Method development & optimization

The overall process is influenced by the nature of the analytes and generally follows the following steps:

1 | Selection of the HPLC method and system
2 | Establish sample prep procedure
3 | Select detector
4 | Selection of initial conditions
5 | Perform preliminary separations
6 | Selectivity optimization
7 | System optimization
8 | Method validation

1 Selection of the HPLC method and system

Method development is not difficult when a literature reference can be found to same or similar needs. Methods are published in pharmacopeia, in column manufacturer application databases and as published scientific studies. This can provide good guidance for the planned work, but what happens when references to the compounds of interest do not exist?

Different approaches are possible, and trial and error is the least successful way forward. A chromatographer normally has access to a wide variety of equipment, columns, mobile phase compositions and operational parameters which make high performance liquid chromatography (HPLC) method development seem complex. In this chapter direction will be given to make your method development intuitive and successful, with emphasis on column selection.

Method goals

Method development is to define needs, set goals, and make experimental plans, then to carry out the practical work and finally validate and put the new method into routine work. For these reasons, method development should be started at the desk, and not in the laboratory. A number of questions should be addressed and answered.

- Is the primary goal quantitative or qualitative analysis?
- If quantitative analysis is requested what levels of accuracy and precision are required?
- Are standards available?
- Do we need to perform detection of one or many analytes?
- Is it necessary to resolve all sample components?
- How many different sample matrices is the method designed for?
- How many samples will be analyzed at one time?
- If qualitative analysis is requested it is important to define whether the method will be used for characterization of unknown sample components or isolation/purification of analytes

These initial questions will direct the chromatographers to define the method goal, and to find out requirements of the new method.

Do you really need high resolution (in separation and detection), short analysis time, maximum sensitivity, long column lifetime, a column with wide pH stability or will the method be used at neutral pH and under non-aggressive conditions. True optimization of a method is a balance between selectivity, speed and efficiency, in order to produce resolution that fits the purpose of the application. Ideally, the development should result in a robust method that gives the laboratory a low overall price-per-injection and ultimately a cost-efficient assay.

Common mistakes in analytical method development

- Inadequate formulation of method goals
- Insufficient knowledge of chemistry
- Use of the first reversed phase HPLC column available
- Use of wrong instrument set-up
- Trial and error with different columns, mobile phases

These mistakes often results in laborious, time consuming projects that lead to methods which fail to meet the needs of the laboratory.

Getting started

After defining the goal of the method development, specific information of the sample and the analytes should be sought. Different sources are available: e.g. scientific journals, chemical databases like **www.pubmed.org** (small molecules) ExPASy Proteomics Server **http://expasy.org** (large biomolecules) and reference books. Listed below are some of the most common parameters.

- Nature of the sample
- Number of compounds/analytes present
- Chemical structure (functionality)
- Molecular weight of the compounds
- pKa values
- log *P* and/or log *D* values (hydrophilicity/hydrophobicity)
- Concentration
- Sample matrix
- Sample solubility

Depending on the method requirements, some steps will not be necessary. For example, if a satisfactory separation is found initially, steps 6 and 7 may be omitted. The extent to which method validation (step 8) is investigated and pursued will depend on the final use of the analysis; for example, a method required for quality control will require more validation than one developed for a one-off analysis.

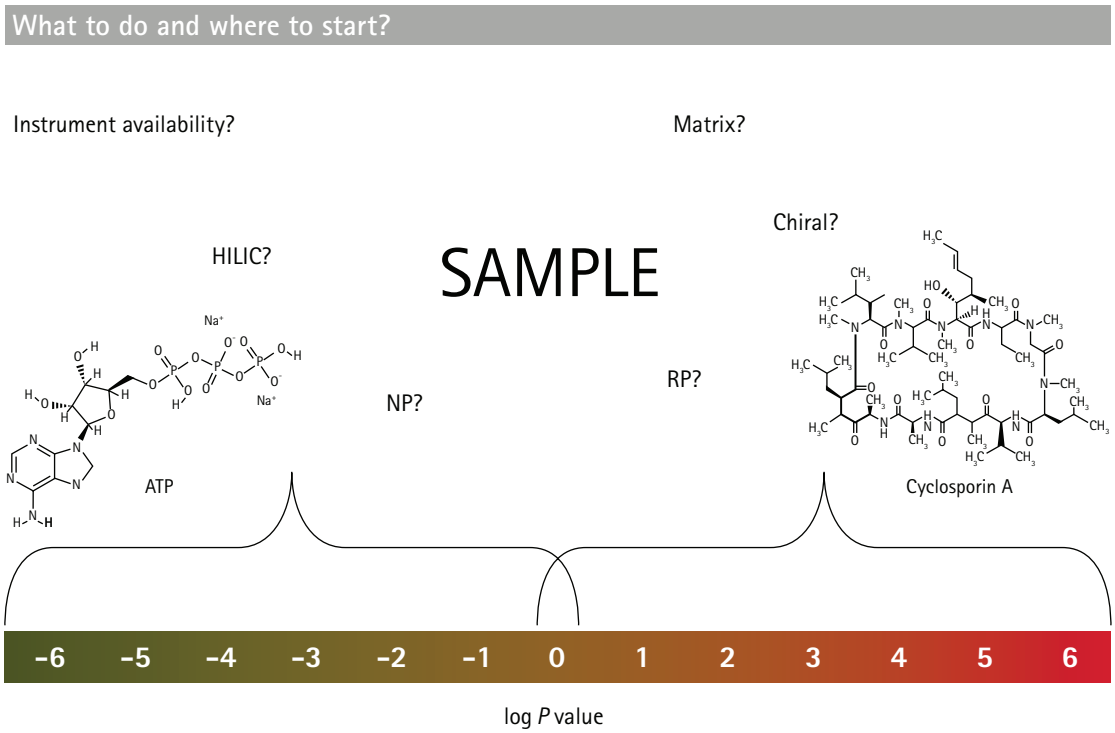
2 Establish sample prep procedure

Consideration must be given to sample preparation. Does the sample require dissolution, filtration, preconcentration or clean up? Is it possible or needed to use solid phase or liquid-liquid extraction procedures, column switching or any other automated on-line techniques? In the following sections, emphasis will be focussed to reversed phase method development. Guidance will be given also to the other modes of liquid chromatography, for which more information can be found at **www.merck-chemicals.com/chromatography**.

Finally, remember to keep it simple, and think of factors that are likely to be significant in achieving the desired resolution.

What to do and where to start?

Think about the sample as being the central part during all steps, as illustrated in figure, since HPLC method development should aim at separating analytes from a defined matrix, and at allowing detection with sufficient sensitivity in a rugged and easy way for the analyst.



More useful and detailed information can be found in the column selection guide on page 152.

Select the detector

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To select the most appropriate detection mode, four important parameters should be taken into consideration; chemical nature of the analytes, potential interferences, limit of detection (LOD) and limit of quantitation (LOQ) required, linearity range, availability and/or cost of detector. Below are some of the most common detection techniques for liquid chromatography presented. Fluorescence, electrochemical or mass detectors should be used for trace analysis. For preparative HPLC, refractive index is preferred because it can handle high concentrations without overloading the detector.

Ultraviolet/Visible absorbance (UV/Vis)

UV detectors are most commonly used. It is a robust, inexpensive and versatile detection technique since most compounds absorb light, especially at low UV wavelengths. It is possible to use a Diode Array Detector (DAD) and allow monitoring at multiple wavelengths simultaneously. The downside is that a UV detector is not analyte specific and requires that the analyte absorb more light than sample matrix at the set wavelength. Choose a detection wavelength that maximizes sensitivity and specificity, but keep in mind that the mobile phase solvents and buffer components may cause slight shifts in UVmax from reference values. Therefore it is advisable to check the analyte absorbance in the mobile phase. Mobile phase solvents and buffer components also have UV cut-off, and make sure to work well above these levels. Otherwise there are likely to be problems with reduced sensitivity and increased system noise (unstable and drifting baseline noise). UV wavelengths below 200 nm should be avoided because detector noise increases in this region. Higher wavelengths give greater selectivity.

Refractive index (RI)

Refractive index is also a common detection technique, and measures the difference in the refractive index of a sample cell versus a reference cell. It is also a non-selective detection technique, being concentration dependent and where the sensitivity is typically 100-1000 times lower than a UV/Vis detector. The benefit over a UV detector is the possibility to quantify analytes with no chromophores in the molecular backbone. The drawback is the sensitivity and the fact that RI detectors can only be used in isocratic mode. It is possible to use with gradients but requires special modifications which makes it less user friendly.

Fluorescence (FL)

Fluorescence detection is very specific and measures only compounds that fluoresce, hence a requirement of this technique. The operation is similar to a UV/Vis detector but where the detector flow cell is used as the sensor through which excitation light passes axially. A photocell is located at the side of the cell to receive radially emitted light. The cell wall is made of special glass to prevent the excitation light or other stray light from reaching the photo cell. When a solute that fluoresces in the excitation light flows through the cell, the molecule excites and fluorescent light passes through the walls of the cell onto the photo cell. The excitation light may be light of any wavelength selected from the light source using a monochrometer. Another monochrometer may also be used to selectively analyze the fluorescent light and, thus a fluorescent spectrum can be produced for excitation light of any specific wavelength and an excitation spectrum produced for fluorescent light of any specific wavelength. To improve specificity of an LC analysis, a fluorescent derivatization reagent can be added (either pre-column or post-column) to form a fluorescent derivative of the substance of interest. This derivative may then be selectively detected from other solutes which, (if they do not fluoresce) need not be resolved from each other by the separation column. Fluorescence detection is up to 1000 times more sensitive than UV/Vis, and also concentration sensitive.

Evaporative light scattering (ELS or ELSD)

ELSD is also a non-selective detection technique, but where the detector is mass sensitive and not concentration dependent. It is an ideal technique for high molecular weight compounds, sugars and less volatile acids. The detector measures the light scattering and where the amount of scattering is related to the molecular mass of the analyte, i.e. the more mass the more scattering will be seen measured. In the detector there are three processes; nebulisation of the mobile phase (1), evaporation of the mobile phase (2) and light scattering by analyte particles. In contrast to RI, it works well in gradient mode. Keep in mind that mobile phase solvents should be volatile for best performance.

Electrochemical (EC)

An electrochemical detector requires that the analytes can be oxidised or reduced by an electrical current. The detector output is an electron flow generated by a reaction that takes place at the surface of electrodes. If this reaction is complete (exhausting all the analyte) the current becomes zero and the generated total charge is proportional to total mass of material that has been reacted. This process is called coulometric detection. If the mobile phase is continuously flowing past the electrodes, the reacting analyte is continuously replaced in the detector. As long as the analyte is present between the electrodes, a current will be maintained, albeit varying in magnitude, and is called amperometric detection. An electrochemical detector requires three electrodes, the working electrode (where oxidation or reduction takes place), the auxiliary electrode and the reference electrode (compensates for changes in the background conductivity of the mobile phase). Electrochemical detection is more sensitive than fluorescence detection, very sensitive but commonly not as selective as fluorescence and generally not compatible with gradient elution.

Mass spectrometer (MS)

Mass analyzers can be quadrupole, magnetic sector, time-of-flight, ion trap or ion cyclotron resonance type. Mass spectrometric detection is rapidly growing in popularity, because of ease of use, better compatibility with liquid chromatography and lower costs. The benefits with MS are that it allows for positive analyte identification and the possibility to discriminate between co-eluting peaks in selected ion monitoring mode. The latter reduces the requirement for chromatographic resolution before detection, but it is always better to have completely resolved peaks to prevent ion suppression or ion enhancement effects. To achieve best sensitivity, mobile phases used should be set at a pH where analytes are ionized, and a rule of thumb is to use neutral to basic pH (7-9) for acids whereas more acidic pH (3-4) is advisable for basic compounds. However, depending if the analyte of interest have multiple pKa values and may change its ionization state, other pH values may be more beneficial both in terms of both ionization of the analyte and behaviour in the separation column. A quadrupole mass analyzer consists of four parallel rods that have fixed DC and alternating RF potentials applied to them. Ions produced in the source of the instrument are then focussed and passed along the middle of the quadrupoles. Their movement will depend on the electric fields so that only ions of a particular mass to charge ratio (m/z) will have a stable path to the detector. The RF is varied to bring ions of different m/z into focus on the detector and thus build up a mass spectrum. Quadrupole mass spectrometers commonly have two configurations when used with liquid-chromatography, either as a simple single quadrupole system or placed in tandem. The latter principle, the triple quadrupole mass spectrometer, enables ion fragmentation studies (tandem mass spectrometry or MS/MS) to be performed.

There are other less commonly used detection techniques possible to combine with liquid chromatography, such as chemiluminescence nitrogen (CLND), radio detectors, charged aerosol (CAD, inductive coupled plasma (ICP), NMR, but these are not dealt with here.

Selection of initial conditions – mode of separation, column and mobile phase

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When selecting the most suitable mode of separation, it is dependent on sample solubility and how the analytes of interest differ from other compounds or matrix in the sample. In reversed phase (RP) mode the mobile phase is polar and the stationary phase is less polar. The major distinction between analytes is their hydrophobicity where samples should be soluble in water or a polar organic solvent. In normal phase (NP), the mobile phase is non-polar while the stationary phase is more polar. This is the same for hydrophilic interaction liquid chromatography (HILIC). In normal phase, the major distinction between analytes is NOT their hydrophobicity, and where the samples should be soluble in a hydrophobic solvent like hexane and the mobile phase is a weak to moderate solvent for the sample.

In HILIC mode, the mobile phases are the same as for reversed phase, but with the opposite elution strength. The major distinction between analytes is their hydrophilicity and the sample should be soluble in a polar organic solvent or organic solvent – water mixtures. For polar and hydrophilic compounds the traditional approach utilized reversed phase ion-pairing and was used for analytes which were ionic or potentially ionic. In this situation the mobile phase contains a buffer, an ion-pair reagent and a polar organic solvent. Typical ion-pair reagents are; alkyl sulfonates (heptane sulfonic acid, octane sulfonic acid) and used for bases; and where quaternary amines (tetrabutylammonium chloride) are used for acids. Today, reversed phase ion-pairing methods can easily be replaced with HILIC with the benefit of having more robust and sensitive methods without the need of using ion-pairing reagent.

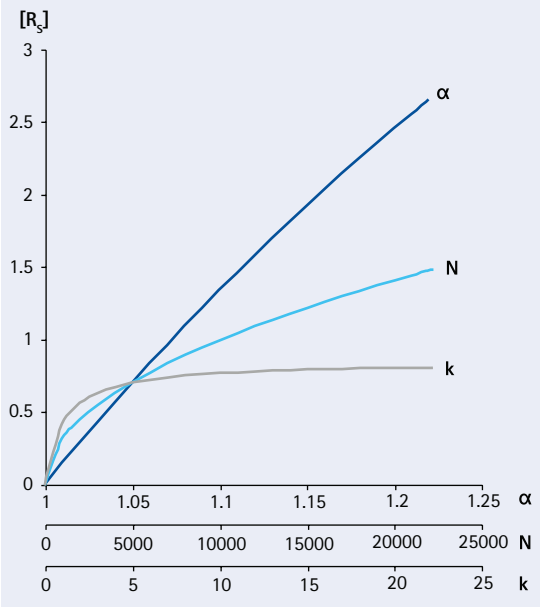
Choose the right HPLC column

Chromatographic resolution is mainly affected by the selectivity (α), as can be seen in the figure below. Changing the mobile phase composition or the stationary phase, is the most powerful way of optimizing selectivity whereas the particle size, pore size, length of the column, temperature, mobile phase strength have much less effect. Therefore, if satisfactorily results are not met, or no retention is achieved, it is better to change to another selectivity using a different column type and/or a different mobile phase.

Resolution is mainly controlled by selectivity

Resolution (Rs or R) can be expressed in terms of three parameters (k, α, and N) which are directly related to experimental conditions.
k is the average retention factor for the two bands,
N is the column plate number and
α is the separation factor (or selectivity factor).

The parameters k and α is determined by the experimental conditions (composition of the mobile phase; stationary phase chemistry and temperature), and where N is affected by column length, particle size and pore size.



Stationary phase selection

After setting the method goals and careful investigation of the analyte structures (hydrophobicity/hydrophilicity; functional groups and potential detection possibilities), select a few bonded phases fitted for its purpose along with a viable detector. The initial column selection is very important and the chromatographer should be advised not to use the first reversed phase HPLC column available. Reversed phase liquid chromatography is indeed a workhorse in most laboratories, and a particulate RP-18 column is often the first choice for many chromatographers, but many methods are unfortunately developed not utilising best or most appropriate selectivity. If the sample is mainly of hydrophobic character, having positive log *P* values and mainly having hydrophobic functional groups, a reversed phase column is advisable. Select a C18 or C8 bonded phase for good retention and resolution. If the method is intended for bioanalysis, analysis of dirty samples in general, or where proper sample preparation is unwanted/not possible, a monolithic reversed phase column (e.g. Chromolith®) is a superior choice over a particulate column. Chromolith® RP-18e is a better choice over Purospher® STAR RP-18e for such purposes as it has very good matrix tolerability and long column lifetime.

If samples are clean or and/or good sample preparation will be included in the final method, and very high peak capacity is needed, a particulate column with small particles and small pores may be more useful. Choose also columns known to have long lifetime at operating mobile phase pH. Choose bonded phases based on high purity, low acidity silica for best peak shape. If the sample is consisting of polar and hydrophilic analytes an orthogonal selectivity to reversed phase should be selected. If chiral resolution is defined in the method goal, a suitable chiral column should be chosen etc. Use analyte specific structure information (chemical structure, log *P* values etc) to choose a proper stationary phase. If acidic or basic analytes are present in the sample; reversed phase ion suppression (for weak acids or bases), reversed phase ion-pairing (for strong acids or bases) or HILIC should be used. For low/medium polarity analytes, normal phase HPLC or HILIC are viable techniques, while HILIC, and particularly ZIC®-HILIC is the most suitable separation technique for polar and hydrophilic compounds.

Polarity scale of analyte functional groups

Polarity	Functional group	Hybridization	Intermolecular forces
Low	Methylene	s	London
	Phenyl	s / p	London
	Halide	s	London, Dipole-Dipole
	Ether	s	London, Dipole-Dipole, H-bonding
	Nitro	s / p	London, Dipole-Dipole, H-bonding
	Ester	s / p	London, Dipole-Dipole, H-bonding
	Aldehyde	s / p	London, Dipole-Dipole, H-bonding
	Ketone	s / p	London, Dipole-Dipole, H-bonding
	Amino	s / p	London, Dipole-Dipole, H-bonding, Acid-base chemistry
	Hydroxyl	s	London, Dipole-Dipole, H-bonding
High	Carboxylic acid	s / p	London, Dipole-Dipole, H-bonding, Acid-base chemistry

Choosing the right column format

Use the column selection guide to find the best column configuration for minimum analysis time with high efficiency and resolution, and match up method goals to make sure that the chosen format has the ability to produce resolution that fits the purpose of the application, e.g. choose right column length, column inner diameter, particle size and pore size. If you have sufficient resolution, it is also possible to speed up the separation by increasing the flow rate or shorten the column length. Silica-based materials are physically strong and will not shrink or swell, being compatible with a broad range of polar and non-polar solvents, and therefore often the initial choice. Most silica based columns are stable from pH 2-7.5, and historically, polymeric packing materials provided better column stability under pH extremes. A polymer-based packing material, like ZIC®-*p*HILIC, is compressible and may shrink or swell with certain solvents. Therefore care must be taken if a polymeric column is used, and the upper back-pressure limit is lower than corresponding silica based stationary phases. Newer high-purity silica based phases, like Purospher® STAR, are stable at pH 1.5-10.5, with the surface functional groups bound to the base silica particle at multiple attachment points via polymeric modification.

Particle size

Smaller particle sizes provide higher separation efficiency and higher chromatographic resolution than larger particle sizes. However, larger particle sizes offer faster flow rates at lower column back-pressure, and are less prone to clogging, and for these reasons are more tolerant to matrix effects. Typical particle sizes range from 3-20 µm, but new 2 µm particle sizes are available to maximize resolution on short Purospher® STAR columns. A 5 µm particle size represents the best compromise between efficiency and back-pressure for most non-high throughput applications.

Pore size

Choose a pore large enough to completely enclose your target molecule. If your molecule is larger than the pore, size exclusion effects will be seen and it will be difficult or impossible to retain. In general, packing materials with a smaller pore size have higher surface areas and higher capacities than packing materials with larger pore sizes. A larger surface area typically indicates a greater number of pores, and therefore a higher overall capacity. Smaller surface areas equilibrate faster, which is important for gradient elution analyses. Larger pores are better for interaction with large compounds, such as proteins.

Carbon load

For silica-based reversed-phase packing materials, carbon load indicates the amount of functional bonded phase attached to the base material. Phases with lower carbon loads are more weakly hydrophobic, which may significantly reduce retention times over phases with higher carbon loads. However, a higher carbon load will give higher capacity and often greater resolution, especially for compounds of similar hydrophobicity. Carbon load is not a relevant parameter for columns used in normal phase or HILIC mode.

Endcapping

Silica-based reversed-phase packing materials have free silanol groups that will interact with polar compounds. Endcapping the bonded phase minimizes these secondary interactions. Choose endcapped phases if you do not want interactions with polar compounds. Choose non-endcapped phases if you want enhanced polar selectivity, for stronger retention of polar organic compounds.

5 Mobile phase selection – solvents and buffers

In a previous chapter, insight has been given to mobile phase recommendations, solvent properties and buffer components. Herein, a summary of starting conditions are presented along with a discussion about the difference among isocratic and gradient elution. The mobile phase solvent strength is a measure of its ability to elute analytes of the column. It is generally controlled by the concentration of the solvent with the highest strength; for example, in reverse phase HPLC with aqueous mobile phases, the strong solvent would be the organic modifier; in normal phase and HILIC, it would be the most polar one. Worth pointing out is that cyano-bonded phases are easier to work with than plain silica for normal phase separations. The aim is to find the correct concentration of the strong solvent. With many samples, there will be a range of solvent strengths that can be used within the aforesaid capacity limits. Other factors (such as pH) may also affect the overall retention of analytes.

Isocratic elution

In partition chromatography, the mobile phase should be a moderate to weak solvent for the samples to achieve peak focusing and not to compromise the actual separation. A good rule of thumb is to achieve a capacity factor (retention factor, k) of 2 to 5 for an isocratic method. In both RP and HILIC mode, the preferred organic solvent is acetonitrile of two reasons; favorable UV transmittance and low viscosity. Methanol is a reasonable alternative, hence it may be worth changing the organic solvent if resolution is not achieved and adjust the percentage organic solvent in the mobile phase to accomplish maximum resolution and retention.

In reversed phase mode, the initial mobile phase pH should be selected with two considerations. Low pH that protonates column silanol groups and reduce their chromatographic activity is generally preferred, especially with non-endcapped columns. Mobile phases having pH 1 to 3 with 20-50 mM buffer (Potassium Dihydrogen Phosphate, TFA or formic acid in water) is advisable depending on detection mode, and to increase temperature to reduce analysis time. Mobile phase solvents should be water miscible, have low viscosity, low UV cut-off, being non-reactive, and for these reasons acetonitrile, methanol and tetrahydrofuran (THF) are used with RP columns. Not all RP methods are suitable under acidic conditions, and other pH intervals may provide different selectivity. At mid pH, dipotassium hydrogen phosphate or ammonium acetate (not a true buffer, but rather a pH adjustable salt) are viable alternatives depending on detection mode. At high pH, dipotassium hydrogen phosphate and ammonium carbonate can be used as buffers to maintain pH above 8. Keep in mind that working at high pH, only columns with wider pH tolerability should be used, and for this purpose Purospher® STAR is an excellent choice.

Gradient elution

Often it is not possible to elute all analytes with a single mobile phase (isocratic) in the desired k' (2-5) range. It is therefore advisable to use gradient elution where the mobile phase strength, and sometime also pH and ionic strength, changes over time. Effectively this means that early in the gradient the mobile phase elution strength is low, and where the elution strength is increasing with time according to a defined program that maximises the number of peaks that can be resolved with a given resolution. This results in of the constant peak width observed in gradient elution, compared to isocratic elution where the peak width increases in proportion to retention time. Gradient elution is used to solve the general elution problem for samples containing mixtures of analytes with a wide range of polarities. Gradient elution will also give greater sensitivity, particularly for analytes with longer retention times, because of the more constant peak width (for a given peak area, peak height is inversely proportional to peak width). Common practice in method development is to run a broad gradient first to decide whether to use isocratic or gradient elution.

If $\Delta t/tG \geq 0.25$ use gradient elution If $\Delta t/tG \leq 0.25$ use isocratic elution

Where Δt is difference in the retention time between the first peak and last peak in the chromatogram, tG is the gradient time; the time over which the solvent composition is changed. For most samples (unless they are extremely complex), short columns (10–15 cm) are recommended to reduce method development time. Such columns afford shorter retention and equilibration times. A flow rate of 1-1.5 mL/min should be used initially.

Gradient method development

Good laboratory practice is to not allow gradients going from 100% aqueous to 100% organic. For method ruggedness reasons (to get better mixing, to prevent precipitation of salt and to provide more robust gradient profiles) it is advisable to keep minimum 5% of each phase in both mobile phase bottles. In practice for a reversed phase method, this means mobile phase A contains 5% organic solvent and 95% aqueous, while mobile phase B contains 95% organic solvent and 5% aqueous.

It is advisable to initially run a wide scouting gradient (0 - 100% B) over 40-60 minutes. From this run decide whether isocratic or gradient elution is best for the application. If gradient mode is a more appropriate alternative, eliminate sections of the gradient and try to compress the analyte peaks in space as much as possible prior to the first and last eluting peak. To further improve the gradient profile and to shorten overall cycle times (including re-equilibration) try to reduce the gradient and total run time. Keep in mind that a segmented gradient can be an effective tool to improve the separation. Seldom is a linear gradient the best solution. If there is a need to improve the separation of two closely eluting peaks; change the solvent strength by varying the fraction of each solvent (gradient shape and steepness); change column temperature; change the mobile phase pH (in small units); use different mobile phase solvents and/or buffer components; and/or use a different selectivity by changing the stationary phase.

6 Selectivity optimization

In HILIC mode, gradient elution is accomplished by increasing the polarity of the mobile phase, by decreasing the concentration of organic solvent, i.e. in the "opposite" direction compared to RPLC separations. With charged HILIC stationary phases, like ZIC®-HILIC there is also a possibility of increasing the salt or buffer concentration during a gradient to disrupt electrostatic interactions with the solute. After a gradient run the column has to be equilibrated with the starting concentration of the mobile phase before the next sample can be injected. It must be emphasized that HILIC stationary phases are less tolerant to fast gradients and short equilibrium times compared to RPLC phases. This is because the water in the aqueous layer within the stationary phase originates from the eluent and therefore is depending on its composition.

It is also worth mentioning that the column back-pressure will increase during the gradient. Failure to properly equilibrate columns will cause drifting retention times and poor reproducibility. In some cases, however, it is possible to reach a dynamic equilibrium with stable retention times if fast gradients are run repeatedly for a longer period of time, but this situation can be tricky to obtain and reproduce.

The direct disadvantages with gradient elution are the need of a more complex HPLC system, and the separation column requires re-equilibration after every analysis which makes injection-to-injection lengthier than for an isocratic method. It is not compatible with all detectors (i.e. RI and EC), more variables to control for reproducibility and system dwell volume (gradient delay volume) becomes important especially in scaling a separation or whenever transferring a method between instruments and/or laboratories. Therefore be aware that delay volumes will vary from instrument to instrument.

System optimization

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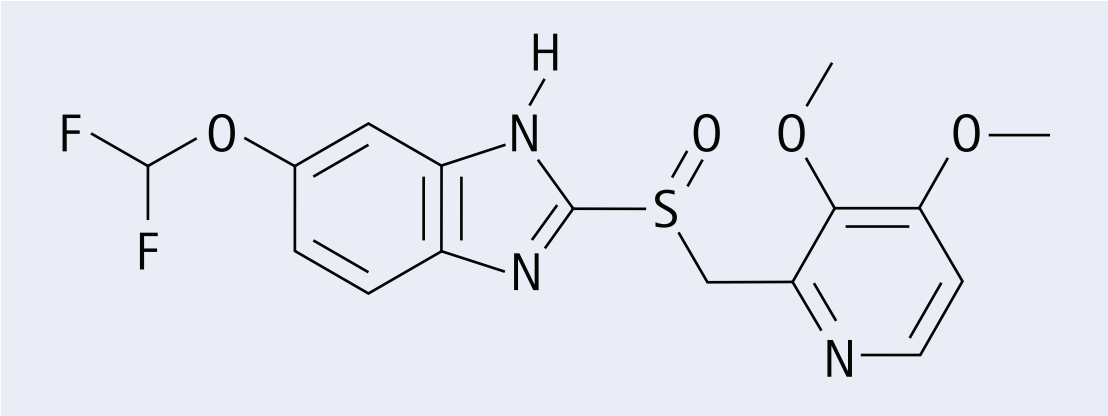
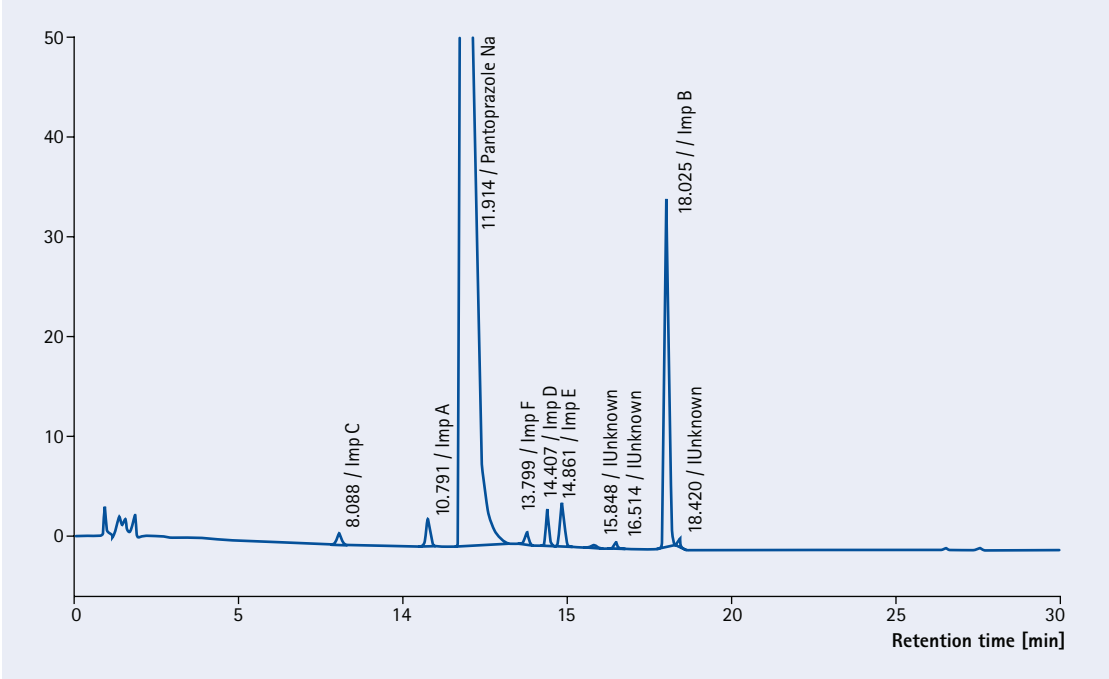
To find the desired balance between resolution and analysis time after satisfactory selectivity has been achieved, parameters such as column dimension, particle size and flow rate should be optimized. With a truly scalable stationary phase, these parameters may be changed without affecting capacity factors or selectivity. With the introduction of smaller particle sizes and narrower column inner diameters, also optimization of the complete HPLC instrumentation is needed, and sometimes it is necessary to replace the whole system. An example of a successful complete system optimization is shown for Pantoprazole sodium (Pantoloc, Protium, Pantecta and Protonix; a proton pump inhibitor drug that inhibits gastric acid secretion).

The chemical structure of Pantoprazole sodium



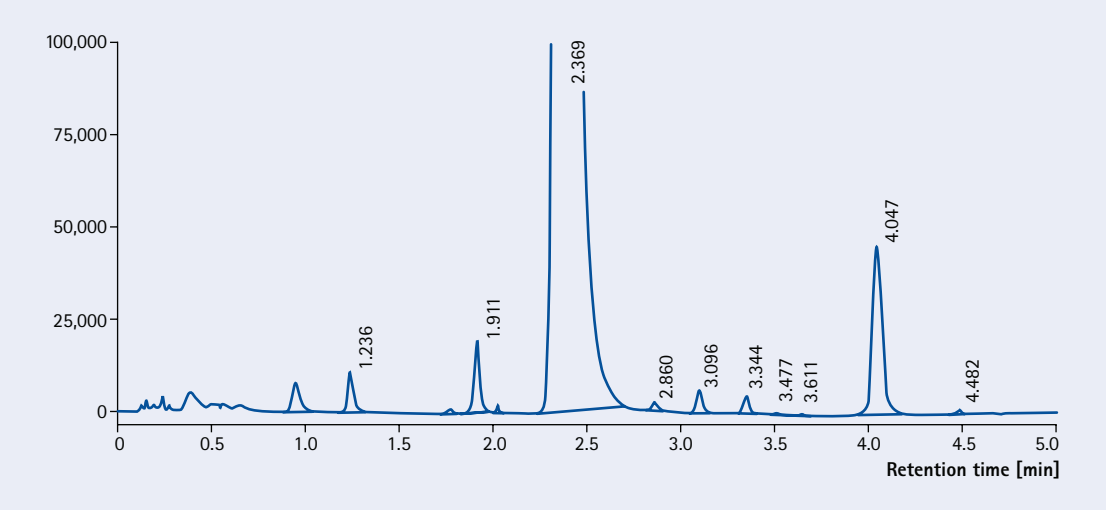
The original method was developed on a Purospher® STAR RP-18 endcapped 150 x 4.6 mm column with 5 µm particles with a total cycle time of 50 minutes. By changing to a Purospher® STAR RP-18 endcapped 50 x 2.1 mm column with 2 µm particles, lowering the flow-rate, and altering the gradient profile, it was possible to reduce the total analysis time from 50 to 5 minutes, maintaining sample peak profile (with improved resolution), at low back-pressure and high separation efficiency.

Chromatogram showing the purity profile of Pantoprazole sodium on a Purospher® STAR RP-18 endcapped 150 x 4.6 mm column with 5 µm particles



Column	Purospher® STAR RP-18 endcapped 150 x 4.6 mm, 5 µm
Mobile phase	A: 1.74 gram dipotassium hydrogen phosphate in 1000 mL water, adjusted pH to 7.0 with dilute phosphoric acid (330 gm/L) B: Acetonitrile
Gradient	initial composition 80% A and 20% B linear increase to 20% A and 80% B in 40 min followed by a 10 min re-equilibrium at initial composition
Flow rate	1 mL/min
Detection	UV 290 nm
Column temperature	40°C
Injection volume	20 µL
Sample	460 ppm in 1:1 mixture of ACN and 0.001 N NaOH

Chromatogram showing the purity profile of Pantoprazole sodium on a Purospher® STAR RP-18 endcapped 50 x 2.1 mm column with 2 µm particles



Column	Purospher® STAR RP-18 endcapped 50 x 2.1 mm, 2 µm
Mobile phase	A: 1.74 gram dipotassium hydrogen phosphate in 1000 mL water, adjusted pH to 7.0 with dilute phosphoric acid (330 gm/L B: Acetonitrile
Gradient	initial compostion 80% A and 20% B linear increase to 28% B in 1.5 min followed by an increase from 28 to 40% B between 1.5 and 4.0 min finally a re-equilibrium at initial composition for 1 min
Flow rate	0.6 mL/min
Detection	UV 290 nm
Column temperature	40°C
Injection volume	7 µL
Sample	460 ppm in 1:1 mixture of ACN and 0.001 N NaOH

8 Method validation

Proper validation of an analytical method is important to ensure that it will provide similar results, today-tomorrow-next week-next year, i.e. over a long period of time, in different laboratories independent of the analyst. Not only because of requirements from regulatory authorities, but rather to ensure good manufacturing practice (GMP) and good laboratory practice (GLP). It is especially important for pharmaceutical analysis when assurance of the continuing efficacy and safety of each batch manufactured relies solely on the determination of quality. Guidelines for the validation of analytical methods can be found at the International Council on Harmonization (ICH). The US Food and Drug Administration (FDA) and US Pharmacopeia (USP) both refer to ICH guidelines.

Keep in mind that analytical method validation should be isolated from the initial selection and development, which actually are only the first steps in establishing a routine analytical method. Validation means testing of the method to find out allowed variability for each method parameter. Routine quality control methods should guarantee that the analytical results of raw materials, excipients, intermediates, bulk products or finished products are viable.

In this section the most widely applied validation characteristics are explained; accuracy, precision (repeatability and reproducibility/intermediate precision), specificity, limit of detection, limit of quantitation, linearity, robustness and stability of analytical solutions.

Accuracy

An analytical method is accurate if it gives the right numerical value for the analyte (either mass or concentration) and can be described as the degree of closeness of measurements of a quantity to its actual value. A method almost never gives the exact same results for replicate analyses, which means that the result is presented as the mean or average. A pragmatic way to express accuracy is to present it in terms of the standard error, which is the difference between the observed and the expected concentrations of the analyte. To determine accuracy, a common practice is to analyze a known amount of standard material under different conditions in a formulation, bulk material or intermediate product to ensure that nothing interferes with the method.

Precision

Precision, this is also referred to as reproducibility or repeatability, defines how reproducible the acquired results are and gives you assurance in the attained data. Repeatability is the measure of how easy it is for an analyst in a given laboratory to attain the same result for the same batch of samples (normally by injecting the same samples repeatedly at different concentration levels) using the same method and using the same equipment and reagents. Reproducibility or intermediate precision measures the variations within or between days, analysts and equipment. High precision quantitative results should be expected, but depending if it is a pharmaceutical assay or a bio-analytical method, different acceptance criteria govern. In pharmaceutical quality control there are much more stringent method requirements and less variation amongst samples compared to analysis of patient plasma- or serum samples. For any assay, the relative standard deviation (RSD) or coefficient of variation (CV) is used as indication of the imprecision of the method. From a practical perspective, six to ten replicate injections will give you a good idea of the precision of the method. An analytical method can be accurate but not precise, precise but not accurate, neither, or both.

Specificity

Specificity is an important parameter to test in a validation program as it verifies the ability of the method to accurately measure the analyte response in the presence of all potential sample components. The analyte response from a solution containing only the analyte is compared with test samples containing the analyte and all potential sample components (placebo, synthesis intermediates, excipients, degradation products and impurities). For pharmaceuticals, stress conditions such as heat, light, acid, base and oxidant are typical. For formulated products, heat, light and humidity are commonly used to stress the samples. The analyte peak is evaluated in all test samples for peak purity and resolution from the nearest eluting peak.

Limits of detection and quantitation

The limit of detection (LOD) is defined as the least amount of an analyte in a sample that can be detected, and commonly expressed as the concentration level that is able to provide a signal-to-noise ratio of three (S/N=3). Limit of quantitation (LOQ) is defined as the lowest analyte concentration level that can be quantified with good precision and accuracy, and providing a signal-to-noise ratio of ten (S/N=10). LOD and LOQ can also be determined based on the standard deviation of the response and the slope of the calibration curve.

Linearity

The linearity of an analytical method is the capability to generate results that are directly proportional to the concentration of analyte in the sample. It is commonly illustrated as the interval between the upper and lower analyte concentration levels that may be determined with precision and accuracy. Linearity data is often calculated using the calibration curve correlation coefficient and the y-intercept. The relative standard deviation (RSD), intercept and slope of the calibration curve should also be calculated.

Robustness

The method robustness is a measure on how well an analytical method remains unaffected by small variations in the experimental conditions, but also how reliable the method is during normal use. Important parameters to monitor are changes in the mobile phase composition; mobile phase pH; changes in the gradient profile; changes in the buffer concentration; column temperature; and injection volume.

Analytical solution stability

Analytical solution stability can be divided into different sections; recovery; dilution; internal standard addition, etc. If an extraction process is used (either liquid-liquid or solid phase extraction) it must provide proper analyte recovery. A method with low analyte recovery and/or where the analyte is degraded during the sample preparation is not tolerable for routine quality control. Internal or external standards (reference substances) should be prepared in such way that they maintain their potency, and produce same response over time. Samples and standards should be tested for stability to verify stability over a normal analysis cycle. A rule of thumb is that the sample solutions, standard solutions and HPLC mobile phase should be stable for minimum 24 hours under defined storage conditions.

Further reading

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