Javier Gotta
Jaiver Osorio Grisales
Mario R. Reta*
Cecilia B. Castells

División Química Analítica y CIDEPINT, Facultad de Ciencias Exactas, Universidad Nacional de La Plata, La Plata, Argentina

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Research Article

Fast RPLC analysis of pharmaceutical compounds at intermediate temperatures by using a conventional instrument

Recent developments in HPLC methods have focused on various strategies in order to increase the speed of analysis. One area of impressive growing is column technology. Today, analytical methods that propose the use of short columns packed with sub-2 μm particles installed in ultra high-pressure LC instruments are not uncommon. Another strategy consisted of heating thermally resistant columns to temperatures well above of 100°C in order to reduce eluent viscosities and, therefore, column backpressure. We discuss experimental conditions for achieving high-throughput analysis using standard instruments with a few simple modifications. The chromatographic performance of two particulated and a silica-based monolithic column operated at moderate temperatures and flow rates are compared. The monolithic column proved to be stable over several thousands column volumes at 60°C. More important, its resistance to mass transfer at this temperature was significantly reduced. Very fast separations of two different mixtures of pharmaceutical compounds, anti-inflammatory drugs and β-blockers, were achieved with the three columns at 60°C by using ACN/buffer at 5 mL/min. Excellent peak shapes of basic solutes and quite reasonable resolutions were achieved in very short analysis times with columns operated at temperatures moderately higher than the usual room temperature.

Keywords: β-Blockers / Fast RPLC analysis / NAIDS / Temperature
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1 Introduction

The optimization of a given LC separation includes a reduction in the analysis time. A decrease in the particle diameter of packed columns along with the column length has long since been known to improve both resolution and analysis time [1]. Such an approach, however, is accompanied by disadvantages. Smaller particles, with small interstitial voids, lead to a decrease in the column permeability, whose property causes the fluids to require a higher pressure in order to be transported along the bed at a given velocity. However, the reduction of particle diameter imposes an instrumental limit with respect to pressure requirements. Recent advances in HPLC columns have used various approaches that focus on increasing the speed of analysis. Ultra high-pressure LC-based columns packed with sub-2 μm particles have dramatically improved separation speed and efficiencies compared with the performance of conventional columns packed with larger particles [2]. This approach requires equipments with the capability of providing a mobile phase at regular flow rates but at extremely high pressures (up to about 1000 bar) [3]. In addition to the requirement for a specialized and expensive instrument, an important drawback of working with such small particles is the more frequent blockage of the system with a resulting reduction in column lifetime.

A more recent column technology aimed at shortening analysis time, and increasing efficiency was the introduction of a solid 1.7 μm core particle fused to an outer shell of porous silica of about 0.5 μm [4] The reduced intraparticle volume results in a significant decrease in diffusion and, consequently, in column theoretical plate numbers close to those achieved with sub-2 μm particles at regular linear velocities but at significantly smaller backpressure. This technology leads to improved column ruggedness as compared with the use of smaller particles. [5].

On the other hand, the introduction of monolithic materials created new possibilities [6, 7]. Inorganic monoliths consist of a single piece of silica gel with a skeleton of biporous structure; one pore type being mesopores of diameter greater than 100 Å, the other macropores of 1–3 μm in diameter, where the mobile phase flows through [8]. The retention takes place mainly in the mesopores.

Correspondence: Professor Cecilia B. Castells, División Química Analítica y CIDEPINT, Facultad de Ciencias Exactas, Universidad Nacional de La Plata, 47 y 115 (1900) La Plata, Argentina
E-mail: castells@isis.unlp.edu.ar
Fax: +54-221-4271537

*Additional correspondence: Professor Mario R. Reta
E-mail: mreta@quimica.unlp.edu.ar

These structures have external porosities of about 60% for conventional-size monoliths and of about 80% for those synthesized within capillaries, which is much larger than external porosity of typical particulate beds (~38–40%). The higher permeability of these monoliths compared with conventional particulate columns allows an operation at relatively high flow rates so as to reduce analysis time without a significant pressure drop. Another favorable characteristic is that flat curves for plate height *versus* linear velocity are usually observed with monolithic silica columns, mainly because of the low mass transfer resistance compared with conventional particle-packed columns. Thus, the increase in flow rate does not lead to a substantial loss in efficiency.

A very different strategy for speeding up separations consists in elevating column temperature in order to reduce the mobile-phase viscosity and, consequently, the column back pressure and at the same time increasing solute diffusion velocities [9–13]. Inordinately, high temperatures (*e.g.* in excess of 100°C), however, lead to other instrumental requirements: column heating needs a GC oven or other heating devices; the incoming eluent must also be heated, and in such circumstances a longer connecting capillary tube is necessary. In this case, there is a tradeoff between peak broadening due to the thermal difference and due to the extra volume added. With respect to peak detection at very high temperatures, sophisticated nonconventional means such as a flame ionization detector are necessary [14]. With UV–vis or diode-array detectors, by far the most common in HPLC laboratories, the detection cells have both temperature and pressure limits. Therefore, the outgoing eluent must be cooled down before reaching the cell by passing through another capillary tube of sufficient length to assure cooling. In order to avoid vaporization, a back-pressure regulator has often to be added, but pressure over certain limit can affect the cell integrity. Other drawbacks of working at very high temperatures are the possibility of sample degradation during chromatographic runs and decreased column lifetimes as well [15]. By contrast, moderate column temperatures (not higher than 70–80°C) can be easily set with very few simple operative precautions. Favorable changes in the hydrodynamics of the process come about at such moderate temperatures. In addition, important and *predictable* changes in the equilibria participating in the chromatographic process take place. Usually, retention of neutral molecules depends on temperature according to a typical van't Hoff equation within a given temperature range [16]. The retentive behavior and selectivity factors of ionizable analytes as pH of the mobile phase and temperature are changed can also be predicted based on the knowledge of the thermodynamics that rule the equilibria [17–19]. The retention of several drugs in a typical RPLC column as pH was changed in the range of 2.5–11 and temperature in the range of 20–60°C has been successfully predicted [11].

At all events, with both monolithic and particulate beds more favorable hydrodynamic conditions are possible if the column temperature is raised. That is, through the use of particulate or monolithic materials in standard instruments at high flow rates and moderately elevated temperatures, efficient and predictable separations can be practicable.

This study is intended: (i) to optimize experimental conditions for achieving higher throughput analysis by using a conventional HPLC apparatus with a few modifications, (ii) to study the overall chromatographic performance of particulate and monolithic columns under conditions that allow fast separations of acidic and basic drugs and finally (iii) to separate compounds that belong to two families of pharmaceuticals: nonsteroidal anti-inflammatory drugs (profens) and β-adrenergic blocking agents isocratically within a short time.

2 Materials and methods

2.1 Reagents

HPLC-grade ACN was obtained from Baker (NJ, USA). Water was purified by means of a Milli-Q Purification System (Simplicity, Millipore, MA, USA). Mobile phases consisted in mixtures of ACN/water or ACN/phosphate buffer pH = 3.0 (measured in pure water). All the solutes were purchased from Sigma or Aldrich (Sigma-Aldrich, St. Louis, MO, USA), and solutions of 50–100 ppm were prepared in ACN/water mixtures. All the samples were filtered through 0.22 μm nylon membrane filters before injection.

2.2 Instrumentation and columns

HPLC runs were carried out with a conventional HPLC instrument (HP1100, Agilent, Palo Alto, CA, USA) equipped with a vacuum degasser, a binary pump, a Rheodyne 7125 manual injector and a variable wavelength UV detector. In order to achieve higher linear velocities for the van Deemter experiments, a binary pump (Shimadzu LC10, Japan) was used instead of the original Agilent.

A low volume 1-μL microcell replaced the standard cell into the UV detector. Detection was set at 210 nm. To keep extra-column effects to a minimum, the injection volume was 5 μL, and all connections were made with 125 μm id capillary tubes. The time constant of the UV detector was set below 60 ms, and acquisition rate at 100 Hz. The data were collected by the workstation CSW Data Apex (Prague, Czech Republic) and peak parameters of the asymmetric profiles obtained without column were calculated by numerical integration of the signal (first and second moments). Peak maximum and width at the half height were used for all other efficiency estimations.

Three different columns have been compared: (i) a conventional small particle size column Zorbax Eclipse XDB-C8 (75 mm × 4.6 mm id, 3.5 μm average particle size), (ii) a high-temperature resistant column Blaze200 C18 from

Selerity Tech. (100 mm × 4.6 mm id, packed with 3 μm spherical particles) and a Onyx C18 monolithic column from Phenomenex (100 mm × 4.6 mm id). The characteristics of the three columns are summarized in Table 1. Column temperatures were maintained by means of a water jacket and circulating water from a Messgerate Werk Lauda (Lauda, Western Germany) bath. A mixture of ethylene glycol and water was used in the bath to keep column temperature at 80°C. A 20-cm (125 μm id) stainless steel capillary tube was connected between the injector and the column. This tube length, immersed in the water jacket, allows the preheating of the incoming eluent and of the sample without increasing significantly the extra-column broadening [20, 21]. For experiments conducted at 60 and 80°C, the eluent was somewhat cooled before the detection cell with a capillary coil immersed in a ice-water bath. The hold-up time was measured by injection of a solution of potassium bromide under each chromatographic condition. The extracolumn volume, measured at room temperature by injection of potassium bromide solution and calculated from the first peak moment, was 67.0 μL (±0.4). The columns were equilibrated with mobile phase for more than 30 min at 1 mL/min after the temperature of the bath or the mobile-phase composition had been changed.

Benzene, toluene and ethylbenzene were selected for the column efficiency tests (van Deemter plots). The elution strength of the mobile phase was modified to keep roughly the same solute retention factor at different temperatures. The curve fittings of the van Deemter plots were carried out by the SigmaPlot (version 4.01) software. Each data point is an average of at least three replicates.

3 Results and discussion

We have investigated the use of a standard HPLC equipment operated at moderately high temperatures and flow rates in order to speed up separations under isocratic conditions. The first step was to evaluate a monolithic column and to compare its performance with particulate-based ones under such moderately high-temperature conditions. Monolithic columns were introduced for their potential use at high mobile-phase velocities. The higher porosities of these monoliths as compared with traditional silica particulate columns result in less stress in the chromatographic system (*i.e.* lower backpressures), a property compatible with the higher flow rates [22]. The entire hydrodynamics and the retentive performance of these monolithic columns were critically compared with the corresponding properties exhibited by two different standard packed HPLC columns. These three columns were chosen since they were expected to provide similar column efficiencies.

Several precautions have been taken to avoid unnecessary peak width. First, since a large difference in temperature at the center of the column relative to the column walls will lead to band broadening, heat dissipation must be efficient [23]. Thus, a water bath was used instead of heating by air convection or a metal-block heater close to the column. Additionally, the incoming eluent must have a temperature close to that of the column. An appropriate length of a narrow capillary tubing, located between the column and the injector for preheating the mobile phase, was used [20].

Second, all other extracolumn volumes were carefully decreased to a minimum: the shortest and narrowest possible capillary tube was set at the exit of the column, and a 1-μL detection cell was used instead of the usual standard detection cell (14-μL for the HP1100 instrument). Finally, a detector time constant and acquisition rate were set to deal with peaks of a few seconds in width (Section 2).

3.1 Column thermal stability

The limiting temperature for the monolithic column indicated by the manufacturer is 45°C. In this study, several experiments were planned well above that temperature. Thus, the stationary phase stability at those higher temperatures was checked before carrying out the formal study. For this purpose, several profens were injected repeatedly over several days at a constant column temperature of 60°C. The observed variations in retention factors during this heating proved to be negligible, as shown in Fig. 1. Indeed, no deterioration in retention time occurred during the experiments with this column over the several months in which temperature was alternatively kept at 60°C, or even at 80°C, for very short intervals.

We did not check the thermal integrity of the two packed columns since the experimental temperatures did

Table 1. Columns used in this study: geometric characteristics and stationary phase properties

Column	Dimensions ($L \times$ id, mm)	Particle size (μm)	%C	Bonded phase coverage (μmol/m^2)	Endcapping	Surface area (m^2/g)	Temperature limit (°C)	Pressure limit (bar)
Monolithic Onyx C18	100 × 4.6	Monolith	18	3.6	Yes	180	45	200
Blaze 200 C18	100 × 4.6	3.0	–[a)]	–[a)]	–[a)]	–[a)]	200 (pH > 2)	400
Zorbax Eclipse XDB-C8	75 × 4.6	3.5	10	3.4	Yes – double	–[a)]	80	400

a) Not available.

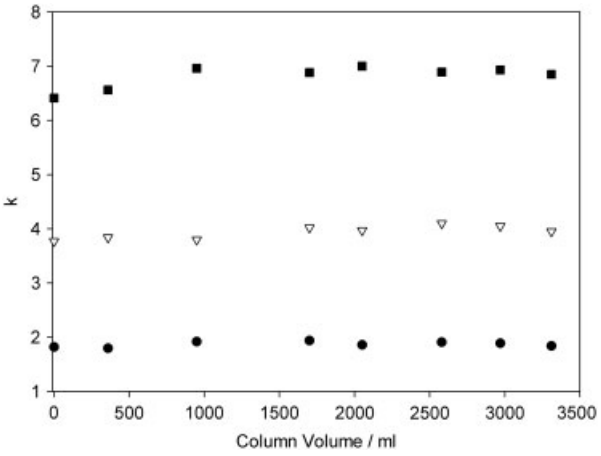

Figure 1. Column stability test for the Onyx Monolithic C18 column. Mobile phase: (60:40) ACN/buffer phosphate, pH 3; flow rate, 3 mL/min; temperature, 40°C. Symbols: (●), ketoprofen; (▽), fenoprofen and (■), ibuprofen.

not exceed the recommended limits: up to 80°C for the Zorbax and 200°C for the Blaze column.

3.2 Column permeability

Plots of pressure drop at several linear velocities for the three columns are compared in Fig. 2. Each plot represents the pressure drop values corrected for column length and fluid viscosity ($\Delta P/\eta L$, where ΔP is the pressure drop along the column, η the dynamic viscosity coefficient of the mobile phase at the experimental temperature and L the column length) as a function of linear velocity, u_0. Column backpressures were obtained by subtraction of the system pressure (without column) from the total pressure drop. These values have been measured at 20 and 40% v/v ACN in water at every flow rate and at 20, 40, 60, and 80°C. The corrections were not appreciable for the packed columns but were critical when the low-resistance monolithic column was used: at the lower temperature the corrections amounted to more than 50% of the total pressure drop. The mobile-phase viscosity values at these compositions and temperatures were taken from the literature [24, 25].

The data corresponding to each column have been plotted and the coefficients of the linear relationship between u_0(m/s) and $\Delta P/\eta L$ were calculated. The results of the linear regressions are summarized in Table 2. The values of the specific permeability of each column, as estimated by the product between the slope of these regressions and the total porosity, are also listed in Table 3.

The pressure drop exhibited by the 100×4.6 mm monolithic column was about 3.5 times lower than that imposed by the 3.0 μm-packed (Blaze) column of the same length and was 2.9 times lower than the backpressure found with the 3.5 μm-packed Zorbax column. The permeability value obtained is slightly smaller than that reported by Tanaka *et al.* (7×10^{-14} m^2) for a monolithic capillary

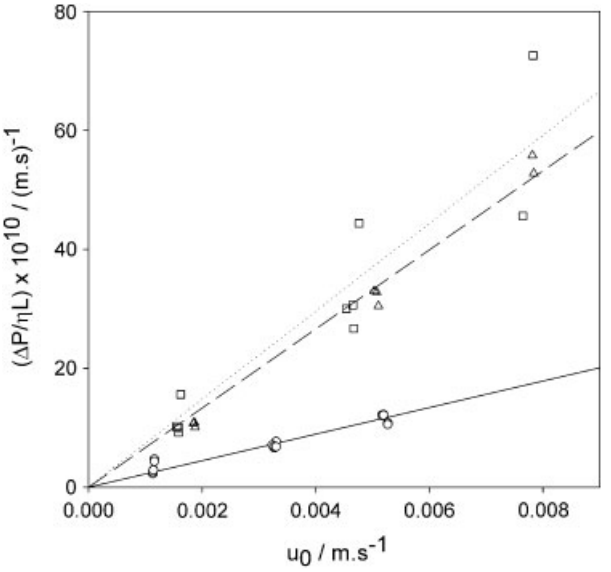

Figure 2. Plots of ($\Delta P/\eta L$) *versus* u_o for the monolithic Onyx column and the particulated Zorbax and Blaze columns. Mobile phases: 40:60 and 20:80 mixtures of ACN/water; temperatures: 20, 40, 60 and 80°C. Viscosity values of 20:80 ACN/water mixture at 20, 40 and 60°C are 1.10, 0.70 and 0.53 cP, respectively; values for 40:60 ACN/water mixtures are 0.99, 0.65 and 0.49, respectively [24].

Table 2. Results of the linear regression between linear velocity, u_0, and ($\Delta P/\eta L$), chromatographic permeabilities and chromatographic particle size

Column	Onix	Zorbax	Blaze
Slope $\times 10^9$	4.6 ($\pm$ 0.2)[a)]	1.37 ($\pm$ 0.05)	1.1 ($\pm$ 0.2)
Intercept	−0.0003 ($\pm$ 0.0002)	0.0005 ($\pm$ 0.0002)	0.0007 ($\pm$ 0.0006)
n[b)]	16	8	8
$B_0 \times 10^{14}$ m^2	4.6 ($\pm$ 0.2)	1.37 ($\pm$ 0.2)	1.1 ($\pm$ 0.2)
d_p (μm)	6.8[c)]	3.7	3.3

a) Standard deviations are represented in parenthesis.
b) Number of data points.
c) Estimated from B_0 (see the text).

column (MS-PEEK) [26]. Similarly, Wu *et al.* [27] have obtained B_0 values almost three times higher for a monolithic column than for other 5 μm particulate columns. Indeed, according to Halász concept of so-called "chromatographic" particle size [28], as estimated from the Carman–Kozeny equation ($d_p = 1000\ B_0)^{0.5}$, the packed columns behave as if they had an average particle diameter of 3.8 and 3.5 μm for the Zorbax and the Blaze columns, respectively; and by the same criterion, the monolithic column would exhibit a flow resistance equivalent to a particulate column packed with 6.7 μm average diameter. Tallarek and coworkers [29, 30] introduced a similar concept: the particle diameter, d_{perm}, was used to compare the hydrodynamic features between monolithic and particulate columns. They used angiotensin and insulin as probes, and found that silica monoliths are comparable to a

Table 3. Values of *A*, *B*, and *C* terms from the fitting of *H versus u* for the monolithic column at 40 and 60°C

Column temperature	40°C		60°C		
Parameter	Toluene	Ethylbenzene	Benzene	Toluene	Ethylbenzene
A, μm	6 (± 2)	2 (± 1)	11.8 (± 0.1)	11 (± 1)	11.9 (± 0.1)
B, mm^2/s	$(7.9 \pm 0.3) \times 10^{-3}$	$(13 \pm 1) \times 10^{-3}$	$(8.6 \pm 0.6) \times 10^{-3}$	$(9.1 \pm 0.7) \times 10^{-3}$	$(8.8 \pm 0.3) \times 10^{-3}$
C, ms	2.2 ± 0.5	2.4 ± 0.3	1.5 ± 0.2	0.1 ± 0.02	0.3 ± 0.1

15 μm particulate column. This feature is one of the most important attributes of monolithic columns, since either the flow rate for fast analysis or the column length for improved efficiency can be significantly increased without producing an unacceptably high backpressure. For instance, upon increasing linear velocity from 0.1 to 0.5 cm/s in a 10-cm monolithic column, the pressure drop raises from 32 to 169 bar at 20°C, whereas the 3.5 μm particulate column generates a backpressure of 90 bar at 0.1 cm/s and one of higher than 400 bar at 0.5 cm/s, although the column is shorter than the monolithic. Moreover, separations can be even faster when column temperatures are increased because of the consequent decrease in the viscosity of the mobile phase, which implies lower pressure drops. Higher temperatures additionally produce a faster mass transfer through a more rapid diffusion of the solutes with a consequent increase in the linear velocity. For instance, viscosities of ACN/water mixtures at practically all compositions drop by about 50% when the temperature increases from 20 to 60°C, and thus the flow resistance decreases in the same proportion.

3.3 Column efficiencies and peak symmetries

The effects of temperature and flow rates on plate counts and peak symmetries for the three selected columns were determined. All data were obtained at 1, 3 and 5 mL/min. Fig. 3, for example, compares the plate counts by length unit (N/m) measured with several profens at either 20 or 60°C and at 1 and 5 mL/min.

Although different compounds exhibited individual behavior, a general pattern was observed where the increase in temperature to 60°C and a flow rate of 1 mL/min improved plate counts of the Blaze column (16% in average), the Onyx column (5% average) and, less evident, the Zorbax (2% average) column. This finding is not unexpected upon consideration of the decreased eluent viscosity and the increased solute diffusion rates in both the mobile and the stationary phases at that temperature rise. Over the same temperature increase at a flow rate of 5 mL/min, however, we observed a decrease in plate counts measured with all the analytes. The differences in *N* measured with different solutes (in the three columns) are hard to explain. Not only *N* decreases, but also the observed trends in *N* as *k* increases are contrary to the expected values. At 5 mL/min, we would expect that plate heights would be determined mainly due to the resistance to mass transfer in mobile phase; and under this assumption, HETP should slightly increase as the retention increases. The rather unexpected behavior compelled us to make a critical evaluation of the calculation of plate heights. First, we had estimated *N* considering Gaussian peaks, but since most of them show slight asymmetries, we recalculated all *N* through the ratio between the first moment (to describe t_R) and the second moment (variance of the profile). The results were identical: *N* increases with *k*. Second, we had ignored the extracolumn incidence in t_R and in σ^2. Again, we recalculated *N* considering the extracolumn effects on retention and on total dispersion, $N = (t_R - t_{extracol.})^2/\sigma^2_{total} - \sigma^2_{extrac}$ where t_R is corrected by the extracolumn time, $t_{extracol}$, and the variance of the eluted peak is also corrected by the dispersion in the extracolumn space. This last quantity was obtained as the second moment of the profiles collected during the extracolumn volume measurements. In this case, the quantity was converted to a time scale at 5 mL/min. The *N* corrected in this way is about 10% larger than the original one for the less retained solutes, but only a 1% larger for the more retained solutes. Thus, the extracolumn effects cannot explain the observed trend and the approximations used to estimate *N* (as if peaks were symmetrical) were valid.

An insufficient temperature equilibration at the higher flow rates [20] would explain not only the decreased efficiencies as compared with those at 1 mL/min and 60°C, but also this apparent dependence of *N* with *k*. If the length of tubing used to preheat the mobile phase was insufficient when the flow rate was 5 mL/min, the less retained analytes would be much more strongly affected by this possible thermal mismatch. For the more retained ones, the band becomes spread out, farther down the column, and hence the thermal mismatch effect is partially masked and may not apparently be reflected at the end of the column. That is, the usual chromatographic dispersion of those more retained bands would predominate over the nonthermal equilibrium, and *N* does not decrease as much as those calculated with less retained solutes.

In order to characterize the performance of the monolithic column at 40 and 60°C, the plate heights for benzene ($k = 0.78$), toluene ($k = 1.29$) and ethylbenzene ($k = 1.82$) as a function of mobile phase velocity (van Deemter plots) were calculated and plotted (Fig. 4). The mobile phase consisted of ACN/water (50:50) and the mobile-phase velocities were set between 0.1 and 9 mm/s (which correspond to the maximum column backpressure recommended by the manufacturer). The plots are seen to be similar at both

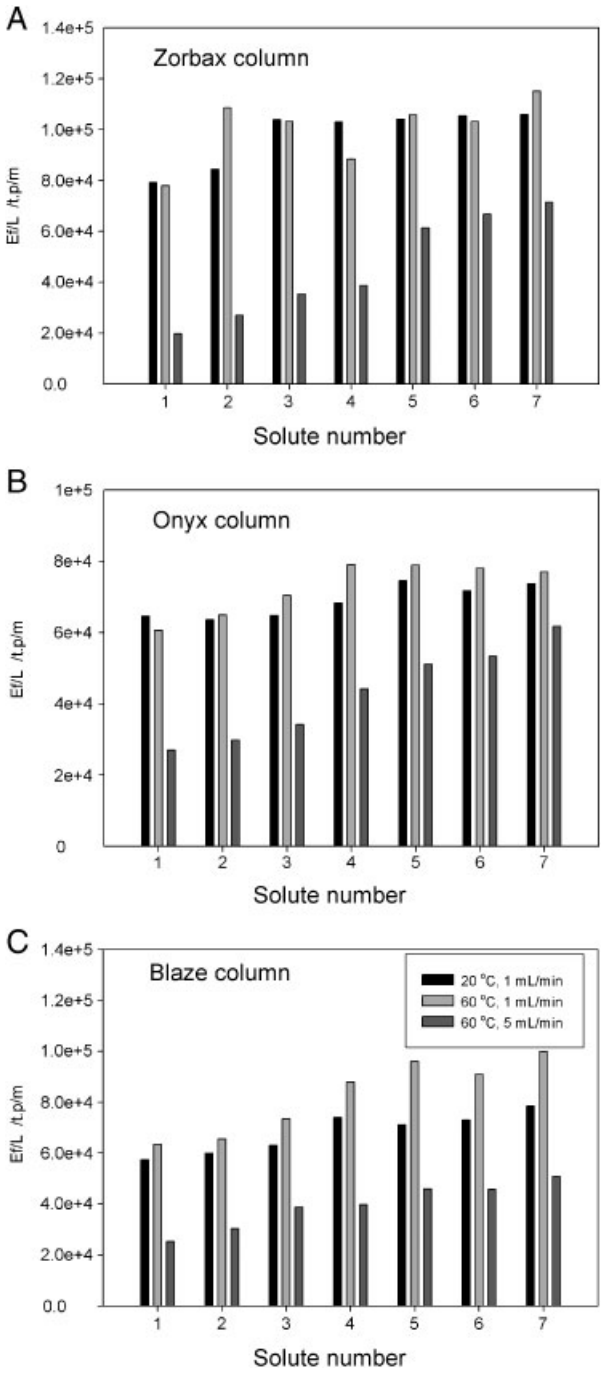

Figure 3. Column efficiencies measured at 20 and 60°C and 1 and 5 mL/min. Mobile phase: (60:40) 30 mM phosphate buffer pH = 3/ACN mixture. (A) Zorbax column, (B) Onyx column, and (C) Blaze column. Solutes: 1, indoprofen; 2, suprofen; 3, ketoprofen; 4, fenbufen; 5, fenoprofen; 6, flurbiprofen, and 7, ibuprofen.

temperatures. The minimum plate heights, which values are expected to be independent of temperature, were 19, 17 and 15 μm at 60°C for benzene, toluene and ethylbenzene, respectively; whereas the corresponding heights were 16 and 13 μm at 40°C for toluene and ethylbenzene, respectively. These values are quite similar to those previously

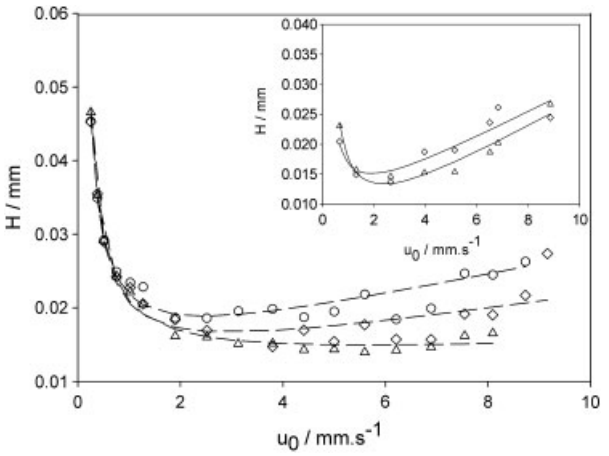

Figure 4. Relationship between plate heights and linear velocity measured for benzene (○), toluene (◇) and ethylbenzene (Δ) in the monolithic C18 column at 60 and 40°C (plot inserted). Mobile phase: 50% ACN/water.

reported in the literature [29, 31, 32]. The plots also indicate that the optimum velocity depends on temperature. Since this optimum velocity is proportional to solute diffusion coefficient in the mobile phase, the shift in the minimum plate heights toward higher velocities would be attributed to a faster rate of diffusion in that phase at a higher temperature. For instance, diffusion coefficients of alkylbenzenes increase about 70% in ACN/water from 25 to 65°C [33].

We determined the coefficients A, B and C by fitting each data set to a van Deemter equation (Table 3). Although the parameter A for monolithic column does not have a physical meaning related to particle diameter as with particulate columns, the data have nevertheless been fitted to typical van Deemter plots previously [34]. Leinweber and Tallarek [30] found that van Deemter plots for different silica rods can be fitted to a typical particulate column of 3.0 μm in particle diameter. Similar ''apparent'' particle diameters can be calculated from the coefficient *A* obtained at 40°C, but almost twice the values are obtained at 60°C. Leinweber *et al*. [29] compared the plate heights for monolithic columns at temperatures between 16 and 32°C and observed that the coefficients *C* and *A* decrease slightly as temperature increases. Siouffi [22] compared the *C*-term measured for different monolithic columns from several sources. These *C*-data span over a wide range of values (about two orders of magnitude) although most of the figures, and mainly those measured by the author, were close to 1.5 ms for silica rods with typical molecules as probes. These values are in excellent agreement with the coefficients *C* obtained at 40°C in this study; but in our studies, a significant reduction in the *C*-values was found at 60°C, especially for the most retained compound, ethylbenzene. This is a quite important characteristic for the purpose of increasing the speed of analysis: flow rate can be increased with a negligible loss in efficiency.

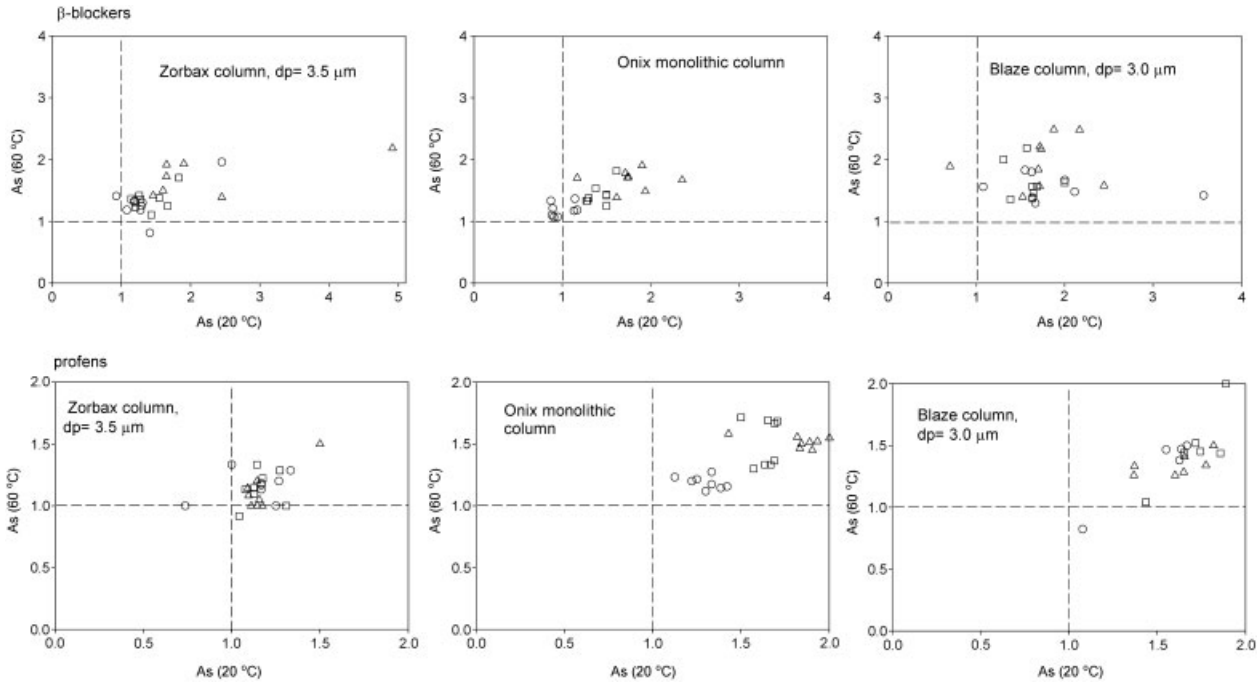

Figure 5. Comparison of asymmetry factors at 20°C with those at 60°C for β-blockers and profens. Symbols correspond to flow rates of 1 mL/min (○), 3 mL/min (□) and 5 mL/min (Δ).

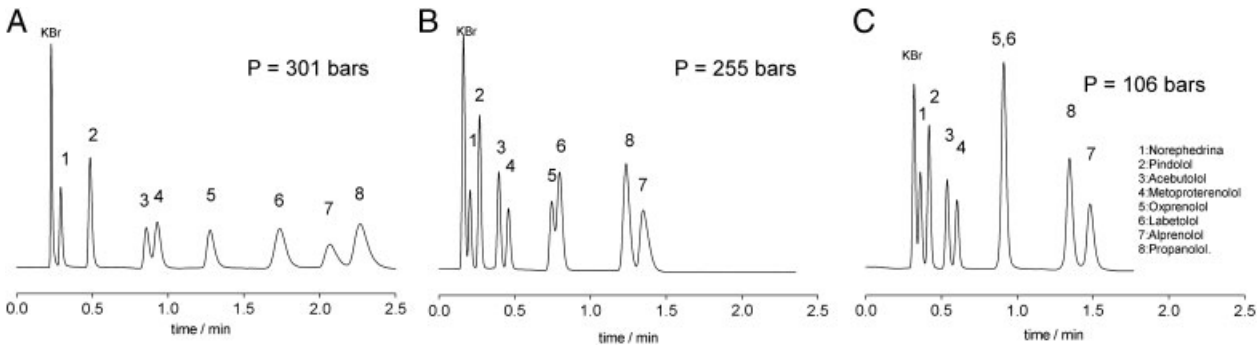

Figure 6. Chromatograms showing the separation of a mixture of β-blockers. Columns: (A) 10-cm, 3-βm Blaze column, (B) 7.5-cm, 3.5-μm Eclipse XDB column, and (C) 10-cm Onyx monolithic column. Temperature, 60°C; flow rate, 5 mL/min and λ, 210 nm. Mobile-phase compositions: (12:88) ACN/30mM phospate buffer (plot A) and (20:80) ACN/30mM phospate buffer, pH 3, mixture (plots B and C).

Figure 5 shows the peak symmetries for several β-blockers (Plot A) eluted from the three columns at three flow rates and at two different temperatures. First, the plots demonstrate that peak symmetry is flow rate dependent. A higher speed significantly improves the peak shapes of the solutes eluted from the three columns. Acceptable symmetries for all these basic solutes at higher flow rates are achieved with the conventional Zorbax column as well as with the monolithic column, whereas more peak tailing is observed for several solutes eluted from the Blaze column. Second, diminished asymmetry factors are obtained at 60°C compared with those at room temperature for these basic compounds upon elution from the three columns (note the less scattering on the *y*-axis). The interactions of some basic solutes with silanol groups in the silica support that usually lead to peak tailing is reduced by increasing the analytical temperature. Other authors have also reported peak tailing with monolithic columns [27, 35]. Wu *et al.* [27] found that peak symmetries are greater in packed columns for acidic, basic and neutral compounds at room temperature. Kele and Guiochon [36] suggested that the latter behavior would be attributed to column-bed heterogeneity. Asymmetry factors for the acidic profens were much less pronounced and were practically the same at both temperatures with low flow rates. Nevertheless, to our surprise, marginally better peaks were observed at 20°C than at 60°C at 5 mL/min. In summary, we observed that the peak symmetry could be improved at high temperatures and flow rates, which is consistent with the objectives of this study as stated in Section 1.

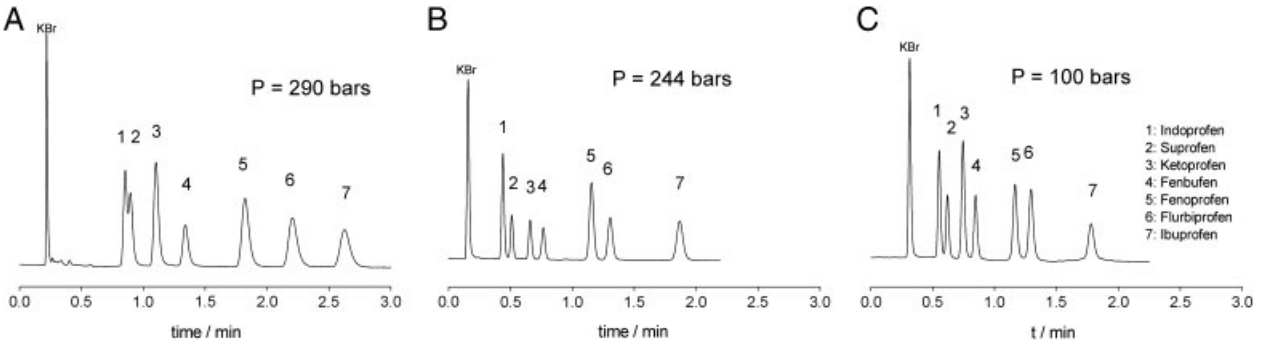

Figure 7. Chromatograms showing fast separations of seven profens. Columns and conditions are the same as described in Fig. 6. Mobile phase: (40:60) ACN/30 mM phospate buffer, pH 3.

3.4 High-speed separations of pharmaceutical compounds

Several examples of impressively fast separations through the use of high flow rates and extreme temperatures have been reported in the literature [37, 38]. For example, a separation of five phenols with 100% water at a flow rate of 12 mL/min and 120°C in less than 30 s was reported [20]. Wenclawiak *et al.* have separated a mixture of four hydrophobic steroids on a ZirChrom-PBD column in less than five min with 100% water at 185°C and a flow rate of 5 mL/min [38]. In contrast to the extreme conditions used in those studies for which, special equipment has to be used, the experiments reported here, by using a conventional equipment with much milder temperatures and only moderate flow rates have been successful in isocratically separating one mixture of eight β-blockers and other containing seven profens within very short analysis time (about 2 min). Figure 6 shows the chromatograms of eight β-blockers eluted from the three columns operated at 60°C and at 5 mL/min. The elution order and selectivity factors are clearly different in these three C18 columns. Under the conditions used, peaks 5 and 6 coeluted from the monolithic column (plot C) and are not baseline separated in the Zorbax column (plot B). On the contrary, at 1 mL/min and 20°C the solutes 3 and 4 were eluted in a single peak from the Blaze column; whereas the solutes 7 and 8 were poorly resolved from the Onyx and the Zorbax columns at the same flow rate and temperature (chromatograms not shown). Similarly, Fig. 7 shows the separation of seven profens from the three columns in less than 2 min. The resolution is only little affected by using fast elution conditions. Moreover, the resolution of the critical pairs is possible ($R_s > 1.4$) even at 5 mL/min and with a marked effect on the analysis time, it having been reduced practically one order of magnitude relative to typical chromatographic runs at room temperature.

4 Concluding remarks

Temperature is one of the easiest and most straightforward parameters to modulate in a chromatographic separation. An increase in temperature within a practical range can be very helpful in improving analysis time. With the use of particulated beds, there is an instrumental limit in attainable flow rates at low temperatures. High flow rates, however, can be easily achieved at relatively higher temperatures owing to the strong concomitant reduction in column backpressure.

With monolithic columns, separation can be achieved with a standard instrument and one-third or one-fourth of the pressure necessary with particulated columns as a result of their high permeabilities (comparable to columns packed with 7 μm particles). The combination of the use of these monoliths together with moderate increases in temperature, usual instrumental settings and a few simple modifications can speed up analysis time by about an order of magnitude.

A fast separation of two different mixtures of pharmaceutical compounds, anti-inflammatory drugs and β-blockers, was achieved with two typical particulate columns and a silica-based monolithic one with a mobile phase of ACN/buffer at 5 mL/min and a temperature not higher than 60°C. The separations show excellent peak shape and quite reasonable resolutions in markedly short analysis time with columns operated at temperatures moderately higher than the usual room temperature but, nevertheless, applicable to conventional equipment.

These moderate temperatures furthermore resulted in improved peak symmetries of basic solutes. Thus, the degree of ionic strength in the buffered mobile phase usually required for satisfactory peak shapes of these kinds of solutes would be decreased, thus benefiting both column lifetime and the chromatographic system, in general.

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