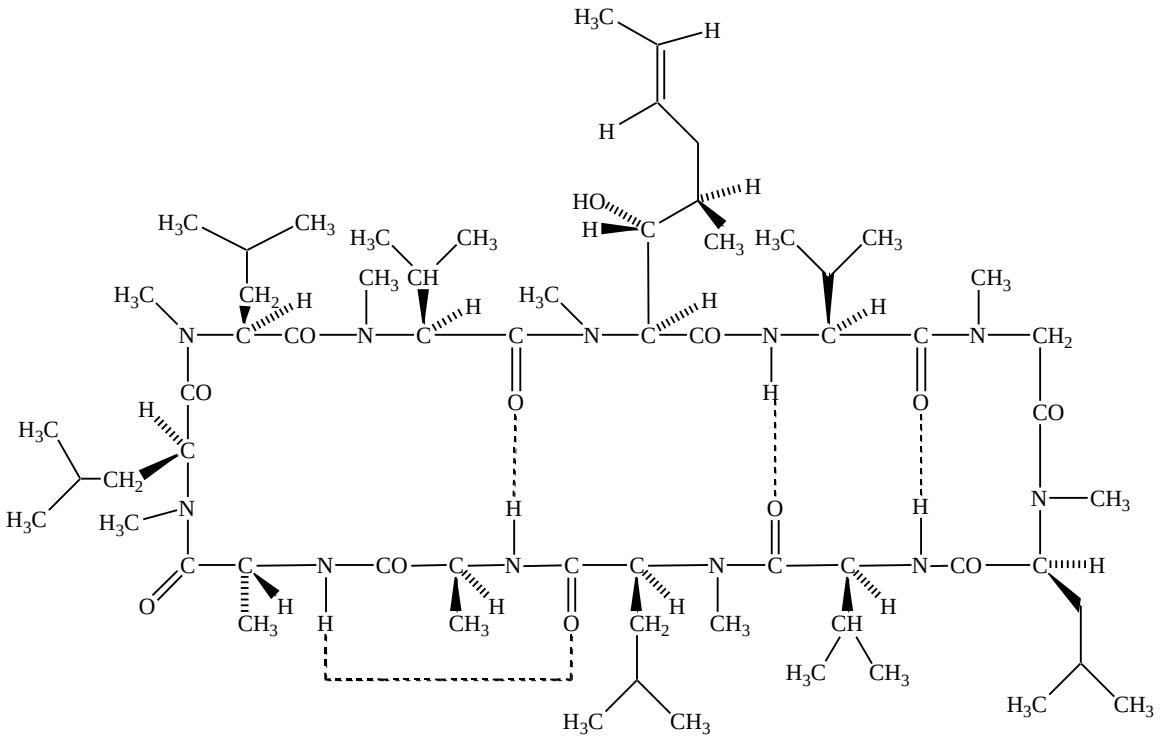


Primaquine



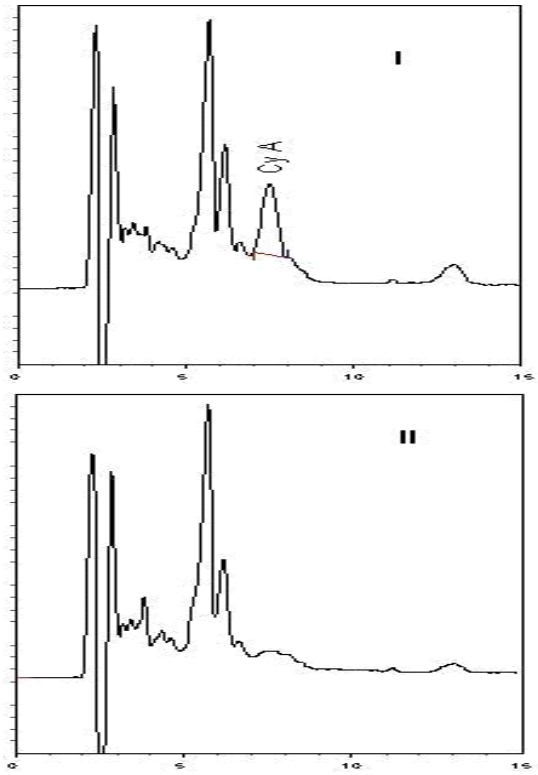
Separation of chloroquine, primaquine and 80/53 [Mobile phase 0.01M sodium acetate buffer pH 5.6 and acetonitrile (45:55). RP select-B C_8 endcapped]



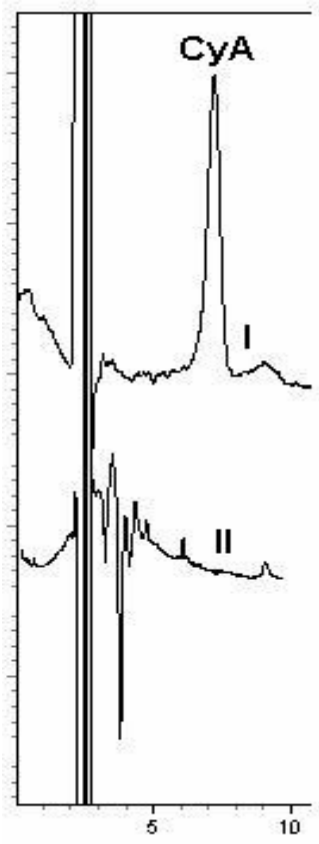


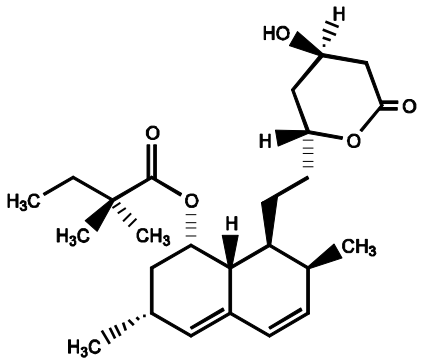
Chemical Structure of Cyclosporine

Chromatogram of extracted serum (I) Cyclosporine spiked serum; (II) blank serum

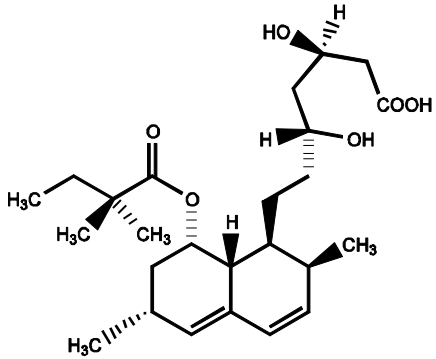


Chromatogram of Formulation (I) Cyclosporine Loaded; (II) Blank Formulation




HO
H
O
H
O
O
H3C
H3C
CH3
O
H
CH3
H3C

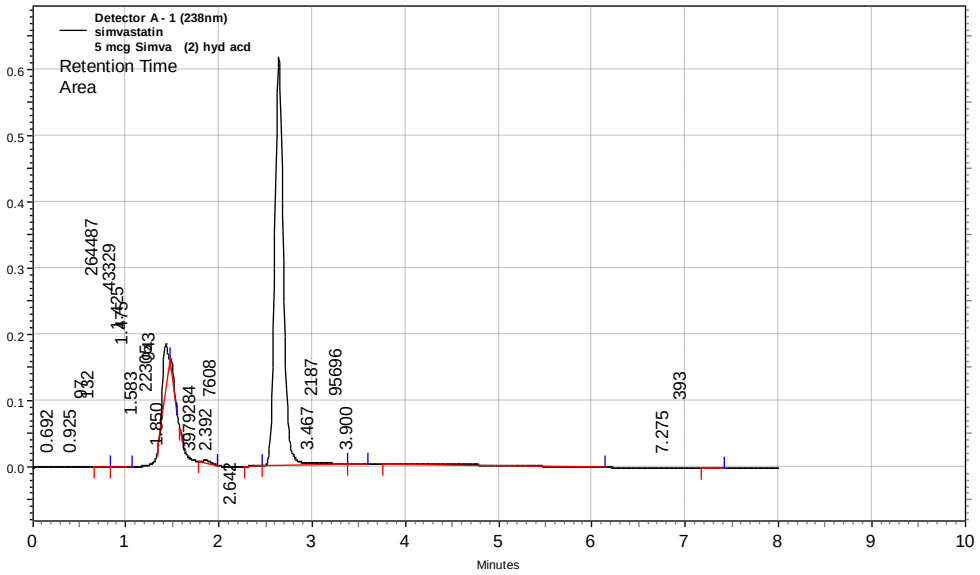
a.


HO
H
COOH
H
OH
O
H3C
H3C
CH3
O
H
CH3
H3C

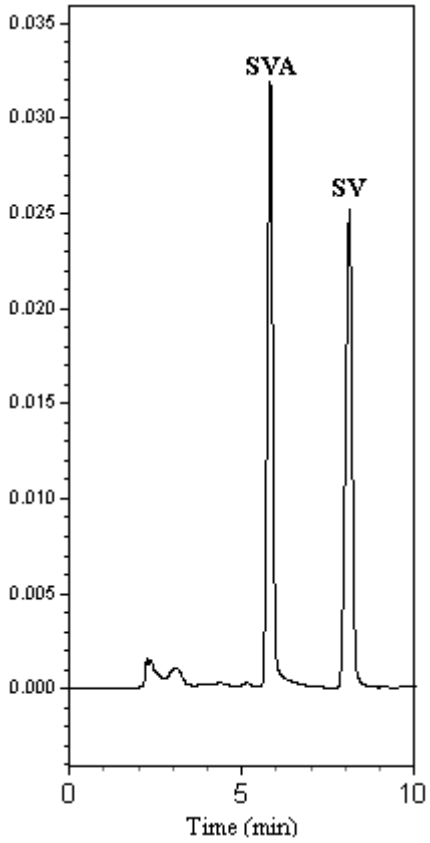
b.

Column C-18

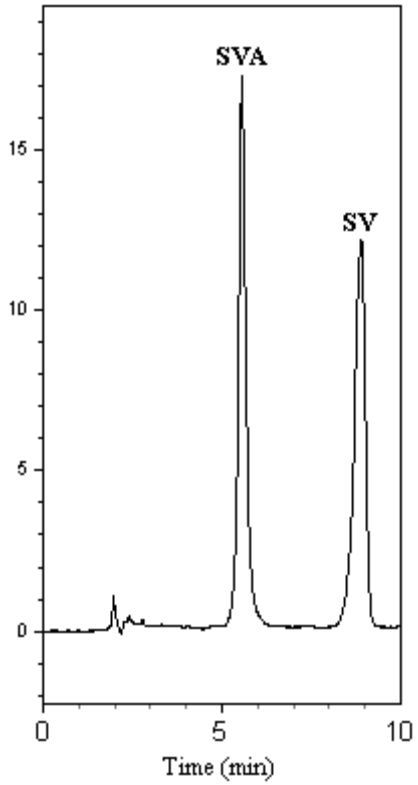
Mobile phase - Phosphate buffer pH 7.4 and ACN (3:7)



Column C-18
Mobile Phase 0.05 M OSA (pH 4.0) and acetonitrile (20:80 v/v)

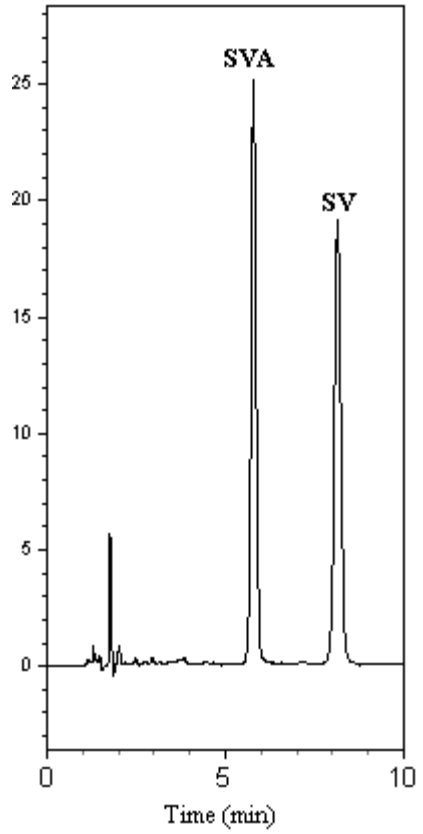


Column C-8
Mobile Phase 0.05M OSA (pH 4.0) and acetonitrile (40:60 v/v)



Column CN

Mobile Phase 0.2% TMAH solution (pH 3.0) and ACN (60:40 v/v)



Possible Problems Uncovered During Method Development and Validation

Problem	Comment
Low Plate numbers	Poor choice of column, secondary retention, poor peak shape effects.
Column variability	Poor choice of column, secondary retention effects.
Short column life	Poor choice of column, need for sample pretreatment, 3>pH>7.
Retention drift	Insufficient column equilibration, need for sample pretreatment, loss of bonded phase.
Poor quantitative precision	Need for better calibration, identification of sources of error.
New interference peaks discovered	Initial separation inadequate or initial samples not representative.

Column Contaminated:

- Peak Symetry distorted
- Base line noise increases
- Base line drift

Regeneration of column:

Disconnect the detector and flush with:

Solvent	**Volume through Column (**ml)
Water	200
ACN:water (1:1)	50
ACN	50
Chloroform	50
Hexane	50
Chloroform	50
ACN	50
ACN:water (1:1)	50

Return to the fresh mobile phase.

How to increase the life of a column:

- Check column efficiency before commissioning and during its service.
- Use only filtered solvents.
- Inject only filtered samples.
- Always use a pre column.
- For normal columns pH range is 2-8.
- Keep temperature below 80^0C.
- Donot subject column to mechanical wear and tear. (Impact, Sudden pressure change or rapid change of eluent).
- Never let the column dry.
- Separate column for separate application.
- Optimum concentration of salts in buffers is 0.0001-0.2M. Column should be carefully cleaned after use of buffers.

Trouble shooting for HPLC column

Problem	System	Possible cause	Remedy
Pressure drop too high though the flow rate is constant.	Input pressure rising continuously.	Frit blocked or Column contaminated.	Change the frit. Replace 2 mm bed of the column with the same materialin MeOH or heptane. Avoid it by using precolumn or filtered solvents and sample solution.
Spikes on the baseline/bubbles in the solvent.	Unstable base line. Intermittent air peaks. Eratic flow rate.	Gases dissolved in the solvent.	Degas the solvent.
UV absorption too high.	Low detection sensitivity. Erratic retention times. Interfering peaks during gradient elusion.	Polar/nonpolar impurities in the eluent. Dissolved gases/chemicals in the eluent.	Use special chromotographic solvents. Degas the solvents.

Problem	System	Possible cause	Remedy
Lack of resolution/poor efficiency.	Broad peaks/ Shouldering/ tailing	Column contaminated with strongly retained compounds.	Flush the column with the optimum solvent.
Regeneration does not restore separating efficiency/selectivity	Erratic retintion times. Overlapping peaks. Sudden rise in pressure.	Top of the column is contaminated with irreversibly adsorbed components.	Replace 2 mm bed of the column with the same materialin MeOH or heptane. Avoid it by using precolumn or filtered solvents and sample solution.
Column show lack of efficiency/ Selectivity following prolonged use.	Broad peaks. Possibly tailing. Overlapping peaks. Plate no. lowered.	Residual solubility of the silica in the eluent.	Replace 2 mm bed of the column with the same materialin MeOH or heptane. Column approaches its life. Reverse the direction of column for prolong use.

Problem	System	Possible cause	Remedy
Poor reproducibility	Erratic retention time. Tailing/fluctuating retention times for unmodified silica gel.	Fluctuating temperature. Contaminated column. Fluctuations in the eluent water content.	Thermostat the system. Regenerate the column. Replace 2 mm bed. Always use about 1% of a polar modifier in dry non polar eluents.
In consistant separating efficiencies.	Broad peaks. Overlaps. Plate No. Lowered.	Too high sample concentrations Top of column contaminated.	Reduce the injection volume. Dilute the sample.


Thank You