



Review Article

A TYPICAL REVIEW ON RAPID RESOLUTION LIQUID CHROMATOGRAPHY (RRLC)**R.N.U.R.R.P.Kumar.N^{1*}, B.Krishnamoorthy^{1*}, M.Muthukumaran, Amreen Nishat.**Montessori Siva Sivani Institute of Science & Technology College of Pharmacy, Mylavaram, Vijayawada,
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(Received: 11 December 2012; Accepted: 24 December, 2012; Published: 29 December, 2012)

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Abstract: This review article should focus on the Rapid Resolution Liquid Chromatography (RRLC) has become an increasingly useful approach to achieve higher throughput, improve sensitivity and reduce costs. The potential of high-speed analyses by Rapid Resolution Liquid Chromatography (RRLC) on 1.8 μm porous particles packed into short columns operated at high flow rate was investigated and compared to the performance of 5 μm porous particles packed into conventional column. In order to display the practicality of RRLC separations, the isocratic analysis of pesticides and the gradient analysis. Often higher temperatures are employed to minimize system back-pressure. With the widespread adoption of RRLC comes the question of HPLC detector compatibility.

Key words: RRLC, HPLC, Analysis

Introduction

Recently, RRLC analysis has become a routine method in the pharmaceutical industry. However, such a fast analysis technique is not only for the pharmaceutical field. In this study, we apply to the fast RRLC technique to a more general field in the analysis of chemical compounds. RRLC method has become one of the most frequently applied approaches especially in the field of pharmaceutical analysis. It holds excellent peak shapes, enhanced reproducibility, high sensitivity, high-speed detection with reduced analysis cost, and is valuable for the quality control of herbal medicines.¹ The separation resolution and reduction of analysis time has continually improved in High Performance Liquid Chromatography (HPLC). Since then, HPLC using smaller particles has become more popular.⁴ For further improvement, column efficiency must be increased. The relationship among separation efficiency, the mobile phase linear velocity and particle size was investigated in detail in the early 1970 s. This and other systematic investigations have led to high throughput and high resolution HPLC that we know today. The shortening in analysis time is due to the use of a shorter column length. However, a shorter column may lead to a loss of theoretical plates, hence a decrease in chromatographic resolution that may be required for a complex mixture of compounds. To offset the potential loss of resolution, the use of smaller size particles has resulted in more efficient columns.

Long columns packed with smaller particles result in higher efficiency and higher resolution. This is important for analysis of complex samples from metabolomics or proteomics studies. Also, applications such as impurity profiling can benefit from higher separation power. Even the LC/MS analysis of drugs in biological fluids can benefit from the higher peak capacity, because of the reduced interference from ion suppression. In general, higher separation power provides more confidence in the analytical. However, with new RRLC technology, analysis time can be significantly reduced without losing chromatographic resolution.⁴

In this study, we had two goals: 1) to demonstrate the ease of conversion of a traditional HPLC method to a RRLC method and 2) to investigate the potential of RRLC and RRLC/MS on 1.8 μm porous particles packed into short columns operating at a high flow rate compared to the performance of 5 μm porous particles packed conventional columns.⁴

1. Benefits of columns packed with sub-2-micron particles**1.1 Faster chromatography**

There are several advantages of having shorter run times. High Throughput labs now have higher capacity and can analyze more samples in less time. More samples in less time also means lower costs.³

1.2. Higher resolution 2

Long columns packed with smaller particles result in higher efficiency and higher resolution. This is important for analysis of complex samples from metabolomics or proteomics studies. Also, applications such as impurity profiling can benefit from higher separation power. Even the LC/MS analysis of drugs in biological fluids can benefit from the higher peak capacity, because of the reduced interference from ion suppression. In general, higher separation power provides more confidence in the analytical results.³

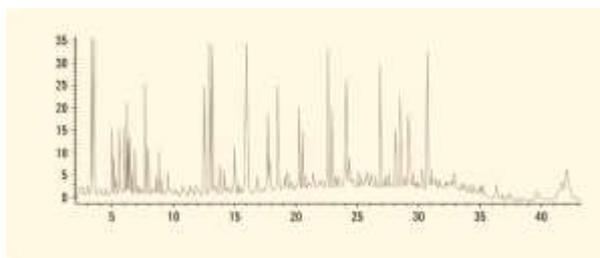


Figure 2

Peak capacities of more than 700 can be achieved using a ZORBAX RRHT SB-C18 column (2.1 x 150 mm, 1.8 μ m) to analyze a tryptic digest of BS.

Frictional heating

Forcing mobile phase through the column at higher pressure and higher flow rates generates heat. The resulting temperature gradients (radial and longitudinal) have an impact on the column efficiency.³

$$POWER = F \cdot p$$

F = Flow rate

P = pressure

Powerful column thermostating (for example, using a water bath) generates a strong radial temperature gradient, which leads to significant loss in column efficiency. Still-air-column thermostating reduces the radial temperature gradient and therefore reduces the efficiency losses, but a higher column outlet temperature has to be accepted. At lower back-pressure, performance losses due to frictional heat are minimized so that 4.6/3 mm inner diameter sub-2-micron columns still deliver superior efficiencies compared with the respective 2.1 mm inner diameter columns.

Apparatus

RRLC analyses were performed on an Agilent 1200 SL HPLC equipped with a binary pump and a micro-vacuum degasser, a multi-wavelength (MW) detector, an auto-sampler, a thermostatted column compartment, and a Luna C18-HST column (2.5 μ m, 100mm x 3.0mm) from Phenomenex (Torrance, CA, US).⁴

2. Design of the Rapid Resolution LC system

The design concept of the 1200 Rapid Resolution LC (RRLC) System was to provide a liquid chromatograph offering ultra fast and high resolution separation capability and yet which retained full functionality for standard HPLC applications. This section looks at how this concept has been implemented to offer the highest flexibility with respect to column dimensions and applications.

The use of sub-two micron (STM) particles means that for high flowrates or long columns additional pressure is required to drive the mobile phase through the column. The RRLC flowpath is optimized to produce minimal backpressure and ZORBAX RRHT columns have an engineered particle size distribution that produces significantly less backpressure than other STM columns. High temperature, up to 100°C on certain columns, allows more selectivity flexibility and reduces solvent viscosity to allow even faster separation. High flow rates up to 5 ml/min can be used for ultra-fast separations. The adjustable delay volume fully supports 2.1 to 4.6 mm i.d. columns. A low dispersion tubing kit and low volume flow cells minimize peak dispersion for narrow bore columns. Detectors with high data rates preserve the resolution of very fast peaks eluting from the RRLC.³

2.1. Delay volume and extra-column volume

The delay volume is defined as the system volume between the point of mixing in the pump and the top of the column. In gradient separation, this volume causes a delay in the mixture reaching the column which effectively means there is an initial isocratic segment in the gradient profile. This becomes more significant at low flow rates and can have a large impact on the transferability of gradient methods. Small delay volumes are important, therefore, for fast gradient separations, especially with narrow bore columns (2.1 mm i.d.) as often used with mass spectrometric detection.

The extra-column volume is defined as the volume between the effective injection point and the effective detection point, excluding the volume fraction of the column containing the stationary phase. Smaller diameter columns require smaller extracolumn volumes to keep peak dispersion at a minimum.³

2.1.1. Optimized configuration for 2.1 mm i.d. columns

In the low delay volume configuration of the Agilent 1200 Binary Pump SL the damper and

mixer are bypassed to reduce the pump delay volume to about 120 μl . Figure 3 shows the flow path connections for this configuration. This provides the shortest gradient delay for ultra-fast gradient separations. In order to take full advantage of the electronic damping control which replaces the physical volume damping it is important to select the respective „enhanced solvent compressibility“ function in the auxiliary screen of the pump menu.³

To minimize peak dispersion the low dispersion kit (**G1316- 68744**) must be installed. This kit includes short 0.12 mm i.d. capillaries and low volume heat exchangers (1.6 μl and 1.5 μl) for the thermostatic column compartment (Figure 6). To maintain resolution in the UV detector a low volume flow cell should be used (e.g., the 2 μl micro flow cell for the diode-array detector.)

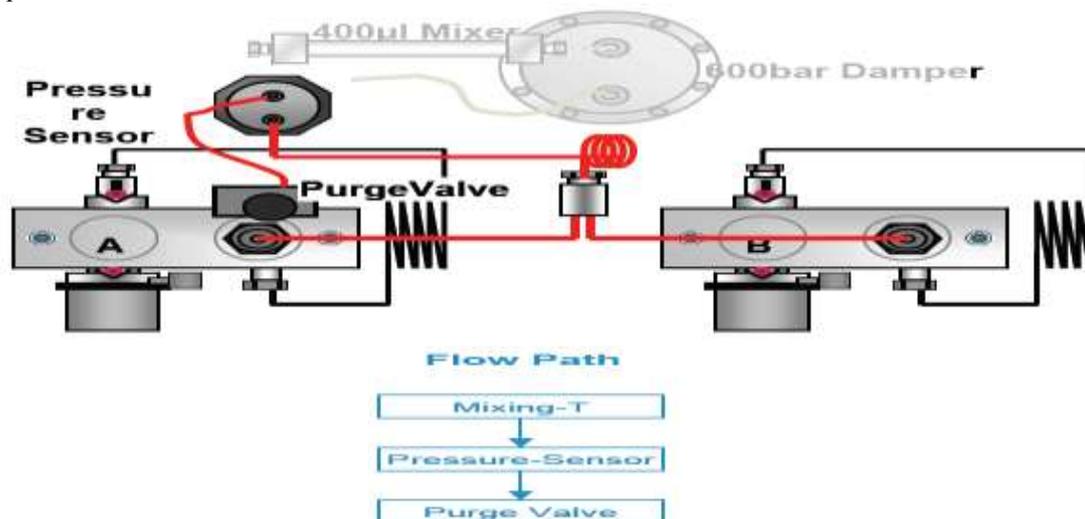


Figure 3 Low delay configuration for 2.1 mm inner diameter columns

It is important to remember to set the correct parameter in the pump auxiliary screen. This ensures that the correct compressibility values are always applied for the mobile phases used. Calibration curves are available for most common solvents. For high sensitivity UV applications an additional 200 μl mixer (**part number 5067-1565**) can be installed to reduce any residual mixing noise. This small mixer gives the lowest UV baseline noise even under extreme gradient conditions. See Figure 4.

The delay volume in the Agilent 1200 Series High-Performance Autosampler SL Plus can be reduced by as much as 140 μl by switching the injection valve from the mainpass position to the bypass position once the injected sample has been flushed onto the column. In practice this can be done a few seconds after injection and is activated by selecting the “Automatic Delay Volume Reduction (ADVR)” function in the autosampler setup menu. This functionality should not be used for carry-over sensitive applications.

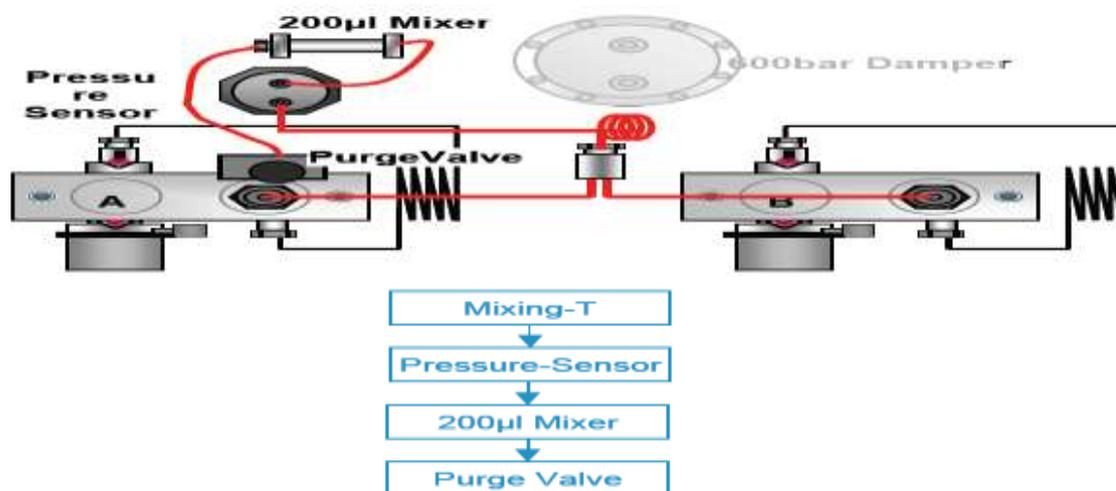


Figure 4 Medium delay volume configuration for 2.1 mm ID columns with highest UV sensitivity.

2.1.2. Optimized configuration for 3 and 4.6 mm i.d. columns

The relative column volumes for 3 mm and 4.6 mm inner diameter columns are about two and five times larger respectively than for the same length

2.1 mm i.d. columns and the flow rates used are also proportionally higher. Therefore, the standard binary pump delay volume will not result in a significantly higher gradient delay.

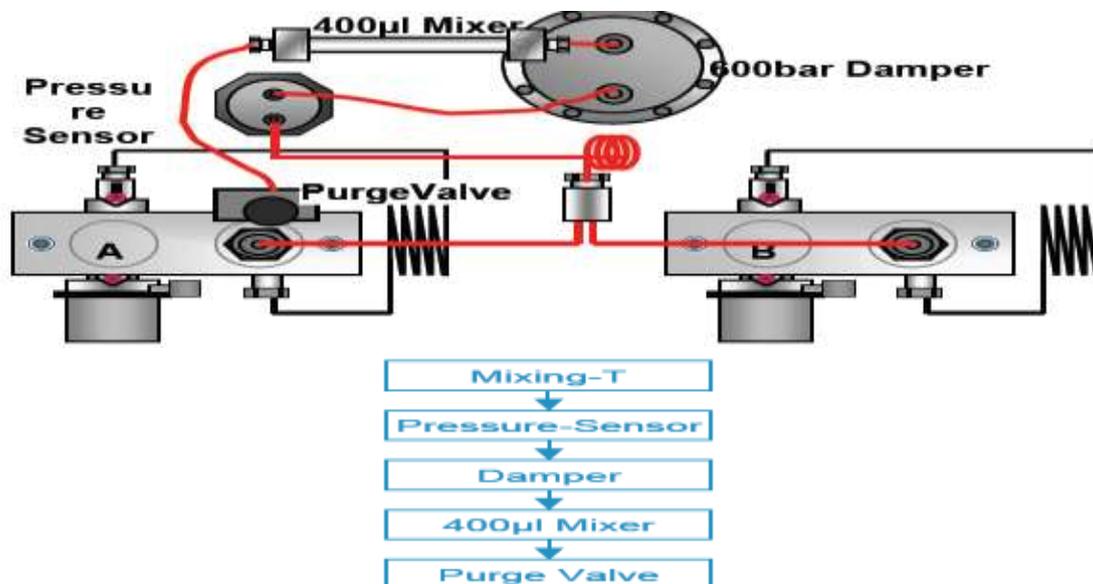


Figure 5 Standard delay volume configuration for 3 and 4.6 mm ID columns with highest UV sensitivity.

The standard delay volume configuration is also the configuration which provides direct method transferability from the Agilent 1100 and 1200 Series LC system to the 1200 Series Rapid Resolution LC system or vice versa. The delay volumes are the same and so no adjustment of the gradient is necessary. This is illustrated by an example chromatogram from a transferred method.

2.1.3. Automatic switching of delay volume

Switching between configurations can be done in two ways:

- manually, by disconnecting and reconnecting capillaries
- automatically, using a 600 bar 2PS/6PT valve (optional) Further design features and benefits are:
- A practical flow rate range from 0.05 up to 5 ml/min up to 600 bar can be used.

- A low dispersion kit for 2.1 mm inner diameter columns is available for use with narrow-bore columns.

- The 1200 Series High Performance Autosampler SL Plus (G1367D) provides short cycle times, about 17 seconds for a 1 µl injection without a wash. The injection volume ranges from 0.1 up to 40 µl and be extended for higher volumes.

- In the 1200 Series Thermostatted Column Compartment SL (G1316B) (Figure 6), different heater (1.6 µl) and cooling elements (1.5 µl) for low extra-column volume can be installed. The temperature is adjustable from 10 °C below ambient up to 100 °C.³



Figure 6 The Thermostatted Column Compartment SL with small heater and cooler installed.

Further:

- Different UV detector flow cells for use with 2.1, 3.0 and 4.6 mm inner diameter columns are available
- Fast UV and MS detectors with data rates up to 160 Hz (1200 Series VWD SL Plus), 80 Hz (1200 Series DAD SL, MWD SL) and up to 40 Hz for MS application are available
- The 1200 Series fluorescence detector (FLD) with 37 Hz data acquisition, and the 1200 Series refractive index detector (RID) are also compatible with the Agilent 1200 Series RRLC System. A stepwise upgrade from 1100 Series to 1200 Series RRLC is possible.

2.2. Shorter cycle times using a second pump and switching valve for column regeneration

Selecting a column

Shorter cycle times can be achieved by selecting a short column with good selectivity. The column dimensions are also determined by the detection system that is used. For MS applications, a good choice is a column with 2.1 mm inner diameter and a flow rate up to 1.5 ml/min. Using these conditions, no flow splitter is necessary in front of

most mass spectrometers. For UV detection, 3.0 mm inner diameter columns are ideal, because here the highest linear velocities can be obtained. With 4.6 mm inner diameter columns, high linear velocities can also be reached, but the maximum flow rate is limited to 5 ml/min.

Selecting the optimum instrument configuration

The pump should be used in its standard delay volume configuration (Figure 3) for 4.6 mm inner diameter and 3.0 mm inner diameter columns. For 2.1 mm inner diameter columns, the low delay volume configuration should be used. In addition, when using 2.1 mm inner diameter columns, the low dispersion kit should be installed to provide lowest extra-column volume. The tubing to the MS should be as short as possible, and of small internal diameter, such as that delivered with the low dispersion kit. For highest UV sensitivity, it is recommended in addition to use the short mixer. (Part number 5067-1565). Even shorter cycle times can be achieved by using a column regeneration valve in combination with a regeneration pump, as shown in Figure 7.

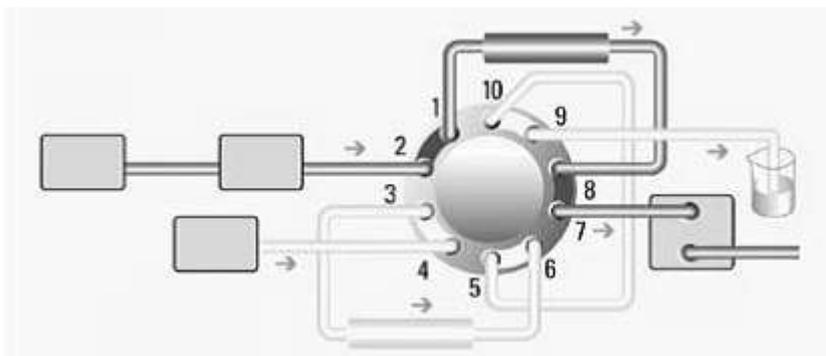


Figure 7 Alternating column regeneration using a 2-position 10-port valve and a second regeneration pump.

Using two columns, two pumps and one 2-position 10-port valve allows switching between these columns for shortest cycle times from injection to injection. Typically, columns of the same chemistry and the same batch provide a retention time precision that allows data processing using the

same calibration table, Figure 8. Also, the stability over several thousand runs is maintained if precautions are taken against blockage of frits due to particles in the sample or mobile phase (algae or/and bacteria).

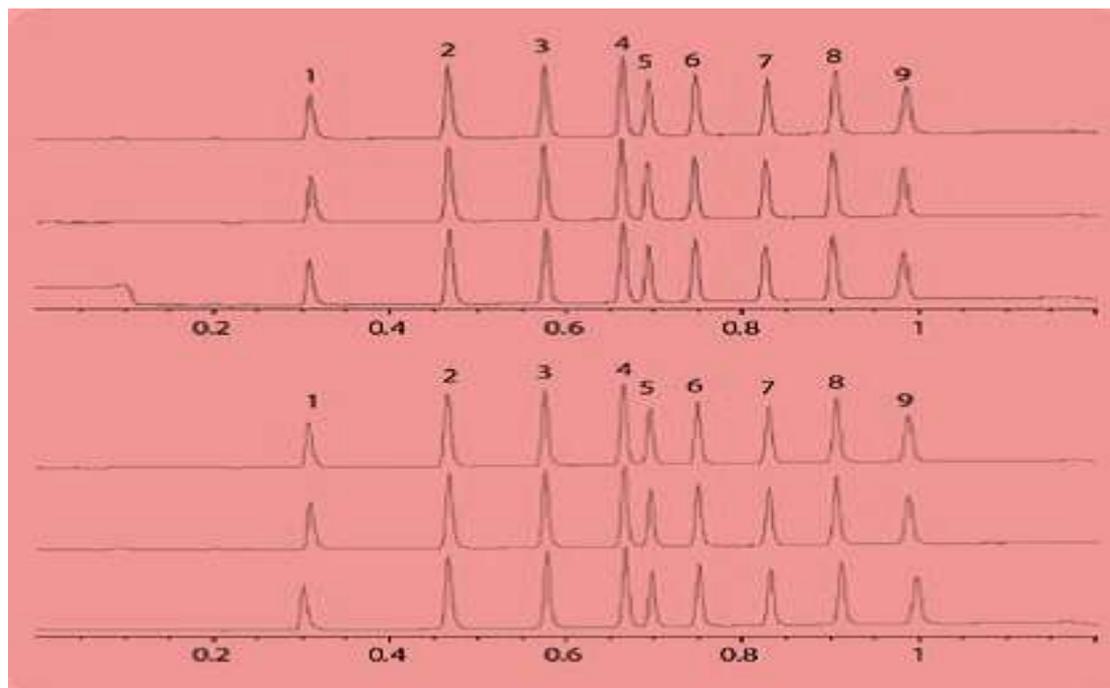


Figure 8 Comparison of the UV chromatograms at 245 nm, 2.3 ml/min and 32 °C using alternating column regeneration. The injections # 1, # 2000 and # 4000 are shown for a) column 1 and b) column 2. Compounds: Alkylphenones test mixture. System configuration with column regeneration.

Flushing and cleaning of the Agilent 1200 Series Autosampler SL Plus to achieve near zero carryover

During the injection routine of the autosampler, the sample loop, the inside of the needle, the seat capillary and the main channel of the injection valve are in the flow path, and remain there throughout the duration of the run. This means these parts are flushed continuously with mobile phase during the complete analysis. It is only during aspiration of the sample that the injection valve is switched out of the flow path. In this position, the pump effluent is led directly to the column. Prior to injection, the outside surfaces of the needle are washed with fresh solvent. This is achieved using the flush port of the autosampler, and prevents contamination of the needle seat. The flush port of the autosampler is refilled with fresh solvent by a peristaltic pump that is installed in the autosampler housing. The flush port has a volume of about 680 μl , and the pump delivers 6 ml/min. Setting the wash time to 10 seconds means that the flush port volume is refilled more than once with fresh solvent, which is sufficient in most cases to clean the outside of the needle.³

Selecting appropriate chromatographic conditions

Chromatographic conditions strongly depend on the compounds that are to be analyzed, but some rules of thumb can be used to achieve short run times:

- The flow rates should be as high as possible, depending on the required resolution, back-pressure and the detection system used.
- Steep gradients should be used.
- High column temperatures are recommended to enable high flow rates to be used, and to shorten run time even further. Zorbax SB columns can be used up to 90 °C, at low pH.³

2.3. How to achieve more resolution

Selecting a column

Resolution depends on the selectivity of the column, the retention of the peak and the plate number of the column:

$$\text{Resolution} = \frac{1}{4} (\alpha - 1) \sqrt{N} \left(\frac{k}{k+1} \right)$$

α = selectivity, N = plate number or Efficiency, k = retention factor of a compound.

- The first step in improving resolution is always to test different stationary phases and to select the column with the best separation. This is the parameter that is of most importance for resolution.
- The second step is to use long columns or even coupled columns to increase the plate number.
- A third step is to shift peaks to higher retention factors. For k values of 5 to 10, the impact is significant. With higher k values, the effect is very

low. In practice, this means that longer columns with appropriate selectivity give better resolution.

Selecting the appropriate instrument configuration

To maintain the high resolution achieved on the column, the extra-column volume, especially after the column, should be as low as possible.

- For 4.6 mm inner diameter columns the standard delay configuration should be used, see Figure 5.
- For UV detection with DAD, the 13 μ l standard cell is recommended.
- For 2.1 mm inner diameter columns, the low delay configuration should be used, and the low dispersion kit should be installed. For highest UV sensitivity, the short mixer is also recommended.
- The 2 μ l UV detector cell is recommended for use with the DAD, MWD and VWD with 2.1 mm inner diameter columns.
- The injection volume is also of importance, especially if the sample is dissolved in an organic solvent. In this case, the gradient should start with a low percentage of organic phase to focus the compounds at the top of the column. This avoids peaks dispersion due to the injection.

Selecting appropriate chromatographic conditions

As already stated previously, chromatographic conditions depend on the compounds to be analyzed. But here also, some rules of thumb exist.

- Moderate flow rates should be used, but recent experiments have shown that elevated flow rates can also be advantageous for improved separation¹⁸. For 4.6 mm inner diameter columns packed with sub-2-micron particles, a flow rate of 2 ml/min is recommended as a starting value. For 2.1 mm inner diameter sub-2-micron columns, 0.4 ml/min is a good starting point.
- Moderate gradients should be used, for example, 2 to 5% gradient change per minute.
- The column temperature is an additional parameter for optimization. Temperature can influence a separation, and should not be overlooked.³

2.4. How to achieve higher sensitivity

The signal-to-noise ratio depends on the peak height and the noise on the baseline. Several parameters have to be optimized to reduce noise and simultaneously increase peak height.

Selection of column length and column id

Using smaller inner diameter columns will generally result in higher sensitivity and is therefore ideal for applications with limited sample amounts. If the same sample amount can be injected on a smaller i.d. column, then the dilution due to column diameter will be less and the sensitivity will increase. For example, decreasing

the column i.d. from 4.6 mm down to 2.1 mm results in a theoretical gain in sensitivity of 4.7 times. This assumes that extra-column effects are minimized and there are no trade offs on the detector side. Some smaller flow cells have shorter path-length or higher detector noise and so the sensitivity gain might not quite be as great as theory indicates. However it is also important to match a small flow cell to a narrow column to preserve resolution. For a mass spectrometer detector, the lower flow rates with narrow columns can result in higher ionization efficiencies and therefore higher sensitivity.³

Selecting the appropriate instrument configuration

- To achieve lowest baseline noise, the standard delay volume configuration is recommended for the 1200 Series RRLC pump module.
- Here also, the injection volume and the sample dissolution solvent are important. Care must be taken that the compounds are focused at the top of the column, to avoid peak dispersion due to the injection, which would cause a reduced peak height. In order to achieve this, the sample should be dissolved in a solvent composition of lower elution strength than the mobile phase.
- The column temperature should not be too low, to avoid long retention of the peaks on the column. This also creates peak dispersion and a lower peak height.
- Selection of the optimum detector cell depends on the id of the column being used. Typically, the longer the path length, the better the signal-to-noise ratio. The data rate of the UV detector should be selected according to the actual peak width. Higher than necessary data rates should be avoided because of higher noise levels.
- Available UV detectors are the Agilent 1200 Series Diode Array Detector SL Plus and the new Agilent 1200 Series Variable Wavelength Detector SL, with data rates of 160 Hz and significantly lower noise and drift levels. For highest sensitivity in the UV range, the new Agilent 1200 Series VWD SL Plus is the optimum choice.³

2.5. Prevention of column blockages

Columns packed with sub-2-micron particles also need frits with small pore size to prevent packing material being swept out. This immediately brings increased risk of blocking these frits with particulates from the sample, mobile phase or/and from the instrument itself. To protect the column, additional small filters (Figure 9) can be used in front of the column. It is also recommended that the sample is thoroughly filtered or/and centrifuged, and that any kind of particulate matter in the mobile phases is avoided.

To ensure best results follow these simple usage guidance:

1. Install and run the column only in the flow direction marked on the column.
2. Use only solvents that are high quality, chromatography grade.
3. Filter all aqueous buffers and all samples through an appropriate 0.2 μm filter before use.
4. Replace bottles of mobile phase buffer every 24-48 hours — do not add mobile phase to the bottle; always use a new bottle.
5. Do not use a high buffer salt mobile phase (> 50 mM) in combination with high acetonitrile concentrations due to possible precipitation.
6. An in-line filter (5067-1551 for 2.1 or 3.0 mm inner diameter columns or 5067-1553 for 4.6 mm inner diameter columns) is recommended to catch particulates and extend column life. Change the filter when the pressure increases by 10 %.

7. Purge the pumps (the connections up to the column) of any buffer containing mobile phases and flush through 5 ml of solvent before attaching the column to the instrument.

8. Flush the column with compatible mobile phase starting slowly at 0.1 ml/min for a 2.1 mm inner diameter column, 0.2 ml/min for a 3.0 mm inner diameter column, and 0.4 ml/min for 4.6 mm inner diameter. Increase the flow rate to the desired flow over 5 minutes.

9. Once the pressure has stabilized, attach the column to the detector.

10. Equilibrate the column and detector with 10 column volumes of the mobile phase prior to use. (1 ml – 5 ml depending on column size.)

11. Avoid over-pressure. Check the pressure range of your gradient which may be 100—130 bar or more before starting any sequence.³



Figure 9 Protection for 4.6 and 2.1 mm id columns packed with 1.8 μm particles, inlet frit with 0.2 μm pore size.

A Typical Diagram of Rapid Resolution liquid chromatography



Figure 10. Rapid Resolution Liquid Chromatography

Conclusion

In this study, RRLC offers improved run times and increased sensitivity over conventional HPLC based methods. We applied the fast RRLC technique to the analysis of general chemical compounds. RRLC separations could be easily achieved from many traditional HPLC methods. Most applications of these small particle columns have been in the area of pharmaceutical analysis. Using a 1200 RRLC system equipped with higher acquisition rate of detector, low dead volume system configuration and combined with a high-pressure HPLC system with 600 bar pressure capability, the ultrafast RRLC analysis could be done with satisfactory analytical precision. In an isocratic example, one cycle analysis time by traditional HPLC/DAD method that required 25 min could be shortened to a RRLC/MS method to 0.8 min, a factor of 31. The same method transfer approach was carried out in a gradient separation. RRLC technique is useful for not only pharmaceuticals but also for chemical compounds currently analyzed by conventional HPLC on 250 mm×4.6 mm i.d. columns. In the future, the fast RRLC technique will be used more widely in other HPLC fields.

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