Optimizing the Agilent 1100 Series System for Higher Sample Throughput on Columns with Internal Diameters of 1 and 2 mm

Technical Note

Introduction

One trend in chromatography today is to run as many samples as possible in a given time. In HPLC analysis, columns with an internal diameter of greater than 4 mm are usually used for high sample throughput. These columns often have a length of 2 to 5 cm and the flow rates used are as high as 3 to 8 ml/min. They are also frequently used in combination with steep gradients.[1, 2, 3] After optimizing the chromatographic method, cycle times of less than 2 minutes can be expected. This is shown in a technical note describing high throughput on 4.6-mm id columns.[4] For some special applications cycle times of less than 30 seconds were obtained.[1] The limiting factor here is the ability of the detector to collect sufficient data points to ensure high precision and a low limit of detection.

Another trend in HPLC is to use columns with small internal diameters and low flow rates.[5] The driving factors here are:

- Lower limits of detection – same mass concentration in smaller peak volume.
- Ease of interfacing HPLC instruments to a mass spectrometer (MSD) – up to 1 ml/min are tolerable for most MSDs.
- Lower solvent consumption – less waste, less costs.

Compared to 4.6-mm id columns, it may appear that columns with internal diameters less than or equal to 2.1-mm id do not allow high sample throughput because of long run times and long equilbration times for gradient analysis.

This technical note describes which parameters need to be optimized to achieve higher sample throughput on narrow-bore and microbore columns with internal dia-meters of 1 and 2 mm. The experiments were run on a standard Agilent 1100 Series system. The Agilent mixer in the Agilent 1100 Series high-pressure gradient pump was, however, replaced by an Upchurch low delay volume mixer.

Two application examples are given, one for gradient HPLC analysis using UV detection and one for LC-MS analysis.

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Equipment

All HPLC experiments were carried out on the Agilent 1100 Series high-pressure gradient system comprising:

- Agilent 1100 Series high pressure pump for lowest delay volume. In this design each solvent is pumped by its own pump assembly, and mixing takes place on the high-pressure side. This means gradient changes reach the column much faster than in low-pressure gradient systems where mixing takes place on the low-pressure side.
  The Agilent mixer was replaced by the A-330 Semi-Prep Filter from Upchurch Scientific to achieve lowest delay volume (see http://www.upchurch.com).
- Agilent 1100 Series vacuum degasser for optimum baseline stability.
- Agilent 1100 Series autosampler for sampling from 2 ml standard vials.
- Optional Agilent 220 micro plate sampler for flexible sampling from deepwell and/or microtiter plates.
- Agilent 1100 Series thermostatted column compartment for highest stability from 10 °C below ambient to 80 °C.
- Agilent 1100 Series diode array detector with standard flow cell (10-mm pathlength, 13-microliter volume).
- Agilent 1100 Series LC/MSD detector for mass and structural information.
- Agilent ChemStation with 3D HPLC single instrument software for instrument control, data handling and sample tracking.

Compounds, chromatographic conditions

For our HPLC experiments we selected

- compounds of different polarity, such as caffeine, primidone, phenacetin, mandelic acid benzylester and bipheny, and
- barbiturates, such as barbital, allobarbital, phenobarbital, butabarbital, butalbital, amobarbital, mephobarbital and flunitrazepam.

For the LC-MS experiments we used

- sedatives, such as clonazepam, flunitrazepam, oxazepam and diazepam.

Optimizing chromatographic parameters

The following parameters have to be adapted for micro and narrow-bore columns to obtain short cycle times, sufficient resolution and good precision over a wide range of compound polarity:

- column length
- flow rate
- delay volume
- gradient
- column temperature

Table 1 roughly shows the importance of selected chromatographic parameters on cycle times. In addition, the limiting factors which hinder further reduction of cycle times are mentioned.

Parameter	Influence on cycle time	Practical limitation
Column length	+++++	peak resolution
Flow rate	+++	backpressure, peak resolution
Delay volume	+++	design of instrument
Gradient	++	peak resolution
Column temperature	+	peak resolution, instrument design

Table 1
Influence of chromatographic parameters and limitations for shortening cycle times (+++++ = strongest influence, + = lowest influence)

Influence of column length and flow rate on cycle times for 1-mm id columns

The reduction of column length is the most important parameter to shorten cycle times. Figure 1 shows the analysis of the same sample on different column lengths. For 1-mm id columns flow rates of 0.01 to 0.05 ml/min are recommended. Higher flow rates can be used, however, the backpressure has to be observed. For long columns of 250 mm, for example, backpressure may already build up at 0.2 ml/min using water and acetonitrile as mobile phases. This is a critical limit where column damage is possible. In our example, a flow rate of 0.1 ml/min was selected because here the backpressure was in the range of 150 bar. A flow rate of 0.2 ml/min already caused a backpressure close to 300 bar. Shorter 1-mm id columns are less critical with respect to higher flow rates. For the 5-cm column even a flow rate of 0.3 ml/min did not cause any backpressure problems.

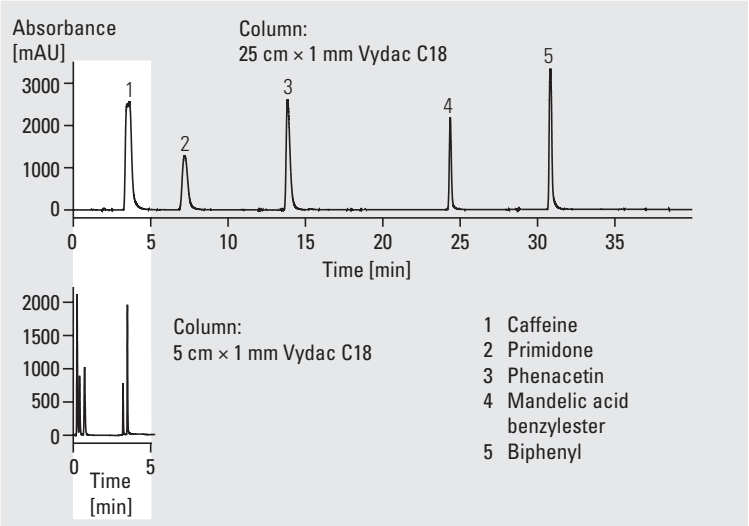

Figure 1
Influence of column length and flow rate on cycle time

	5 cm x 1 mm Vydac C18, 5 µm	**25 cm x 1 mm Vydac C18, 5 µm**
Run time	3.2 min	40 min
Post time	4.3 min	30 min
Injection volume	1 µl, ALS in bypass after 0.12 min	1 µl, ALS in bypass after 0.36 min
Column compartment temperature	60 °C	30 °C
Mobile phase	A = water, B = acetonitrile	same
Gradient	at 0.3 min 19 % B at 2 min 95 % B at 3 min 95 % B at 3.01 min 10 % B	at 0.3 min 10 % B at 30 min 75 % B at 30.01 min 10 % B
Flow rate	0.3 ml/min	0.1 ml/min
Detection	diode array detection, 210/8 nm, reference 360/100 nm, 13-µl flow cell	same

The second parameter which can reduce cycle times significantly is the flow rate. The limiting factor here is backpressure. With 0.5 ml/min flow rate the backpressure is in the range of about 210 bar starting with 90 % water and 10 % acetonitrile. With 0.3 ml/min flow rate the backpressure starts at 170 bar. In our case resolution also suffers from increasing flow rate, as shown figure 2.

Figure 1 shows the influence of reduced column length on cycle times and figure 2 shows the influence of increased flow rate on cycle times. Optimizing both parameters reduced the cycle time by a factor of 10. In addition, solvent consumption could be reduced by 30 % over 10 runs.

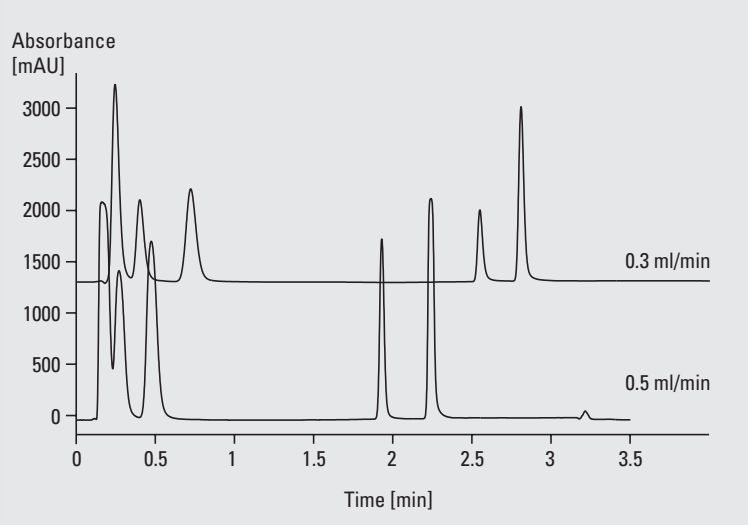

Figure 2
Influence of flow rate on cycle time and resolution

Influence of delay volume on cycle times for 1-mm id column

The lower the flow rate the greater the influence of system delay volume on cycle times. Figure 3 is an example for the influence of mixer delay volume. The lower the system delay volume, the shorter the time in which gradient changes are able to reach the top of the column. Also, equilibration times after a gradient analysis are significantly lower, if the delay volume is minimized.

The mixer is one means to lower system delay volume, another one is the autosampler. The Agilent 1100 Series system offers an injector

Equipment	Agilent 1100 Series HPLC system with binary pump, Agilent mixer or Upchurch mixer
Sample	caffeine, primidone, phenacetin, mandelic acid benzylester, biphenyl
Column	5 cm × 1 mm id Vydac C18 LC packings, 5 µm
Run time with Agilent mixer	4 min
Run time with Upchurch mixer	3.2 min
Post time	4.5 min or 4.3 min
Injection volume	1 µl ALS in bypass after 0.12 min
Column compartment temperature	60 °C
Mobile phase	A = water B = acetonitrile
Flow rate	0.3 ml/min
Gradient	at 0.3 min 10 % B at 2 min 95 % B at 3.1 min 10 % B
Detection	diode array detection 210/8 nm, reference 360/100 nm, pathlength 10 mm, 13 µl flow cell

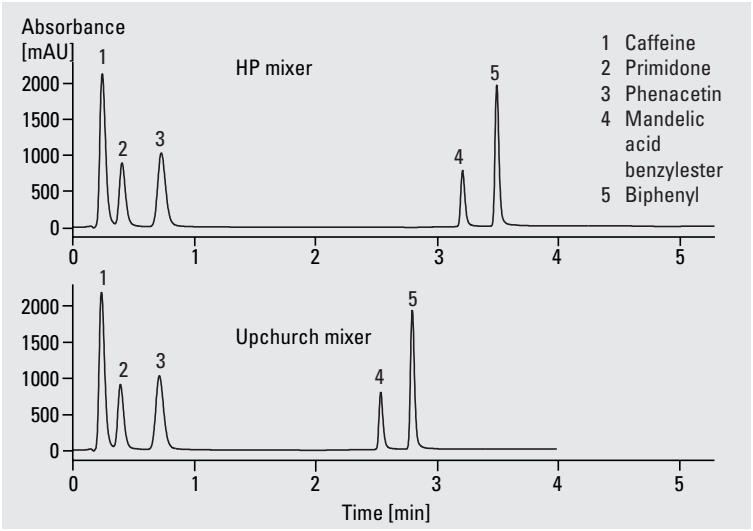

Figure 3
Influence of system delay volume – both runs with ALS in bypass

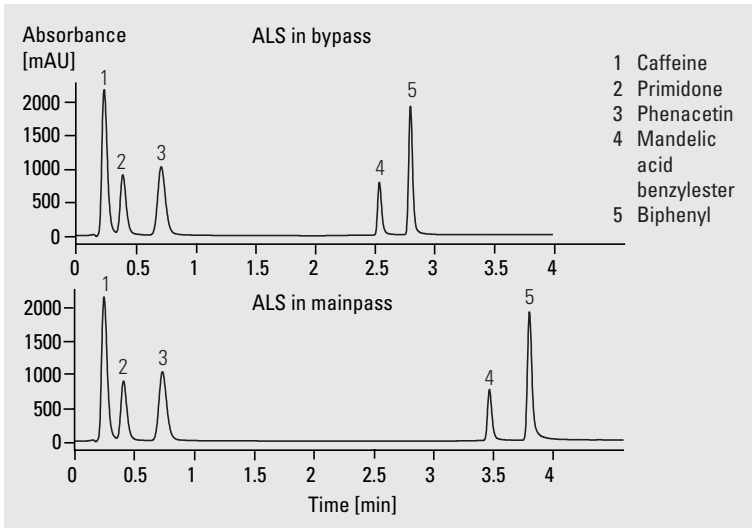

Figure 4
Influence of system delay volume using Upchurch mixer

program which switches the Agilent 1100 Series autosampler into the bypass position after the sample has reached the column. This saves another 300 µl of system delay volume. In figure 4 a comparison of the two autosampler settings is shown. Using the bypass function and the low volume mixer saves another minute.

The following injector program is used:

1 DRAW 1-µl from sample
2 INJECT
3 WAIT 0.12 min
4 VALVE bypass
5 WAIT 2.7 min
6 VALVE mainpass

The wait time before the valve is switched into the bypass position depends on the injection volume, according to the equation:

Wait time = 6 (injection volume + 5 µl) / flow rate.

Influence of gradient steepness on cycle times for 1-mm id columns

The gradient is another key factor, besides column length, flow rate and delay volume, which can shorten cycle times. The limiting factor for the steepness of the gradient is resolution. For the selected compounds, which are of different polarity, the gradient has to start at a low percentage of organic phase and then has to go up to approximately 100 % organic phase to ensure quick elution of the unpolar compounds (figure 5).

The gradient, which is less steep gives better resolution for the first two peaks, whereas the other gradient saves another 0.9 minutes run time. For MS analysis where resolution is less important for precise quantitation the steepest gradient can be selected.

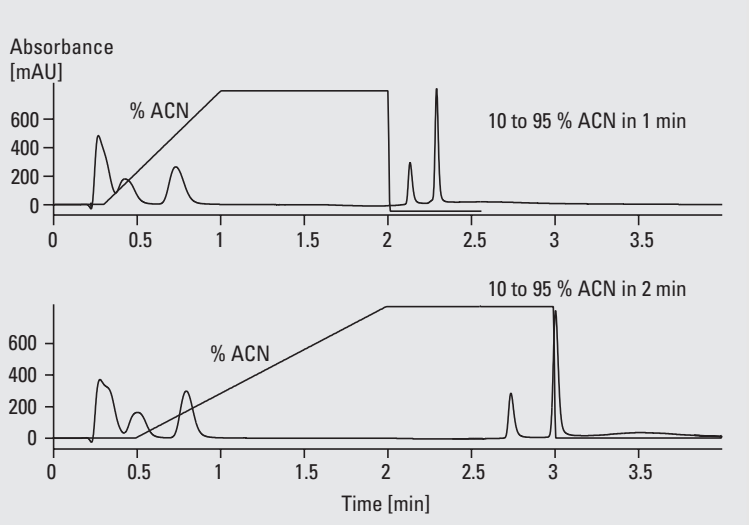

Figure 5
Analyses at two different gradients

Influence of column temperature on cycle times for 1-mm id columns

The influence of column temperature on cycle times is in general very low compared to the influence of the parameters mentioned before. In our example we compared the chromatograms obtained at 15 °C and 60 °C (figure 6).

The difference in cycle time is only about 0.2 min, however, in this case, the influence on peak shape is more important.

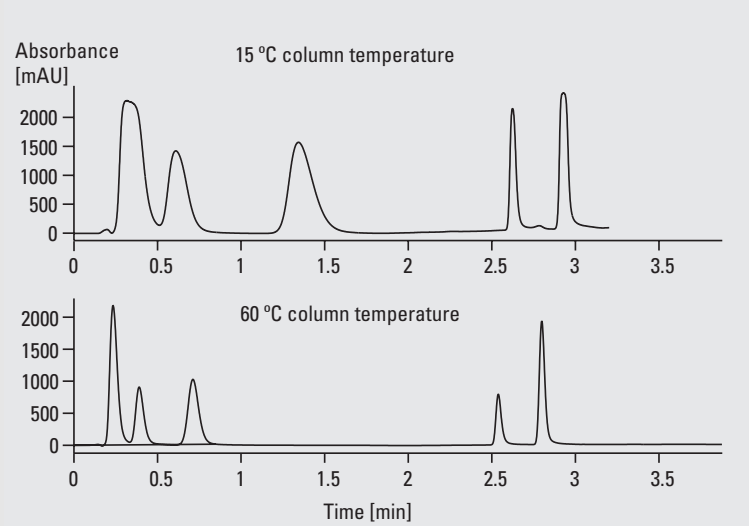

Figure 6
Influence of column temperature on cycle time

Result of cycle time optimization procedure for 1-mm id columns

Having optimized the most important parameters such as delay volume, flow rate and steepness of gradient, cycle times of less than 8 minutes are possible with a 5 cm × 1-mm id column for a compound mixture of different polarity (figure 7). Further reduction of cycle time was not possible due to the relatively long equilibration time which was needed after the run in order to get good precision for retention times (table 2).

Compound	RSD of retention time (10 runs)	RSD of areas
Caffeine	0.28	0.54
Primidone	0.18	0.81
Phenacetin	0.16	0.88
Madelic acid benylester	0.08	1.09
Biphenyl	0.12	1.20

Table 2
Performance on 1-mm id column

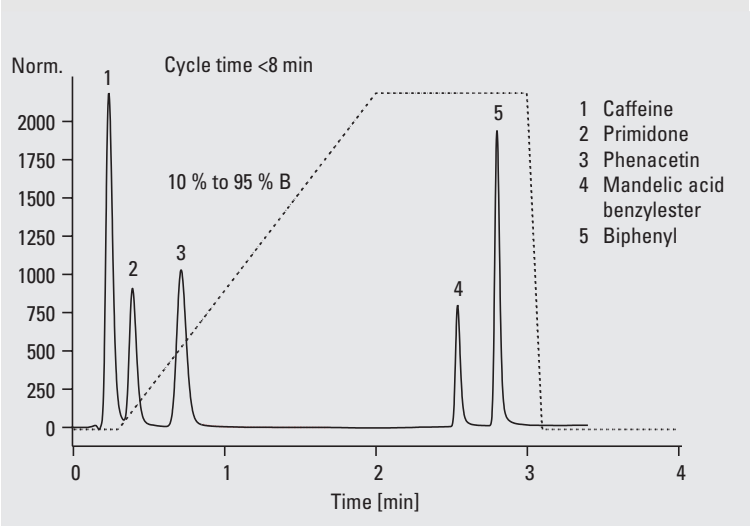

Figure 7
Result of optimization process for 1-mm id column

Equipment	Agilent 1100 Series HPLC system with binary pump, Upchurch mixer
Sample	caffeine, primidone, phenacetin, mandelic acid benzylester, biphenyl
Column	5 cm × 1 mm id Vydac C18 LC packings, 5 µm
Run time	3.2 min
Post time	4.3 min
Injection volume	1 µl ALS in bypass after 0.12 min
Injector program	1 DRAW 1 µl from sample 2 INJECT 3 WAIT 0.12 min 4 VALVE bypass 5 WAIT 2.7 min 6 VALVE mainpass
Column compartment temperature	60 °C
Mobile phase	A = water B = acetonitrile
Flow rate	0.3 ml/min
Gradient	at 0.3 min 10 % B at 2 min 95 %B at 3.1 min 10 %B
Detection	diode array detection 210/8 nm, reference 360/100 nm, pathlength 10 mm, 13-µl flow cell

Influence of flow rate and steepness of gradient on cycle time for 2.1-mm id columns

The influence of column length, flow rate and steepness of gradient on cycle times on 2-1-mm id columns is similar to that of 1-mm columns. First the column length has to be reduced, then the flow rate, the gradient and delay volume have to be optimized (figures 8 and 9). In our example we used a column of 30-mm length. The advantage of short 2.1-mm id columns is that flow rates up to 2 ml/min are possible without producing high backpressures that may damage the column. These relatively high flow rates also help to reduce equilibration times after the run. Consequently, cycle times of less than 2.5 minutes are possible for a compound mixture of different polarity.

Equipment	Agilent 1100 Series HPLC system with binary pump, Upchurch mixer
Sample	caffeine, primidone, phenacetin, mandelic acid benzylester, biphenyl
Column	3 cm × 2.1 mm id Zorbax XDB C18, 3.5 µm
Injection volume	0.5 µl ALS in bypass after 0.12 min
Column compartment temperature	60 °C
Mobile phase	A = water B = acetonitrile
Flow rate	1 or 1.5 ml/min
Gradient	at 0.3 min 10 % B at 2 min 95 %B or 1 min 95 % B
Detection	diode array detection 210/8 nm, reference 360/100 nm, pathlength 10 mm, 13-µl flow cell

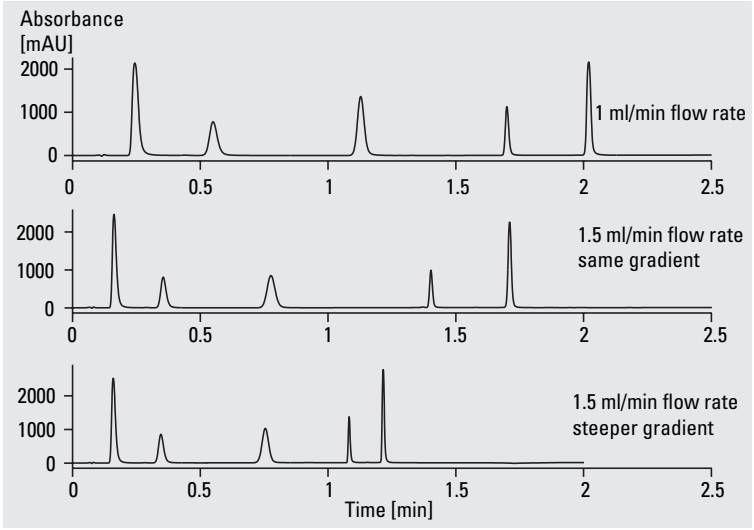

Figure 8
Influence of low flow rate and steepness of gradient on 2.1 mm column

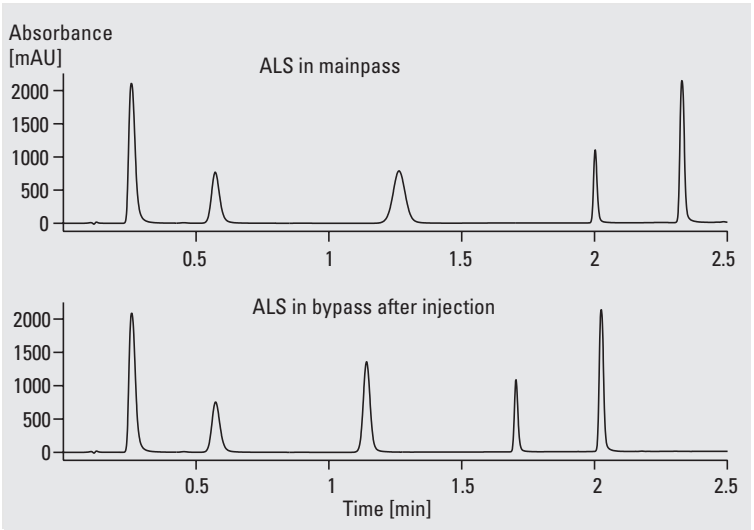

Figure 9
Influence of delay volume on cycle time for 2.1 mm id column and 1 ml/min flow rate

Result of cycle time optimization procedure for 2.1-mm id columns

With 3 cm × 2.1 mm columns cycle times of 2 minutes can be achieved even if analyzing compounds of different polarity in one run. Figure 10 shows the optimized chromatogram. Precision of retention times is listed in table 3.

Compound	RSD of retention time (10 runs)	RSD of areas
Caffeine	0.22	0.61
Primidone	0.15	0.72
Phenacetin	0.12	0.79
Madelic acid benylester	0.06	0.75
Biphenyl	0.04	1.90

Table 3
Performance data on 2.1-mm id column

Equipment	Agilent 1100 Series HPLC system with binary pump, Upchurch mixer
Sample	caffeine, primidone, phenacetin, mandelic acid benzylester, biphenyl
Column	3 cm × 2.1 mm id Zorbax XDB C18 3.5 µm
Run time	1.2 min
Post time	0.8 min
Injection volume	0.5 µl ALS in bypass after 0.02 min
Injector program	1 DRAW 0.5 µl from sample 2 INJECT 3 WAIT 0.02 min 4 VALVE bypass 5 WAIT 1.1 min 6 VALVE mainpass
Column compartment temperature	60 °C
Mobile phase	A = water B = acetonitrile
Flow rate	2 ml/min
Gradient	at 0.3 min 10 % B at 1 min 95 %B at 1.18 min 95 %B at 1.19 min 10 % B
Detection	diode array detection 210/8 nm, reference 360/100 nm, pathlength 10 mm, 13-µl flow cell, response time < 0.01 min

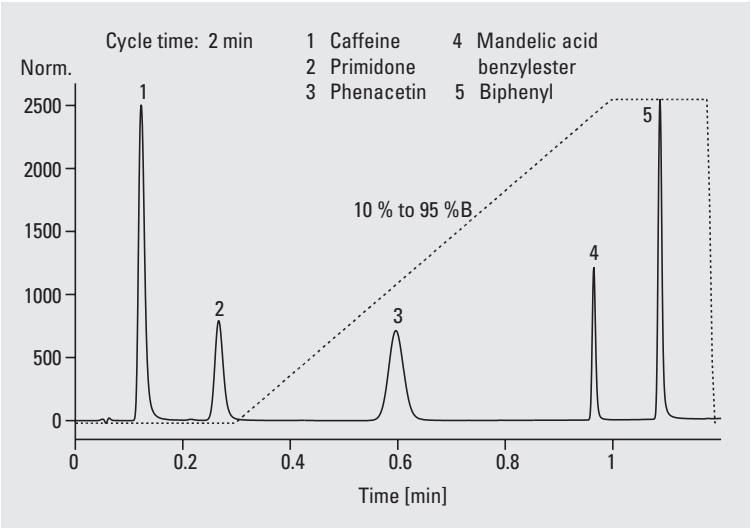

Figure 10
Result of optimization process for 2.1-mm id columns

Influence of column id and flow rate on UV detection

One major advantage of short narrow-bore columns (2.1-mm column id) over short standard-bore columns (4.6-mm column id) is that, for equivalent cycle times, the limit of detection is far better. This is due to less sample dilution. Furthermore, with 1 or 2-ml/min flow rate the detector is able to collect more data points at the selected response rate than at a flow rate of 4 or even 8 ml/min. Figure 11 compares two columns of the same length but different inner diameters and different flow rates, which ensures equivalent elution times. The column material and all other conditions were the same. To get a similiar run and similar cycle times the flow rate for the 2.1-mm id column was 2-ml/min, whereas it was 4.5 ml/min for the 4.6-mm id column. Comparing the area counts for the last two peaks, which have similar retention times and a similar peak shape, showed a loss of counts for the 4.6-mm id column of about 50 %.

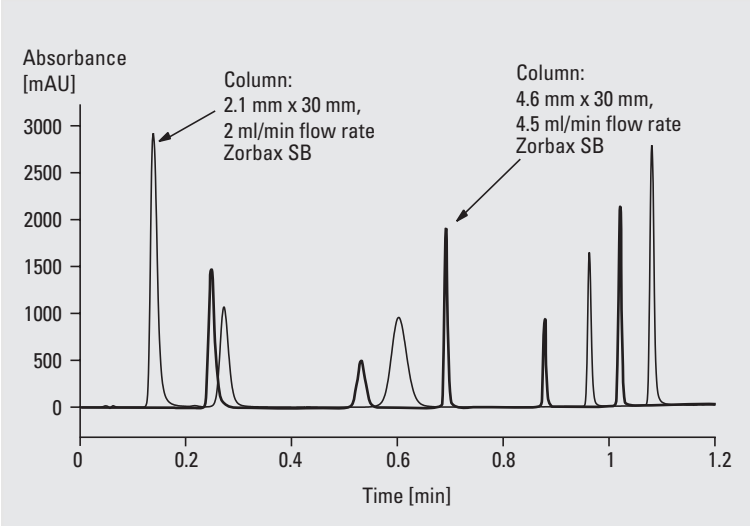

Figure 11
Influence of column id on limit of detection

Application Examples

Analysis of hypnotic drugs on short 1-mm id columns

Several different hypnotic drugs were analyzed using two columns of different internal diameters (figure 12). One set of runs was done on a 125 × 4 mm id ODS Hypersil column, the other set on a 50 × 1 mm Vydac C18 column. It is quite obvious that the resolution is far better on the 4-mm column. On the other hand, for rapid screening the analysis can be done in half the time on the 1-mm column. Furthemore, the analysis can be done with 85 % less consumption of solvent over 10 runs. Considering the above benefits the 1-mm column can be considered as an alternative method. The precision data for both columns is listed in table 4.

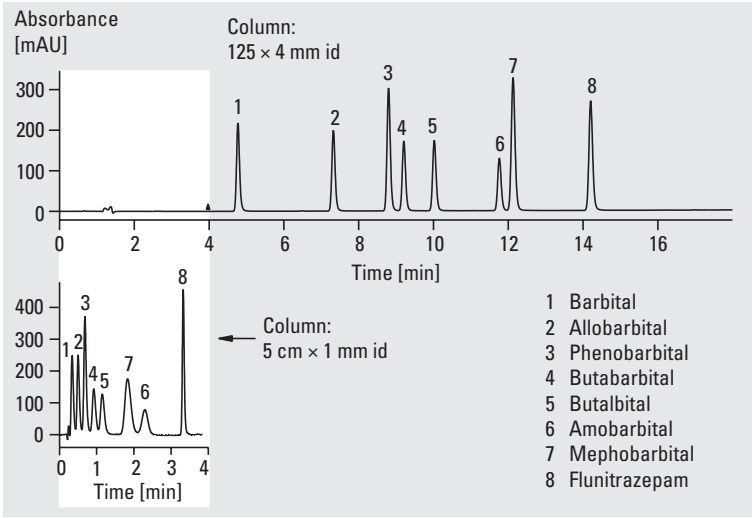

Figure 12
Analysis of barbiturates on 4-mm id and 1-mm id column

	4 cm x 125 mm ODS Hypersil 5 µl guard cartridge 4 x 4 mm Hypersil ODS	**1 x 50 mm Vydac C18, 5 µm**
Stop Time	18 min	4 min
Post time	5 min	10 min
Injection volume	5 µl	1 µl, ALS in bypass after 0.12 min
Column compartment temperature	25 °C	60 °C
Mobile phase	A = water, B = acetonitrile	same
Gradient	10 % B to 50 % B in 16 min 50 % B to 10 % B in 2 min	10 % B for 0.3 min at 3 min 75 % B at 3.51 min 10 %
Flow rate	1.0 ml/min	0.3 ml/min
UV detection	variable wavelength detection 204 nm, standard cell	diode array detection, 210/8 nm, reference 360/100 nm standard cell

Table 4
Performance of HPLC analysis of barbiturates

Compound	**RSD of RT 1-mm column**	**RSD of areas 1-mm column**	**RSD of RT 4-mm column**	**RSD of areas 4-mm column**
Barbital	0.16	0.83	0.05	0.50
Allobarbital	0.14	0.87	0.05	0.38
Phenobarbital	0.20	0.88	0.05	0.36
Butabarbital	0.14	0.87	0.03	0.25
Butalbital	0.13	1.07	0.03	0.25
Amobarbital	0.14	0.88	0.02	0.25
Mephobarbital	0.09	1.08	0.02	0.25
Flunitrazepam	0.06	0.88	0.03	0.23

LC-MS analysis of sedativa on short 2.1-mm id columns

Modern LC-MS analysis is not as dependent on excellent peak resolution as HPLC analysis with UV or fluorescence detection to obtain precise quantitative data. In MS analysis precise results can be obtained even if two or three peaks overlap and are not or only partially separated. The limit of detection in LC/MS is generally in the low pg range (injected amount) which can also be achieved with fluorescence detection, however, only in a few cases with UV detection. Most MS instruments today are also able to handle flow rates of up to 1 ml/min.

The conclusion is that for fast LC/MS analysis short 2-mm columns with 1-ml/min flow rate are ideal for high sample throughput. Figure 13 shows the rapid analysis of four only partially separated sedativa. The cycle time is as low as 4 minutes.

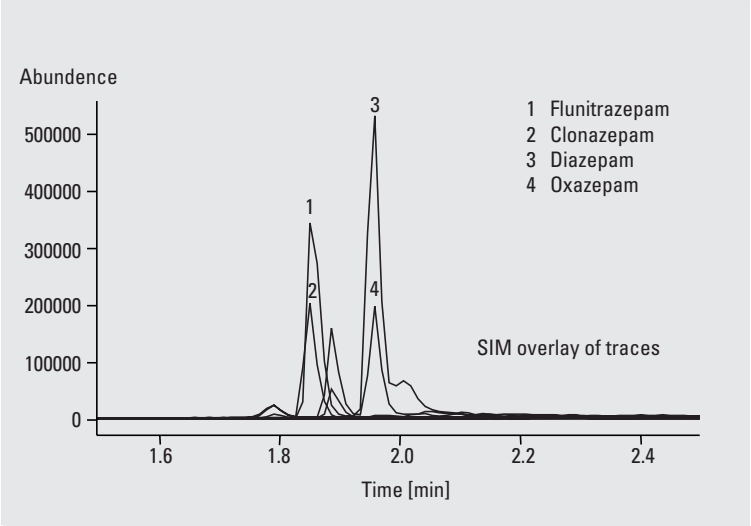

Figure 13
Rapid LC-MS analysis of sedatives on 2.1 mm id column

Equipment	Agilent 1100 Series HPLC system with binary pump	**Column compartment temperature**	50 °C
		Mobile phase	A = water
Sample	clonazepam, flunitrazepam oxazepam, diazepam		B = methanol
		Flow rate	1 ml/min
Run time	3 min	**Gradient**	at 0 min 5 % B
Post time	1 min		at 1 min 95 %B
Column	3 cm × 2.1 mm id Zorbax XDB C18, 3.5 µm		at 2.5 min 95 % B
			at 3 min 10 % B
Injection volume	1 µl ALS in bypass after 0.02 min	**Detection**	MS ESI positive mode, fragmentor: 80 V

Compound	RSD retention time	RSD area (injected amount <2pg)
Clonazepam	0.06	5.48
Flunitrazepam	0.06	5.68
Oxazepam	0.08	4.59
Diazepam	0.04	4.15

Table 5
Performance of LC-MS analysis of sedatives

Conclusion

An increase of sample throughput on 1- and 2-mm id columns by a factor of 10 can be achieved with a significantly reduced column length. The flow rate has to be increased such that the backpressure and resolution stay in acceptable ranges. The gradient should also be as steep as possible to ensure good resolution and fast elution of unpolar peaks. To obtain optimum quantitative data, baseline separation of peaks is always needed, when using an UV detector or/and fluorescence detector. Columns of 5 cm or 3 cm lengths with 1-mm or 2-mm id and flow rates of 0.3 to 2 ml/min are best suited in this case. If an MS instrument is used as detector, quantitative data will be excellent even if two or three peaks are not or only partially separated. Therefore, the cycle time is not limited by resolution. Very short 2-mm columns with 1-ml/min flow rates are ideal in this case.

Table 6 shows the advantages and disadvantages of fast HPLC versus conventional standard HPLC – time saving versus solvent consumption versus gain for the limit of detection is shown for different column ids. In principle, the smaller the column id, the longer the run cycles, and the lower the solvent consumption the higher the limit of detection.

Gradient operation mode	Column dimension	Flow rate	Cycle times	Solvent consumption (10 runs)	Advantage	Disadvantage
Conventional HPLC	4.6 × 100 mm 2.1 × 100 mm 1 × 250 mm	1.5 ml/min 0.4 ml/min 0.1 ml/min	25 min 30 min 70 min	375 ml 120 ml 70 ml	excellent resolution	long cycle times
Fast standard HPLC	4.6 × 50 mm 4.6 × 30 mm	3 ml/min 4.5 ml/min	2.5 min 1.5 min	40 ml 67.5 ml	very short cycle times, low solvent consumption, over a limited series of runs	limit of detection depends on lowest possible data rate of detector[4]/resolution
Fast narrow-bore HPLC (LC/MS)	2.1 × 30 mm	1 ml/min 2 ml/min	4 min 2 min	40 ml 40 ml	short cycle times, low solvent consumption, in principle better limit of detection, best suited for LC-MS	resolution
Fast microbore HPLC	1 × 50 mm	0.3 ml/min	10 min	30 ml	moderate cycle times, very low solvent , consumption, in principle better limit of detection	resolution, backpressure

Table 6
Advantages and disadvantages of "fast" HPLC on micro, narrow and standard bore columns versus conventional HPLC

As a final conclusion we can state that the following aspects have to be taken into consideration if high sample throughput is required:

- Reduce cycle times by using a short column, high flow rates and steep gradients.
- Ensure compatibility of selected flow rate with detector (for example, MS can not handle more than 1 ml/min).
- Limit of detection – peak width should allow collection of 20 to 30 data points per peak.

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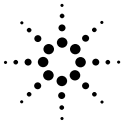

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