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Practical aspects of fast reversed-phase high-performance liquid chromatography using 3 μm particle packed columns and monolithic columns in pharmaceutical development and production working under current good manufacturing practice

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Abstract

The potential and limitations of fast reversed-phase high-performance liquid chromatographic separations for assay and purity of drug substances and drug products were investigated in the pharmaceutical industry working under current good manufacturing practice using particle packed columns and monolithic columns. On particle packed columns, the pressure limitation of commercially available HPLC systems was found to be the limiting factor for fast separations. On 3 μm particle packed columns, HPLC run times (run to run) for assay and purity of pharmaceutical products of 20 min could be achieved. As an interesting alternative, monolithic columns were investigated. Monolithic columns can be operated at much higher flow rates, thus allowing for much shorter run times compared to particle packed columns. Compared to particle packed columns, the analysis time could be reduced by a factor up to 6. However, some compounds investigated showed a dramatic loss of efficiency at higher flow rates. This phenomenon was observed for some larger molecules supporting the theory that mass transfer is critical for applications on monolithic columns. At flow rates above 3 ml/min some HPLC instruments showed a dramatic increase in noise, making quantifications at low levels impossible. For very fast separations on monolithic columns, the maximum data acquisition rate of the detector is the limiting factor.

Keywords: Stationary phases, LC; Monolithic columns; Pharmaceutical analysis; Liquid chromatography, fast

1. Introduction

The drive to submit new drug applications ever faster has put increasing pressure on the development process in the pharmaceutical industry. In drug development, a large number of new drug substances and drug products are developed and have to be tested for their potency and purity. For this purpose, high-performance liquid chromatography is usually employed and a large amount of samples have to be tested for stability. One aspect of the time consumed in the analytical laboratory is the high-performance liquid chromatographic runtime. Today HPLC runtimes of 60 min (run to run) are not uncommon in the pharmaceutical industry, so it takes several hours if not days to get the data needed. Also in quality control and process control it is critical to get analytical data fast to release a batch or to control a process. Therefore, fast analysis is not only important in the development process but a key element throughout the whole life cycle of a product. Several aspects are relevant in the pharmaceutical industry when developing a HPLC method for drug substances and drug products.

(i) Since work has to be done under current good manufacturing practices (cGMP), all methods must be reproducible and validated [1,2].
(ii) Usually, besides the drug substance, by- and degradation products have to be determined down to a level

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of 0.05%. Generally, methods where the assay of the drug substance and the purity can be determined in the same run are preferred. Therefore, it is favorable to use a method with a linear range of 0.05–120% of the working concentration plus adequate sensitivity (signal-to-noise ratio 10:1 at a level of 0.05%).

(iii) In many cases as many as 10–15 components (by-, degradation products, excipients, preservatives etc.), which are often structurally related, have to be separated by the analytical method. To achieve this separation power, a certain number of theoretical plates is required.

(iv) Thanks to the fact that in pharmaceutical development processes are scaled up from the laboratory to production equipment, availability of the sample is usually not limited. There is no need to use micro bore columns and micro HPLC systems.

(v) Most pharmaceutical companies are global companies. This means that methods are often transferred from one site to another requiring that methods can be run on commercially available equipment worldwide. Hence, the limitations of currently available HPLC systems have to be taken into account when developing a new HPLC method.

(vi) Besides assay and purity, other parameters like content uniformity and dissolution rate are routinely monitored. In those cases usually only the main component is of interest and HPLC methods with only limited resolution or spectrophotometric methods are sufficient.

In recent years progress bas been made in the area of fast separations with particle packed columns. In the early 1990s 250 mm × 4.6 mm columns packed with 5 μm particle size were generally used in analytical HPLC. At that time, columns with 3 μm particles became first available and the quality of the columns was a concern [3]. Nowadays many phases from a variety of suppliers are available on the market with 3 μm particle size. Column manufacturers are investigating even smaller particles than 3 μm; for example columns packed with hybrid particles of 2.5 μm are available on the market [4]. The preparation of porous and non-porous silica particles in the range of 0.2–2 μm has been reported [5]. Besides the difficulty of producing uniform particles of such small diameters and packing them in a column, the pressure drop of such a column would be much higher than the pressure limit of most commercially available HPLC systems today.

MacNair et al. have reported the use of ultrahigh-pressure LC using packed capillaries with non porous silica particles of 1–1.5 μm in diameter in isocratic as well as gradient elution mode [6,7]. Mobile phase pressures of up to 5000 bar (72 000 psi) were applied to generate more than 200 000 theoretical plates.

To reduce the backpressure when using columns packed with particles of 3 μm or smaller, often the column length is reduced as well, thus reducing the column volume. As the column volume is lowered, the extra column volume of the HPLC system has to be considered. The extra column volume consists of the injection volume, the capillary connections to the column and from the column to the detector plus the detector cell volume. The higher the extra column volume of the instrument, the more pronounced is the band broadening of the peaks. Band dispersion has been extensively investigated and a vast amount of data has been reported. Knox [8] has recently given a review of the work done.

Other approaches for shortening HPLC run times have also been investigated. One of these approaches uses a new type of stationary phase based on a single piece of porous silica material (monolith) that has a total porosity of over 80% [9]. The silica gel skeleton of these Chromolith columns is constituted of differently sized large and small pores created by a new patented “sol–gel” process [10,11] involving highly purified metal-free alcoxy-silanes [12,13]. The dense network of 2 μm diameter macropores through which the eluent can flow quickly allows much higher flow rates without significant pressure drop compared to particle packed columns, while the fine porous structure of 13 nm diameter mesopores provides the surface required for the separation process. Monolithic columns exhibit flat van Deemter plots comparable to 3 μm particle packed columns [14]. This silica network structure is responsible for excellent resolution at high flow rates due to enhanced mass transfer. The solute reaches the pores by convection and not by diffusion. In this flow regime, the separation process is much faster [15–18].

In this work the potential and limitations of fast RP-HPLC separations for assay and purity of drug substances and drug products were investigated in the pharmaceutical industry working under cGMP using particle packed columns and monolithic columns.

2. Experimental

HP 1100 (Agilent, Palo Alto, CA, USA), Waters Alliance 2695 (Waters, Milford, MA, USA), Shimadzu 2010 (Shimadzu, Kyoto, Japan) and TSP 3000 (Thermo Finnigan, San Jose, CA) HPLC systems equipped with quaternary low pressure gradient systems and standard analytical cells of 10 mm path length were used. Some data were acquired using a high pressure binary pump (HP 1100). HPLC systems were equipped with on-line degassers, autosamplers equipped with temperature controlled compartments for the samples, column heaters, variable wavelength detection (VWD) or photodiode array detection (DAD) systems, as indicated in the result section. For data processing and acquisition, Peak Net 5.101d software (Dionex, Sunnyvale, CA, USA) was used. All reagents used were of analytical grade supplied by Merck (Darmstadt, Germany) or Fluka (Buchs, Switzerland). Water was purified to a quality of ≥18.0 MΩ cm (e.g. Milli-Q system, Millipore, Billerica,

MA, USA). Chromatographic columns were purchased from the respective manufacturer (Waters, Milford, MA, USA; YMC, Kyoto, Japan; Macherey–Nagel, Düren, Germany and Merck). Novartis standards and samples of drug products and drug substances were analyzed. Sample solutions were prepared according to internal Novartis procedures.

The extra column volume of the HPLC instruments was measured by replacing the column by a low volume flow restrictor and injecting an uracil solution at a given flow rate. The retention time of the uracil peak was then multiplied by the flow rate to calculate the extra column volume.

The dwell volume was determined graphically from a step gradient with methanol and a mixture of methanol containing 0.4% of acetone.

Van Deemter plots on particle packed columns were acquired injecting a solution of methyl-, ethyl-, propyl- and butylparaben of 0.015 mg/ml each, the mobile phase consisting of water–acetonitrile (60:40, v/v), the detector wavelength set to 254 nm and the column temperature to 40 °C. The plate height was determined according to European Pharmacopoeia [19].

The baseline noise was determined with Chromolith columns (RP18e, 100 mm × 4.6 mm i.d., Merck) and isocratic elution of 10 mM KH_2PO_4, pH 3.0–acetonitrile (40:60) at 25 °C. The detector signal was monitored at 210 nm with a frequency of 10 Hz. The short-term noise ($dt = 1$ min) was calculated for a time range of 10 min with the American Society for Testing and Materials (ASTM) method based on peak to peak measurement [20].

Chromolith column efficiency was evaluated with isocratic runs by calculating the number of theoretical plates per column with the tangent method [21].

3. Results and discussion

3.1. Fast RP-HPLC methods on particle packed columns

3.1.1. Hardware considerations

The most important hardware factors affecting fast HPLC on particle packed columns are listed in Table 1. The maximum operating pressure is the most important limitation of fast chromatography on particle packed columns. The pressure limit as shown in Table 1 restricts the flow rate on a 150 mm × 3.0 mm, 3 μm particle packed column to about 1 ml/min.

Attention must be paid to the extra column volume (sum of the injection volume, the capillary volume connecting the column from the injector and to the detector and the detector cell volume). Unnecessary components like column switching devices and connectors, that also contribute to the extra column volume, must be avoided whenever possible.

The values given in Table 1 for extra column volume are values obtained after optimization of the HPLC system (use of 0.12 mm i.d. capillaries as short as possible). In the isocratic mode, the extra column volume contributes significantly to the band broadening of the peaks. Critical in this respect is the ratio of the extra column volume to the column volume itself. The smaller the column used, the stronger the influence of the extra column volume of the instrument gets.

Comparing Van Deemter plots of different column dimensions, we concluded that using the standard analytical cell a column void volume (volume of the empty tube) of not less than 1 ml is required otherwise a band broadening for the early eluting peak methylparaben ($k = 2.5$) can be observed. The use of the standard detector cell with 10 mm path length has a sensitivity advantage compared to the micro cell with only 6 mm path length.

The dwell volume is an important factor when running fast gradients. If the flow rate is 1 ml/min, the gradient delay is about 1 min for the instruments listed in Table 1. Of course it is possible to work with a delayed injection so that the injection occurs at the moment when the dwell volume has been flushed, but this does not reduce the overall runtime because the equilibration time is also part of the total runtime. A solution to excessive dwell volume is the use of binary high pressure pumps, which generally have much lower dwell volumes.

3.1.2. Column life time

Columns with 3 μm particles are generally more expensive than columns with 5 μm particles. Therefore, for routine use the column life time is an important factor. A survey conducted in our laboratories investigating the life-time of 3 μm particle packed analytical columns reviewing about 80 columns from different manufacturers (Waters, Macherey–Nagel and YMC) over a 1 year time period showed that under appropriate operating conditions

Table 1
Parameters affecting fast separations of widely used HPLC systems

System	Maximum operating pressure (bar)	Extra column volume (μl)[a]	Dwell volume (ml)[b]
Agilent HP 1100, DAD	400	40	1.1
Waters Alliance 2695, DAD (996 model)	345	60	0.7
Shimadzu 2010, VWD	380	90	1.0
TSP 3000, VWD	400	40	1.0

For extra column volume and dwell volume measurement, see Section 2.

[a] Standard detector cell, 10 mm path length.

[b] Quaternary low pressure systems.

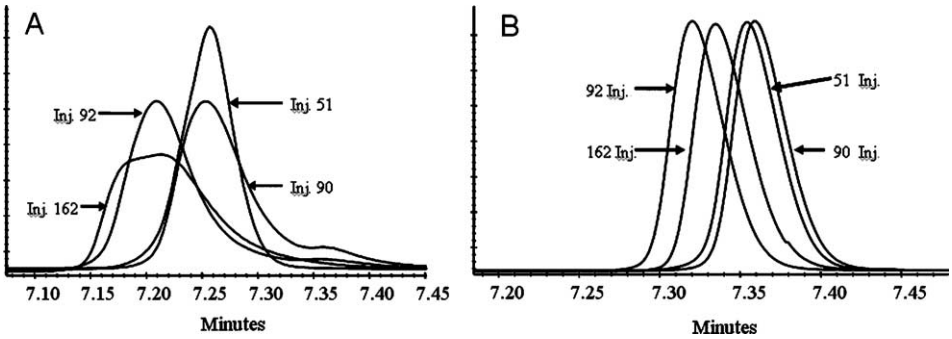

Fig. 1. Column performance deterioration after repeated injection. Column: YMC ODS AQ 150 mm × 3.0 mm, 3 μm, injection numbers 1–50 at 1.0 ml/min (260 bar), injection number 51–200 at 1.2 ml/min (330 bar), acetonitrile/phosphate buffer pH 3, gradient. (A) Commercial column quality, (B) custom made 'high pressure' packing.

(pH, temperature) a column life-time of more than 1000 injections can be reached. Columns packed with 3 μm particles are more sensitive to column damage than conventional columns with 5 μm particles. Precautions have to be taken to avoid clogging. Another reason for column damage is pressure. Some manufacturer pack their analytical columns with 3 μm particles at pressures lower than 400 bar. The rapid deterioration of such a column is shown in Fig. 1. After only a few injections at 330 bar the commercial column showed a drastic deterioration of the peak shape. After opening the column at the column head, a void volume was visible. Operating the column at this pressure compressed the packaging. Most manufacturers today pack at pressures above 400 bar. However, some column manufacturers are reluctant to give details about their packing process.

3.2. Fast RP-HPLC methods on monolithic columns

3.2.1. Hardware considerations

On monolithic columns generally much higher flow rates are applied than on particle packed columns. In this work flow rates as high as 4–5 ml/min were applied, according to the limitations given by the instruments, without encountering pressure limitations. To our surprise we discovered that a widely used HPLC system showed a strong dependence of the baseline noise on the flow rate. To determine by- and degradation products at levels down to 0.05% of the main substance, a low baseline noise is required to obtain an appropriate signal-to-noise ratio ($S/N \geq 10$). Fig. 2 shows the baseline noise as a function of the flow rate for four HPLC systems investigated.

For all HPLC systems except for Agilent HP 1100 systems equipped with a DAD detector, the level of baseline noise is independent from the flow rate (see Fig. 2). The Agilent HP 1100 systems equipped with a DAD detector showed a dramatic increase of the baseline noise for flow rates higher than 3 ml/min. This observation could be confirmed with 6 different HP 1100 DAD systems. All of them showed an increase of the noise at flow rates above 3 ml/min. As a result, the limit of detection obtained with such systems would be out of the requirements for the determination of by- and degradation products when operating at flow rates of greater than 3 ml/min.

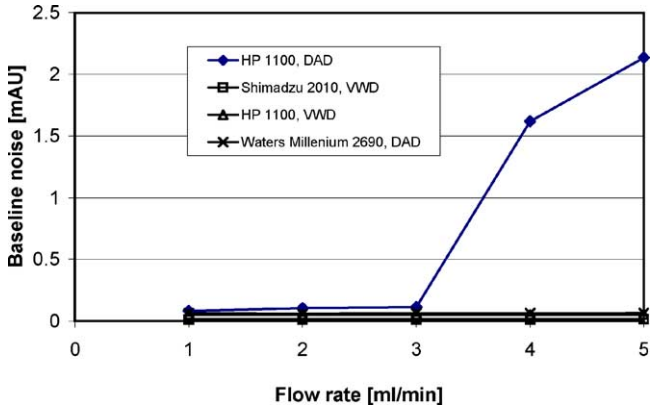

Fig. 2. Baseline noise as a function of the flow rate on Chromolith column using different types of HPLC systems; for experimental conditions see Section 2. DAD: photodiode array detection.

Further investigations focused on the impact of the pump and the detector on the baseline noise at higher flow rates. Fig. 3 shows the baseline noise as a function of the flow rate for HP 1100 binary and quaternary pump systems equipped

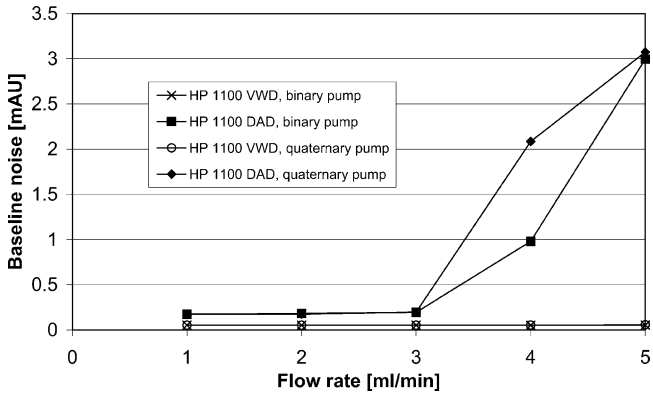

Fig. 3. Baseline noise as a function of the flow rate performed on Chromolith column using HP 1100 binary and quaternary pump systems equipped with HP 1100 DAD or VWD systems; for experimental conditions see Section 2.

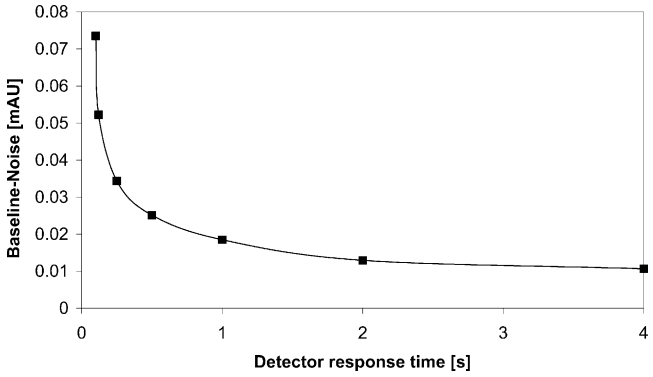

Fig. 4. Baseline noise as a function of the detector response time performed on Chromolith column (RP18e, 100 mm × 4.6 mm i.d., Merck) with a HP 1100 binary pump and a VWD system. Detection UV at 254 nm. Flow rate 5 ml/min. Temperature 25 °C. Isocratic elution of a mixture of acetonitrile and 10 mM KH_2PO_4, pH 3.0.

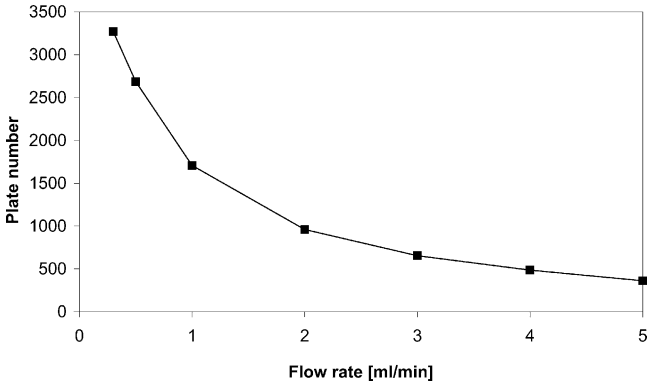

Fig. 5. Plate number of a Novartis development compound ($M_r = 810.5$) as a function of the flow rate on two Chromolith columns (RP18e, 100 mm × 4.6 mm i.d., Merck) in series, isocratic elution with water–acetonitrile–*tert*-butyl methyl ether–phosphoric acid (521:406:73:0.2), detection UV at 210 nm.

with HP 1100 DAD or VWD systems. Fig. 3 shows that the increase of the baseline noise for flow rates higher than 3 ml/min is only observed with the HP 1100 DAD system, independent of the pump system used (binary or quaternary). Comparing the characteristics of the different detector systems tested, a significant difference in the flow cell geometry of the detectors is noticed. Therefore, the most probable origin for the increase of the baseline noise noticed for flow rates higher than 3 ml/min for the DAD systems of HP 1100 is an inadequate geometry of its flow cell or insufficient thermostatisation of the mobile phase before entering the detector cell. Indeed, an inadequate geometry of the cell could generate an interruption of the laminar flow and cause turbulences due to mechanical agitation, and therefore cause an increase of the noise. Although there is no difference of geometry between the standard and high pressure flow cells for the HP 1100, investigations with high pressure flow cells have been carried out and the same increase of the baseline noise at high flow rates has been observed.

While chromatographic peaks become narrower when increasing the flow rate, the number of points per peak for a given data collection rate (response time) decreases. With a too low number of points per peak, narrow peaks are no longer well defined, their heights are reduced and their widths increased. However, assays performed by HPLC have to show a sufficient reproducibility in terms of peak areas. Approximately 30 points per peak are required to define a peak well and to obtain a good reproducibility. Peaks acquired with fewer points are still recorded, but there is a band broadening due to the detector response time. Fig. 4 shows the baseline noise as a function of the detector response time.

Detector response time has to be lowered to ensure a good peak definition, but should be kept as large as possible since baseline noise increases exponentially. It has been noticed that with all HPLC systems used in this work, the detector response time is limited to a minimum of 0.1 s (10 Hz). This is a limiting factor for very fast HPLC methods.

3.2.2. *Efficiency of chromolith columns*

Some of our development compounds showed a dramatic loss of theoretical plates on monolithic columns at high flow rates, thus making a fast separation impossible, as shown in Fig. 5. For this compound, the percentage loss of theoretical plates between the optimum flow (flow where the highest number of plates is recorded) and the flow of 5 ml/min is around 90%. In most cases, this phenomenon was observed for larger molecules (e.g. M_r 800). In order to investigate the influence of the molecular size on mass transfer behavior, Van Deemter plots were evaluated for several model compounds with increasing molecular size (see Fig. 6). For benzene and naphthalene the plate height decreases up to 5 ml/min whereas for anthracene, methyl- and butylparaben the optimum flow rate is around 2–3 ml/min. At higher flow rates the plate height increases slightly. It appears that the smaller the molecule, the higher is the optimum flow rate.

As Van Deemter's theory shows, there is a direct correlation between mass transfer phenomenon, linear velocity and

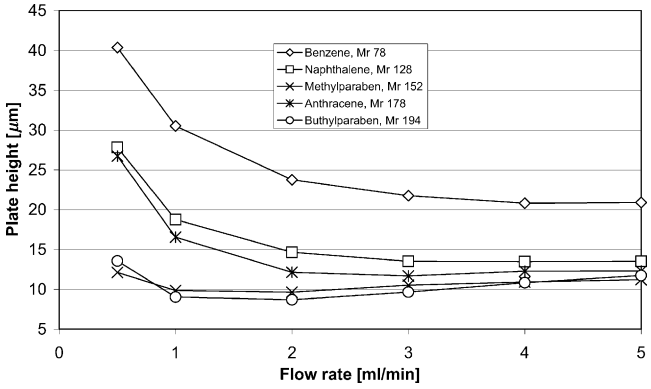

Fig. 6. Van Deemter plots for methyl- and butylparaben, benzene, naphthalene and anthracene, performed on Chromolith (RP18e, 100 mm × 4.6 mm i.d., Merck) column using HP 1100 system equipped with a VWD system and a binary pump. Detection UV at 210 nm, detector response frequency 10 Hz. Injection volume 5 μl. Temperature 40 °C. Isocratic elution of a mixture of acetonitrile and 10 mM KH_2PO_4, pH 3.0.

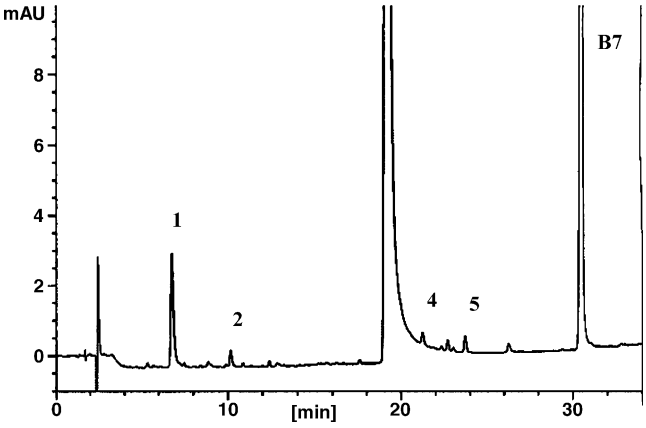

Fig. 7. Separation of a Novartis development compound and its by-products on a LiChrospher 100 column (RP18, 5 μm, 250 mm × 4 mm, Macherey–Nagel), flow 1 ml/min, gradient profile: 10% B initially, from 10 to 80% B in 35 min, new injection: 42 min, detection UV at 310 nm.

the number of theoretical plates at high flow rates [22]. In other words, mass transfer between the mobile and stationary phase is the most relevant factor contributing to band broadening.

This indicates that the increase in plate height with increasing flow rate is dependant on the molecular size. A possible explanation for this observation is that larger molecules, due to their lower diffusion coefficients compared to smaller molecules, have less interaction with the monolithic phase. The mass transfer resistance is probably dependent on the molecular size and rigidity that is opposite to the diffusion of the molecules as well as to the penetration in the mesopores. The effect observed with the model compounds in Fig. 6 is much less compared to the dramatic loss of plate number of the Novartis development compound in Fig. 5. However, the molecular size of the model compounds is also much less than that of the Novartis compound. Further experiments need to be done with a wider range of molecular sizes to clarify this point.

For method development on a monolithic column we generally apply flow rates similar to those also used with conventional particle packed columns (e.g. 1 ml/min) first. If the separation is achieved at this flow rate, the method is speeded up by increasing the flow rate while decreasing the gradient time proportionally.

4. Examples

Elution profiles of a Novartis development compound ($M_r = 330.4$) and its by-products are shown, recorded with the conventional method using a particle packed column (Fig. 7) and with a monolithic column (Fig. 8). The elution order in Figs. 7 and 8 is the same. Compared to the conventional method, the analysis time is reduced by a factor of about 6 while an equivalent resolution is obtained. The

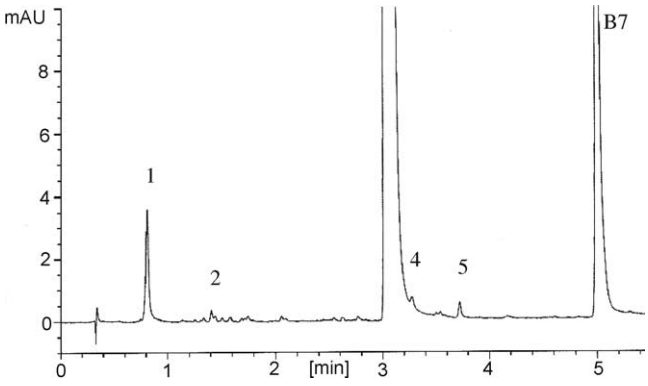

Fig. 8. Separation of a Novartis development compound and its by-products on a Chromolith column (RP18e, 100 × 4.6 mm i.d., Merck), flow 5 ml/min, gradient profile: 10% B initially, from 10 to 80% B in 7 min, new injection: 7.5 min, detection UV at 310 nm.

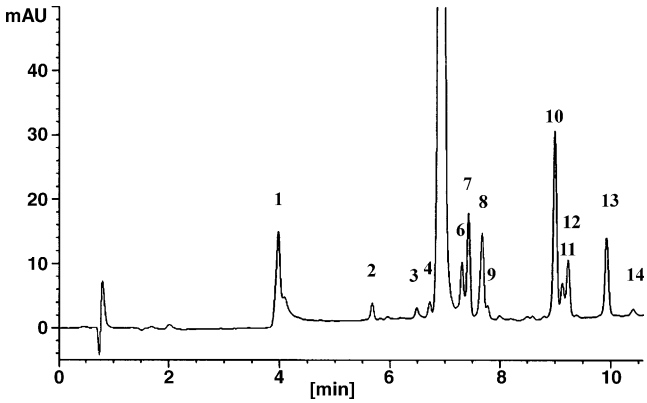

Fig. 9. Separation of a Novartis development compound and its by-products on a Symmetry Shield column (RP18, 3.5 μm, 150 mm × 3 mm i.d., Waters), flow 1 ml/min, gradient profile: 20% B initially, from 20 to 75% B in 10 min, then 75% B for 5 min, new injection: 20 min, detection UV at 210 nm.

separation between the main peak and the impurity number 4 was satisfactory for both methods.

Figs. 9 and 10 depict the elution profiles of an other Novartis development compound ($M_r = 362.9$) and its

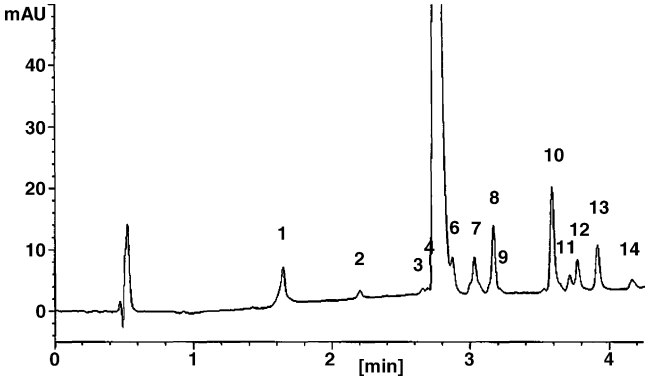

Fig. 10. Separation of a Novartis development compound and its by-products on a Chromolith column (RP18e, 100 mm × 4.6 mm i.d., Merck), flow rate: 3 ml/min, gradient profile: 20% B initially, from 20 to 75% B in 4 min, then 75% B for 1 min, new injection: 5.5 min, detection UV at 210 nm.

by-products recorded with a method using a packed column with 3.5 μm particles and a monolithic column. Both elution profiles of Figs. 9 and 10 are similar. The analysis time can be reduced from 20 to 5.5 min utilizing the monolithic column while acceptable resolution is kept. The separation between the main peak and the impurities 4 and 6 was reduced on the monolithic column but the resolution was sufficient for the purpose of this application.

5. Conclusion

For particle packed columns the pressure limitation of commercially available HPLC systems (usually 400 bar) clearly sets the limit for fast chromatography. But even if this could be overcome, the columns are also damaged by excessive pressure. Nowadays, most column manufactures pack their analytical columns at pressures well above 400 bar. But even today, columns can be purchased which were packed at pressures lower than 400 bar. Based on our experience and the data presented here, all HPLC systems used in this work are suitable for what we consider 'fast separations'. The extra column volume of those instruments is sufficiently small, provided that the column void volume (volume of the empty tube) is not less than approximately 1 ml. With the limitations given by the hardware we could achieve injection times (run to run) of less than or equal to about 20 min for purity methods of drug substances and drug products. To further reduce the run time when using particle packed columns, modifications of the HPLC system and the column would be needed to withstand much higher pressures.

The experiments performed on Chromolith columns showed the clear advantage of monolithic columns compared to particle packed columns. Thanks to their low back pressure, monolithic columns can be operated at much higher flow rates, thus reducing analysis time drastically. The analysis time of the examples shown in this work could be reduced by a factor up to 6. The monolithic columns have demonstrated not only comparable repeatability and reproducibility to particle packed columns, but also a very easy handling on conventional HPLC systems and a very good stability. In addition, it has been demonstrated that the Chromolith columns were suitable to separate most of our development compounds from their by- and degradation products at high flow rates. Some larger molecules, however, showed a high loss of plate number when increasing the flow rate, indicating that the limiting factor for larger molecules is the mass transfer.

On the other hand, the use of monolithic columns has shown some limitations. Fast methods require a high detector response frequency and an increase of the baseline noise level has been recorded when raising the frequency. Moreover, some instruments showed a dramatic increase of the baseline noise at high flow rates. This phenomenon hinders the determination of the impurities at low levels. The major draw back of silica based monolithic columns is that they are available only in very limited dimensions and modifications and up to date only one company is able to supply them. In order to become more popular in the pharmaceutical industry, monolithic columns should be available in more dimensions, more modifications and from alternative suppliers.

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