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High-efficiency hydrophilic interaction chromatography by coupling 25 cm × 4.6 mm ID × 5 μm silica columns and operation at 80 °C

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ABSTRACT

Recently, hydrophilic interaction chromatography (HILIC) has emerged as a valuable orthogonal tool to reversed-phase liquid chromatography (RP-LC) as it allows for resolution of highly polar ionisable compounds. The relationships between separation efficiency, column length and speed of analysis for 4.6 mm ID × 5 μm silica particle columns in HILIC are demonstrated using kinetic plots. The kinetic plots constructed for conventional pressure systems operating at 350 bar and at 30 °C and 80 °C are confirmed using experimental data for different column lengths. Efficiencies of more than 130,000 theoretical plates could be achieved by connecting up to six columns of 25 cm. As expected, a significant gain in analysis speed without loss of efficiency could be obtained by operating at 80 °C compared to 30 °C. The advantages of using long columns in HILIC in combination with elevated column temperature for the pharmaceutical industry are illustrated using test mixtures comprised of commercially available ionisable compounds (including some containing functional groups with potential genotoxic typical structural alerts) as well as real polar ionisable pharmaceuticals.

1. Introduction

Reversed-phase liquid chromatography (RP-LC) is by far the most widely employed analytical tool in drug discovery and development. In recent years, the efficiency of RP-LC has been drastically increased through various approaches including using sub-2 μm particles in combination with ultra high pressure LC (UHPLC) [1–3], increasing the column length and operation at elevated temperature (ETLC) [4–6] or by combining both UHPLC and ETLC [7,8]. These developments have certainly facilitated the development of generic methodologies for RP-LC for separations of active pharmaceutical ingredients (API's) and impurities [5]. Furthermore, these advances have helped separation scientists to exploit early fundamental theories around the principles of separation optimisation with respect to efficiency and speed by utilisation of kinetic plots. For the pharmaceutical industry, which will certainly still have a plethora of conventional HPLC instrumentation in its good manufacturing practice (GMP) laboratories, kinetic plots can certainly serve a purpose in directing how conventional instrumentation and columns can be used more appropriately in combination with ETLC and long columns. This can help to optimize the use of conventional particle diameters (5 μm) to yield either maximum efficiency and to compare to sub-2 μm particles, or to increased the speed of analysis.

Since these developments, a chromatographic technique that has been enjoying recent attention in the pharmaceutical industry is hydrophilic interaction chromatography (HILIC). HILIC has become a valuable addition to RP-LC in drug analysis, due to its orthogonality to the latter separation mode [9–13]. HILIC is particularly useful for the analysis of highly polar and ionisable compounds [14–18]. When confronted with complex mixtures of the latter, high-efficiency HILIC can be the key to achieving adequate resolution. The separation efficiency can be increased by reducing the size of the particles (sub-2 μm) and/or by increasing the length of the column. Coupled HILIC columns packed with superficially porous particles have been used by McCalley to increase efficiency [19]. When using long columns packed with 5 μm totally porous particles however, the drawback is the resulting increased analysis times. In several studies comparing different LC formats and approaches e.g. the use of monolithic supports, sub-2 μm particles or elevated column temperature, the latter is the most attractive option when efficiencies higher than 70,000 plates are required [20–22]. The use of UHPLC in combination with high temperature, would intuitively be the superior approach. However it has already been shown that due to instrumental limitations, the full potential of sub-2 μm particles cannot be achieved at elevated temperatures [7].

After taking all of these considerations into account, this work focused on the exploitation of elevated temperatures to increase efficiency of HILIC using conventional LC columns and HPLC instrumentation (ΔP_{max} = 400 bar). A commercial column oven large

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enough to contain 6 columns and equipped with active preheating and post-column cooling is thereby used.

The immediate advantages of using elevated temperatures for any analysis is of course increased speed of analysis and decreased mobile phase viscosity, which circumvents the issue of high back-pressure generation when using long columns. The only additional requirement is an appropriate mobile phase and column heating system [20,22–24]. The use of elevated column temperatures in RP-LC to achieve high separation efficiencies have been investigated extensively [5,25–30] and indeed have recently been reviewed [18]. An in-depth study was conducted in our laboratory [31] and will be published elsewhere. The main conclusion of this study was that bare silica columns such as Ascentis Si (Supelco, Sigma–Aldrich, Bornem, Belgium) can be used at temperatures of 80 °C under HILIC conditions without deterioration. Higher temperatures are not recommended because of the polymeric frits. Columns with metal frits like Zorbax RSil (Agilent Technologies, Brussels, Belgium) can be used at 90 °C and XBridge HILIC (Waters, Waver, Belgium) at 100 °C.

Kinetic plots [26,32] and other related plots [7,20,33–37] have been employed with increasing frequency to compare the performance of e.g. different chromatographic supports and the influence of experimental conditions, such as column temperature and pressure, on chromatographic performance. All of these studies have focused on RP-LC. In this contribution, using the kinetic plots derived from Van Deemter data, the relationships between separation efficiency, column length and speed of analysis at 30 and 80 °C and ΔP_{max} = 400 bar (350 bar column pressure and 50 bar system pressure) were established. From these plots the column length needed to generate more than 100,000 plates in the shortest possible time was obtained. The calculated values were verified experimentally for different column lengths.

2. Experimental

2.1. Materials

HPLC gradient grade acetonitrile and water were purchased from Sigma–Aldrich (Bornem, Belgium). Formic acid was obtained from Acros Organics (Geel, Belgium). Ammonium formate, toluene, adenine, ketoprofen, diphenhydramine, nortriptyline, nicotine, procainamide, naphthylamine, aniline, 5-aminoindole, 4-aminobenzoic acid, 2-aminopyridine, 3-aminopyridine, 3-aminobenzoic acid, 4-nitrobenzoic acid, 5-amino-2-methylpyridine, benzylamine, nitroterephthalic acid, 4-aminopyridine, 4-amino-2-methyl-pyridine were purchased from Sigma–Aldrich. The active pharmaceutical ingredient (API) was provided by Pfizer (Sandwich, UK). The structure is benzodiazepine-like with a molecular weight of ca. 400 Da. Stock solutions of analytes were prepared by dissolving each in an appropriate mixture of water and acetonitrile. Mixtures for analysis were prepared at ca. 0.1 mg/mL, except for diphenhydramine and benzylamine which were prepared at 0.2 mg/mL and 0.4 mg/mL, respectively, using 100% acetonitrile for the final dilutions. Injection volume for all analyses was 2 μL. All mixtures contained 0.2 mg/mL of toluene as a void marker. The mobile phase consisted of a 5 mM ammonium formate buffer at pH 5 in 95% acetonitrile (pH of the stock aqueous solution at 100 mM adjusted with formic acid before mixing with acetonitrile), mixed on-line with an aqueous 5 mM ammonium formate buffer at pH 5 (prepared from the same stock solution). Analyses of the test mixture (API, ketoprofen and adenine) were performed with a mobile phase adjusted to 90% acetonitrile, while a mobile phase containing 85% acetonitrile was used for the isocratic analysis of the mixture of pharmaceuticals and potential genotoxic compounds. The gradient used for the analysis of the latter mixture was 0 min 87% acetonitrile, 0.1 min 87–85% acetonitrile (linear over 3 min) then held at 85% for 32 min.

2.2. Instrumentation

An Agilent 1100 HPLC system (Agilent Technologies, Waldbronn, Germany) consisting of a quaternary pump, degasser, auto sampler and variable wavelength detector (VWD) set at 254 nm was used for the chromatographic analyses. The temperature of the column or column series was accurately controlled with a Polaratherm 9000 series oven, with mobile phase preheater and post-cooling of the column effluent (SandraSelerity Technologies, Kortrijk, Belgium). Agilent Chemstation Software (Rev. B.03.01) was used for instrument control and data acquisition. Supelco (Sigma–Aldrich, Bornem, Belgium) Ascentis Si, 250 mm × 4.6 mm I.D. columns with 5 μm particles were used for the analyses described in this paper. A bare silica guard column 4.0 mm ID × 3.0 mm long was connected to the inlet. Columns were connected in series with 10 cm × 0.12 mm ID stainless steel tubing (Agilent Technologies). A Valco zero dead-volume union employed for obtaining the system pressure values required for calculation of ΔP_{col} in the kinetic plot construction (see Section 2.3) was obtained from VICI (Schenkon, Switzerland).

2.3. Construction of Van Deemter and kinetic plots: experimental determination of H, u_0 and K_{v0} values

Experimental plate height, H, and mobile phase linear velocity, u_0, values were obtained using a 25 cm column at column temperatures of 30 °C and 80 °C. The test mixture composed of toluene (void marker), ketoprofen, adenine and the API, was analysed at mobile phase flow rates from 0.25 to 5 mL/min at each column temperature. The theoretical plates (N) for each test mixture analyte were calculated from the peak widths at half height using the Chemstation software. The u_0 and H values were calculated from $H = L/N$ and $u_0 = L/t_0$, where t_0 is the void time, the elution time of an unretained solute, in this case toluene. Van Deemter data for a 6 × 25 cm coupled columns system was similarly established at column temperatures of 30 °C and 80 °C. The test mixture was analysed at flow rates from 0.25 to 1.25 mL/min and 1.95 mL/min at 30 °C and 80 °C, respectively.

The column permeability, K_{v0}, was calculated from:

$$K_{v0} = \frac{u_0 \eta L}{\Delta P_{col}} \quad (1)$$

where ΔP_{col} is the pressure drop of the column in Pa, and u_0 in m/s is the corresponding mobile phase linear velocity [33] obtained experimentally by operating one 25 cm column at a mobile phase flow rate of 5 mL/min, the maximum flow provided by the Agilent 1100 HPLC pump. When performing this at column temperatures of 30 °C and 80 °C, ΔP_{col} were 226 bar and 140 bar, respectively. The system pressure values needed for the calculation of ΔP_{col}, are determined at each temperature by replacing the column with a Valco zero dead-volume union. The mobile phase viscosity η at each temperature was calculated as 4.15×10^{-4} kg m^{-1} s^{-1} and 2.73×10^{-4} kg m^{-1} s^{-1}, respectively from the Chen and Horvath equation [38]:

$$\eta(\phi, T) = \exp\left[\phi\left(-3.476 + \frac{726}{T}\right) + (1-\phi)\left(-5.414 + \frac{1566}{T}\right) + \phi(1-\phi)\left(-1.762 + \frac{929}{T}\right)\right] \quad (2)$$

where ϕ is the fraction of acetonitrile in the mobile phase and T is the temperature in Kelvin. Using these values, the calculated column permeabilities are 3.02×10^{-14} m^2 and 3.30×10^{-14} m^2 at 30 °C and 80 °C, respectively. The somewhat lower value measured

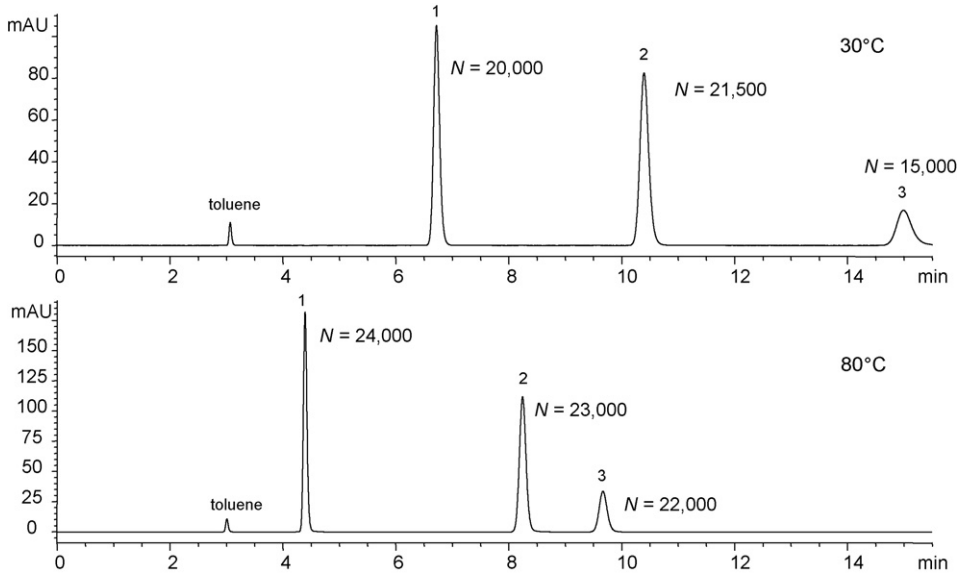

Fig. 1. Analysis of the test mixture using a 25 cm column at (A) 30 °C and (B) 80 °C. Mobile phase flow rate 1 mL/min of 5 mM ammonium formate pH 5 in 90% acetonitrile. Detection: UV at 254 nm. Peaks: 1 = ketoprofen; 2 = adenine; 3 = API.

at 30 °C (K_{v0} should be independent of temperature) is related to frictional heating occurring in the 30 °C experiment (at 5 mL/min and 226 bar), lowering the viscosity and therefore the permeability measurement.

2.4. Experimental determination of diffusion coefficients, D_m

The diffusion coefficients for ketoprofen, adenine and the API was determined by the Aris–Taylor open tube method as described by Li and Carr [39]. Solutions of 0.1 mg/mL of each compound were injected (2 μL) separately into PEEK tubing of 3 m length with 0.508 mm ID at 30 °C and 80 °C with a mobile phase flow rate of 0.1 mL/min and the band broadening was measured. The band broadening contribution of the system was determined by replacing the PEEK tubing with a zero dead volume union and repeating the experiments. The diffusion coefficients were calculated from

$$D_m = \frac{t_0 d_t^2}{96\sigma_t^2}$$

where σ_t is the peak standard deviation, t_0 is the elution time of the solute and d_t is the internal diameter of the tube.

2.5. Experimental verification of the kinetic plots

Experimental u_0 and H values were obtained by analysing the test mixture using different column lengths. The test mixture was analysed using 3 coupled 25 cm columns at 30 °C and 6 coupled 25 cm columns at 30 °C and 80 °C. For each analysis the appropriate mobile phase flow rate was used to generate a column pressure of 350 bar, thereby allowing for a system pressure of 50 bar.

3. Results and discussion

3.1. Increasing the speed of HILIC separations by operation at elevated column temperature

The study of the influence of temperature and the possibilities this offers in terms of speed and efficiency was performed with 25 cm × 4.6 mm × 5 μm columns, as the robustness of this column format ensures broad applicability. A test mixture for HILIC was composed such as to contain a basic and an acidic pharmaceutical compound (adenine and ketoprofen, respectively) and a benzodiazepine like API. The chromatograms of the test mixture analyses using a 25 cm column at temperatures of 30 °C and 80 °C and a flow of 1 mL/min are shown in Fig. 1. Increasing the column temperature from 30 °C (A) to 80 °C (B) results in a decrease of the retention for all compounds. The relationship is described by the Van't Hoff equation [22,41]. Because retention is an exothermic process ($\Delta H < 0$) and generally entropy is lost upon transfer from the mobile to the stationary phase ($\Delta S < 0$), an increase in temperature leads to a shift of the equilibrium towards the mobile phase and therefore to a drop in retention [27,31,40,41]. Overall, however, the retention of the test compounds is relatively low at 80 °C ($k_{API} < 2.3$). Temperature also induces selectivity (α) changes as illustrated by the shift of adenine towards the API at 80 °C ($\alpha = 1.3$) compared to 30 °C ($\alpha = 1.6$).

At a flow of 1 mL/min, the analysis at 80 °C yielded a greater number of plates for each test compound than at 30 °C. Most pronounced is the increase for the API from 15,000 to 22,000 plates. The Van Deemter curves for the different solutes at flow rates from 0.25 to 5 mL/min are given in Fig. 2. The first observation is that at 1 mL/min (1.4 mm/s), the velocity is above u_{opt} for all solutes at 30 °C while close to u_{opt} at 80 °C. The fact that higher temperatures generate higher optimal mobile phase velocities is well documented in the literature [22,26,31,35] and the result of the interplay between an increased B-term and a decreased C-term.

When comparing the Van Deemter plots of one compound to those of the others, the different slopes of their C-terms are prominent, especially at 30 °C. This can be explained by the difference in solute diffusivity. The experimentally determined diffusion coefficients of ketoprofen, adenine and the API, using the Aris–Taylor method as described by Li and Carr [39], are 1.47×10^{-9} m^2/s, 1.76×10^{-9} m^2/s and 1.30×10^{-9} m^2/s respectively at 30 °C, and 3.03×10^{-9} m^2/s, 3.69×10^{-9} m^2/s and 2.62×10^{-9} m^2/s respectively at 80 °C. The implication of this is for instance that at 30 °C no minimum in the plate height is reached for the API while this is the case for ketoprofen (at 0.5 mL/min) and adenine (at 0.75 mL/min). At 80 °C the difference between the C-terms of the different compounds becomes less pronounced due to their increased diffusivity, and the optimal flow is ca. 1 mL/min (1.4 mm/s). The lower opti-

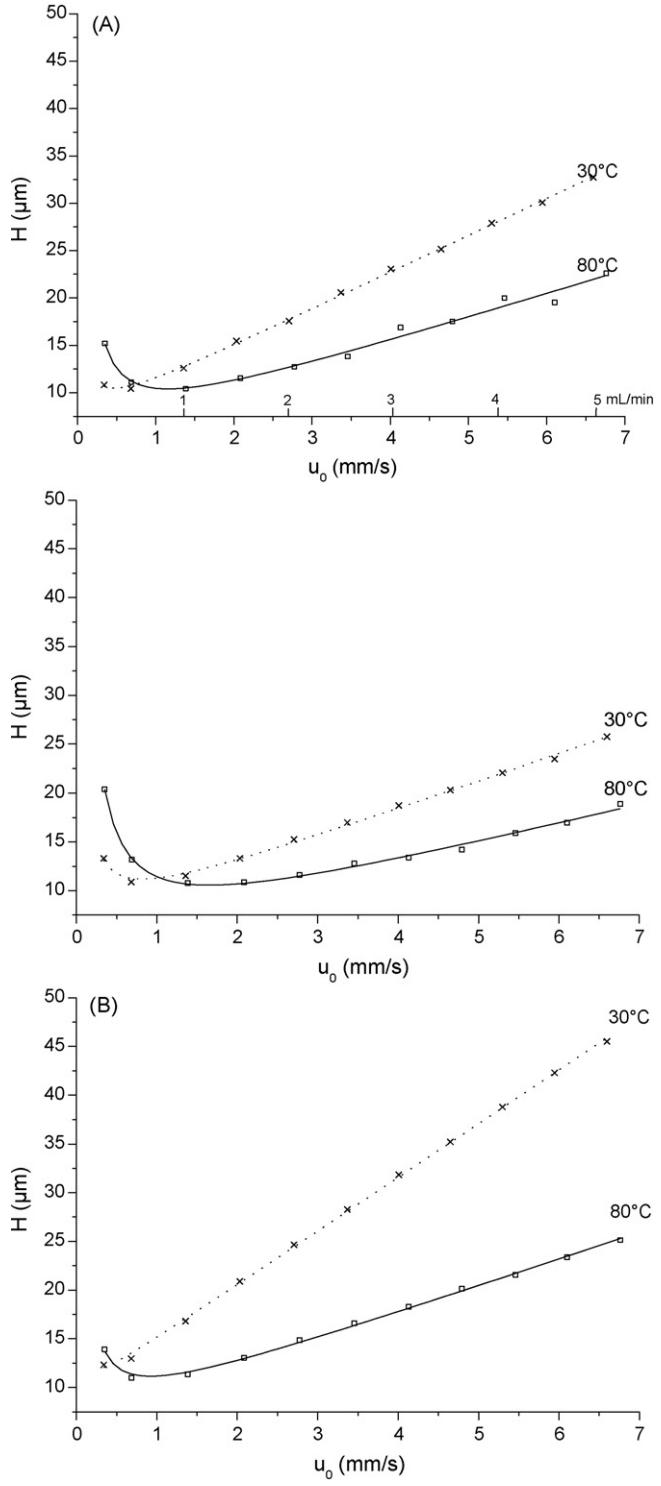

Fig. 2. Van Deemter plots for (A) ketoprofen, (B) adenine and (C) the API using a 25 cm column at 30 °C and 80 °C. Mobile phase: see Fig. 1.

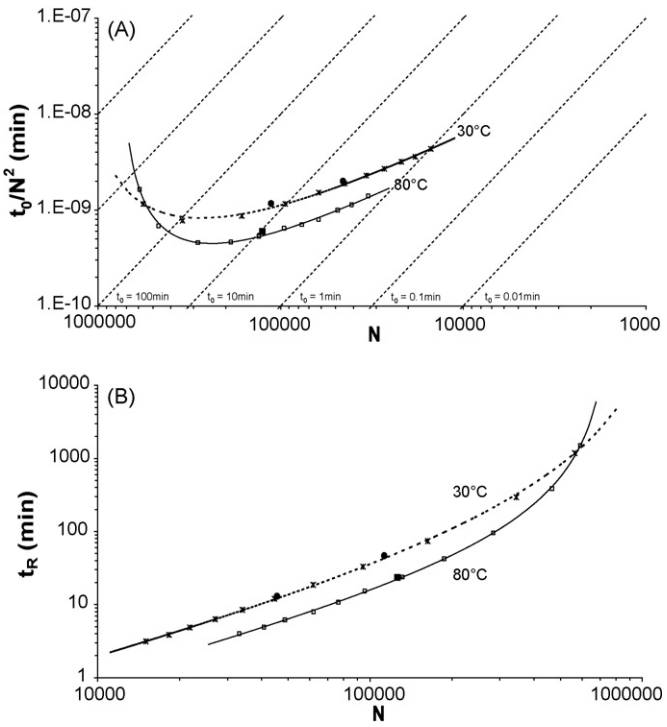

Fig. 3. Kinetic plots at 30 °C and 80 °C, (A) t_0/N^2 versus plates and (B) retention time of adenine versus plates, calculated from the Van Deemter data of adenine. The kinetic plots were verified experimentally using 3 coupled 25 cm column systems at 30 °C (●) and 6 × 25 cm columns at 30 °C (●) and 80 °C (■). ΔP_{col} = 350 bar; $K_{v0,30°C} = 3.02 \times 10^{-14}$ m^2 and $K_{v0,80°C} = 3.30 \times 10^{-14}$ m^2; $\eta_{30°C} = 4.15 \times 10^{-4}$ kg m^{-1} s^{-1} and $\eta_{80°C} = 2.73 \times 10^{-4}$ kg m^{-1} s^{-1}.

mal linear velocity for APIs and the influence of temperature has recently been discussed for RP-LC separations [42].

3.2. Using kinetic plots to establish the column lengths needed to generate 100,000 theoretical plates in the shortest possible analysis times

The kinetic plot method is a useful tool for comparing the performance of different chromatographic systems, for instance when comparing chromatographic support type (i.e. porous and superficially porous particles, monoliths, etc.), support size and the temperature and pressure at which these supports are operated [33–36]. It has also been illustrated how this method can be used as a tool to design coupled column systems that produce 100,000 theoretical plates in the shortest possible analysis times [20]. In the kinetic plot method, Eqs. (3) and (4) are used to transform the (H,u_0) Van Deemter data into (N,t_0) data, calculated at the maximum obtainable column pressure, ΔP_{max}:

$$N = \frac{\Delta P_{max}}{\eta}\left[\frac{K_{v0}}{u_0 H}\right]_{exp} \quad (3)$$

and

$$t_0 = \frac{\Delta P_{max}}{\eta}\left[\frac{K_{v0}}{u_0^2}\right]_{exp} \quad (4)$$

where the viscosity (η) is calculated and the plate height (H), linear velocity (u_0) and column permeability (K_{v0}) are experimentally obtained values, generated with one 25 cm column at each column temperature, 30 °C and 80 °C as described in section 2.3. Each of the (N,t_0) data points are representative of a different column length at a given column pressure, as opposed to the Van Deemter data points (H,u_0) where the column length is constant and the pressure is different for each flow rate at which a data point is generated. The kinetic plot method can therefore be used to establish what column length is needed to generate a chosen number of theoretical plates, e.g. 100,000 and what the fastest achievable linear velocity (and consequently the shortest analysis time) would be, if the column is operated at the maximum achievable column pressure.

Considering the fact that conventional HPLC equipment can operate at a maximum pressure of 400 bar, kinetic plots were con-

structed at 350 bar thereby allowing for 50 bar system pressure. For adenine (N,t_0) data were calculated from its Van Deemter data (H,u_0) for each column temperature, by using Eqs. (3) and (4), with the column permeability and viscosity values determined as described in Section 2.3 and with $\Delta P_{max} = 350 \times 10^5$ Pa. Plotting t_0/N^2 against N for 30 and 80 °C and, implementing the x- and y-axis modifications as described by Desmet [36], plots resembling Van Deemter curves were obtained, i.e. with B- and C-term regions in the same positions on the graph (Fig. 3A). In this display format there is an increase in column length when following the plot from right to left, while there is an increase in analysis time, diagonally from the bottom right hand corner, to the top left hand corner of the graph, as indicated by the time lines. In Fig. 3A the effect of temperature on these curves can be observed. The t_0/N^2 values are lower at 80 °C in the C-term region up to the region where the curves approach their optimum, confirming that lower values for t_0 (higher u_0) are obtained for a given number of plates at higher temperature.

An even more practical approach is constructing kinetic plots of the retention times, t_R (calculated from $t_R = t_0\,(1 + k)$) vs. N (Fig. 3B), which can directly be applied to establish the shortest possible analysis time in which a chosen number of theoretical plates can be obtained, at a given column temperature. The column length needed to achieve this can then simply be calculated from t_R, using Eq. (4) and $L = u_0 t_0$. For instance, to achieve 100,000 plates at 30 °C, the analysis of adenine will be 36 min on a 1.3 m column operated at 350 bar. At a column temperature of 80 °C, 100,000 theoretical plates can be generated in 16 min using a 1.2 m column operated at the same pressure. When evaluating the effect of elevated temperature on the kinetic plots of ketoprofen and the API (plots not shown), similar conclusions were reached.

Since the plots are constructed using Van Deemter (H,u_0) and column permeability (K_{v0}) data using one 25 cm column only, it makes sense to verify if the plots are accurate when longer columns are used in practice. The highlighted data points in Fig. 3 are experimental points obtained by operating a 75 cm column (3 × 25 cm coupled columns) at 30 °C and a 150 cm column (6 × 25 cm coupled columns) at both 30 °C and 80 °C and in all cases at a column pressure of 350 bar. Due to the decreased mobile phase viscosity at 80 °C, data points could not be generated at 350 bar for the 75 cm column (maximum flow of 5 mL/min reached at $\Delta P_{col} < 350$ bar). It was concluded from Fig. 3B, that at 30 °C a column of at least 130 cm in length should be used to obtain 100,000 theoretical plates, therefore the use of a 150 cm column should be more than sufficient to generate above 100,000 plates at both column temperatures. To generate column pressures of 350 bar, the 75 cm column was operated at 2.25 mL/min at 30 °C, while the 150 cm column was operated at 1.25 mL/min and 1.95 mL/min for column temperatures of 30 °C and 80 °C, respectively. The experimental points generated with the coupled column systems correspond very well with the kinetic plots constructed from data generated using only one 25 cm column, demonstrating that in HILIC, just as in RP-LC, the kinetic performance of a given support type under given conditions at a certain column pressure drop, can be extrapolated to different column lengths.

3.3. Increasing efficiency and speed of HILIC separations by combining coupled columns with elevated column temperature

Fig. 4A shows the chromatogram at 30 °C and 1 mL/min flow of the test mixture on a 150 cm long column. Compared to the chromatogram in Fig. 1A, the analysis times and efficiencies are

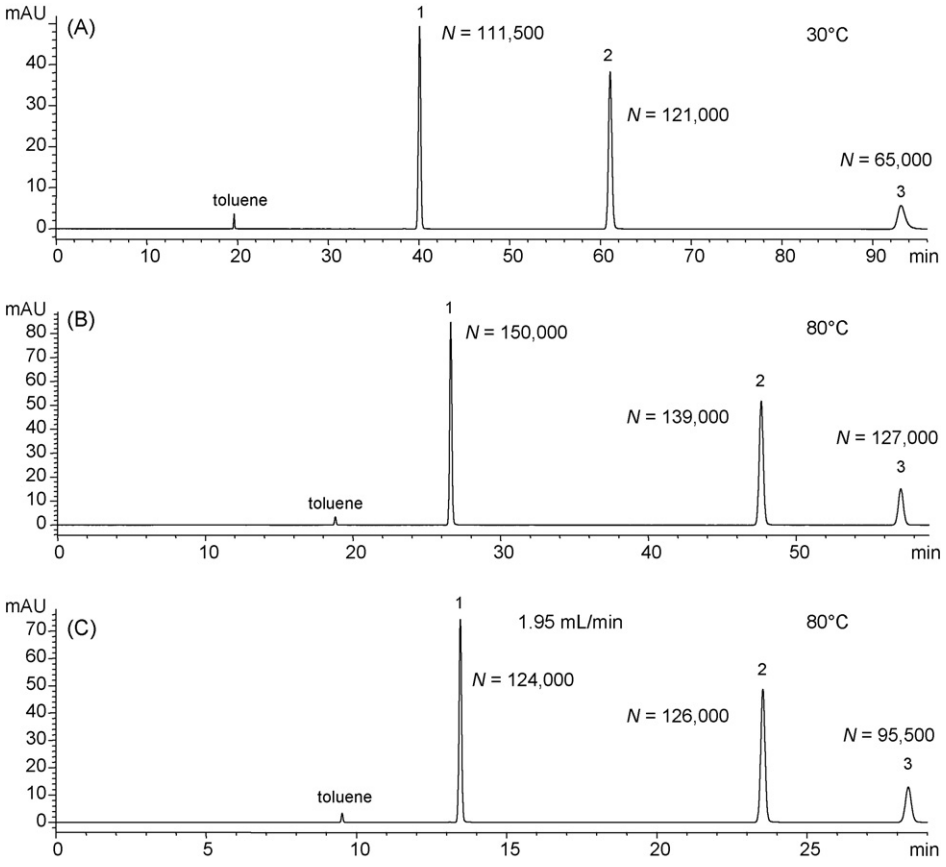

Fig. 4. Analysis of the test mixture using a 150 cm column at (A) 30 °C and (B) 80 °C with a mobile phase flow rate of 1 mL/min and (C) at 80 °C with a flow rate of 1.95 mL/min ($\Delta P_{col} = 350$ bar). Other conditions: see Fig. 1.

increased by a factor of ca. 6 with the exception of the plate number for the API (factor of ca. 4). At 80 °C and 1 mL/min flow (Fig. 4B), both retention times and efficiencies increased by a factor of 6, because at this temperature 1 mL/min is closer to u_{opt}. Because the increase in temperature reduces the viscosity of the mobile phase and consequently the pressure drop over the column, a higher mobile phase flow rate can be used, without excessive sacrifice of efficiency, whereby the analysis time can further be reduced. At 80 °C the 150 cm column could be operated at 1.95 mL/min (ΔP_{col} = 350 bar), resulting in an analysis time below 30 min (Fig. 4C). Approximately the same resolution is obtained as with 1 mL/min for the adenine-API pair. Note, however, that this is also due to the low retention of the 3 analytes at 80 °C.

For completeness, Fig. 5 gives the *H* versus *u* plots for the 150 cm column. A similar behaviour is noted as for the 25 cm long column with the exception of the slopes at 30 °C which are much steeper for the 150 cm long column compared to the 25 cm long column. For the API, *H* at 1 mL/min was 16.5 μm (Fig. 2C) whereas 23 μm is measured for the 150 cm long column (Fig. 5C).

The increased compressibility of the mobile phase consisting of >85% acetonitrile reduces D_m of the analytes in the coupled column system and increases the C-term. In addition, the optimum plate height for the API is significantly higher at 30 °C than at 80 °C. This implies that for this compound the kinetic plot constructed at 30 °C, using data generated with one 25 cm column, cannot be extrapolated to longer column lengths. This anomaly could be related to the increased compressibility of the HILIC mobile phase (containing high amounts or acetonitrile which is much more compressible than the aqueous mobile phases used in RPLC), combined with the low diffusion coefficient of the compound. Alternatively, some frictional heating taking place in the coupled column system could also affect the apparent pH and protonation of the API, the small drop in retention factor of the API on the 1 or 6 column systems (2.23 and 2.05, respectively) could attest for this. This phenomenon warrants further investigation of the applicability of the kinetic plot method, which is currently under way. The importance of experimentally verifying kinetic plots at the desired column length, for all compounds of interest, is self evident from these results. Perhaps this also highlights an advantage of constructing kinetic plots at elevated temperature (thereby increasing diffusion), which eliminates this effect. Kinetic plots should be validated for such compounds in the future, and ways of compensating for this effect to improve the calculated plots, should be investigated.

3.4. High-efficiency analysis of a mixture of pharmaceuticals and potential genotoxic compounds

To illustrate the potential of HILIC on a long column operated at elevated temperature, Fig. 6 shows the analysis of a mixture of pharmaceuticals and commercially available compounds including some with functional groups that are typical genotoxicity alerting on a 25 cm and a 100 cm column (4 coupled 25 cm columns connected in series). Monitoring potentially genotoxic impurities is of growing importance in the pharmaceutical industry and RP-LC often is inadequate [43]. Genotoxic impurities can be introduced into pharmaceutical formulations during the synthesis of an API, either as a starting material or a by-product, or they can be formed by degradation of the API or other components of the formulation. On a 25 cm column operated at 80 °C a decrease in analysis time, as well as changes in selectivity can be observed compared to the analysis at 30 °C (Fig. 6A and B). Increasing the column length to 100 cm increases the resolution drastically e.g. baseline separation of 1/2, 8/9, 15/16 which is due to the increased efficiency as the selectivity is unaffected (Fig. 6C). In order to halve the analysis time, the flow was doubled to 2 mL/min (Fig. 6D). A slight loss of resolution was noted for peaks 6/7 (Fig. 6E) and this could be solved by applying a shallow gradient from 87% to 85% acetonitrile in 3 min (hold 32 min). Note that under these conditions, peaks 8 and 9 reversed while peak 14 moved towards peak 15. Other gradient compositions, starting with higher amounts of ACN were also tried, but these were not successful as this was leading to overlapping peaks because of a shift of nitroterephthalic acid (peak 14).

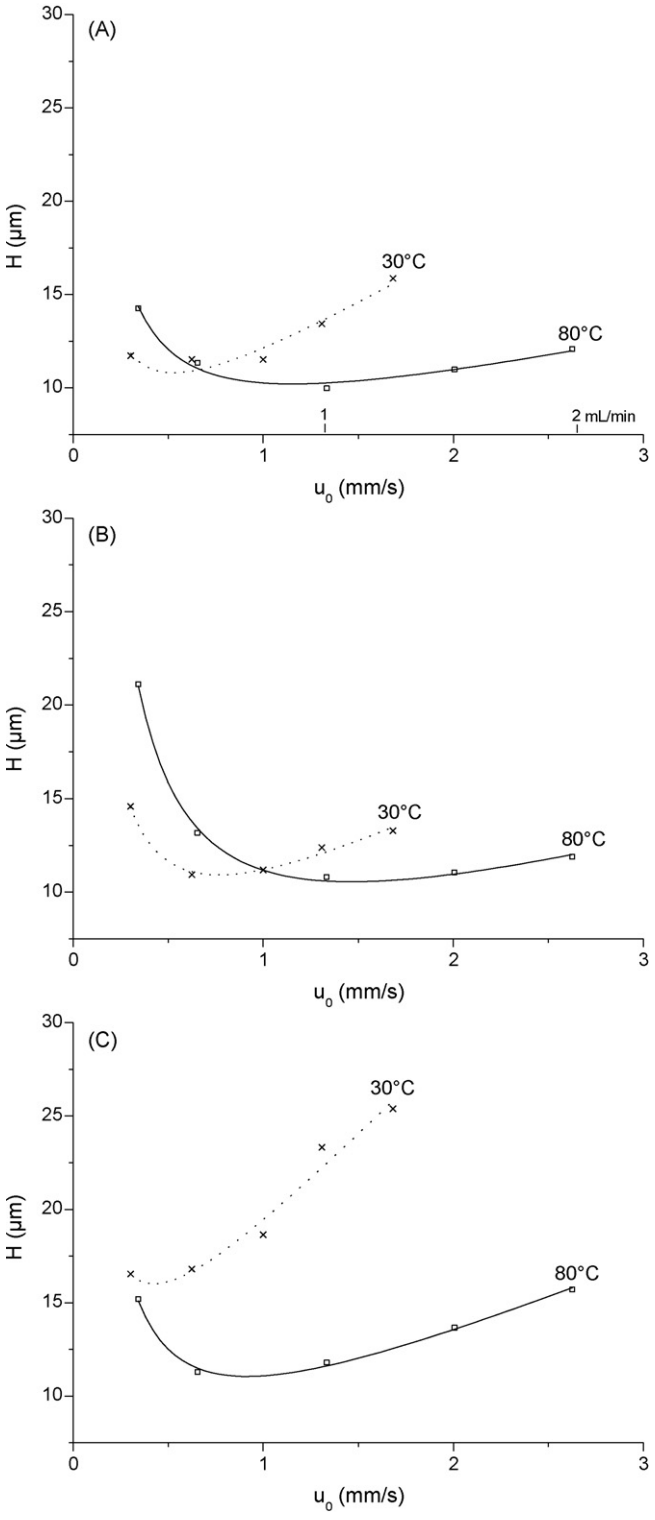

Fig. 5. Van Deemter plots for (A) ketoprofen, (B) adenine and (C) the API, using a 150 cm column at 30 °C and 80 °C. Mobile phase: see Fig. 1.

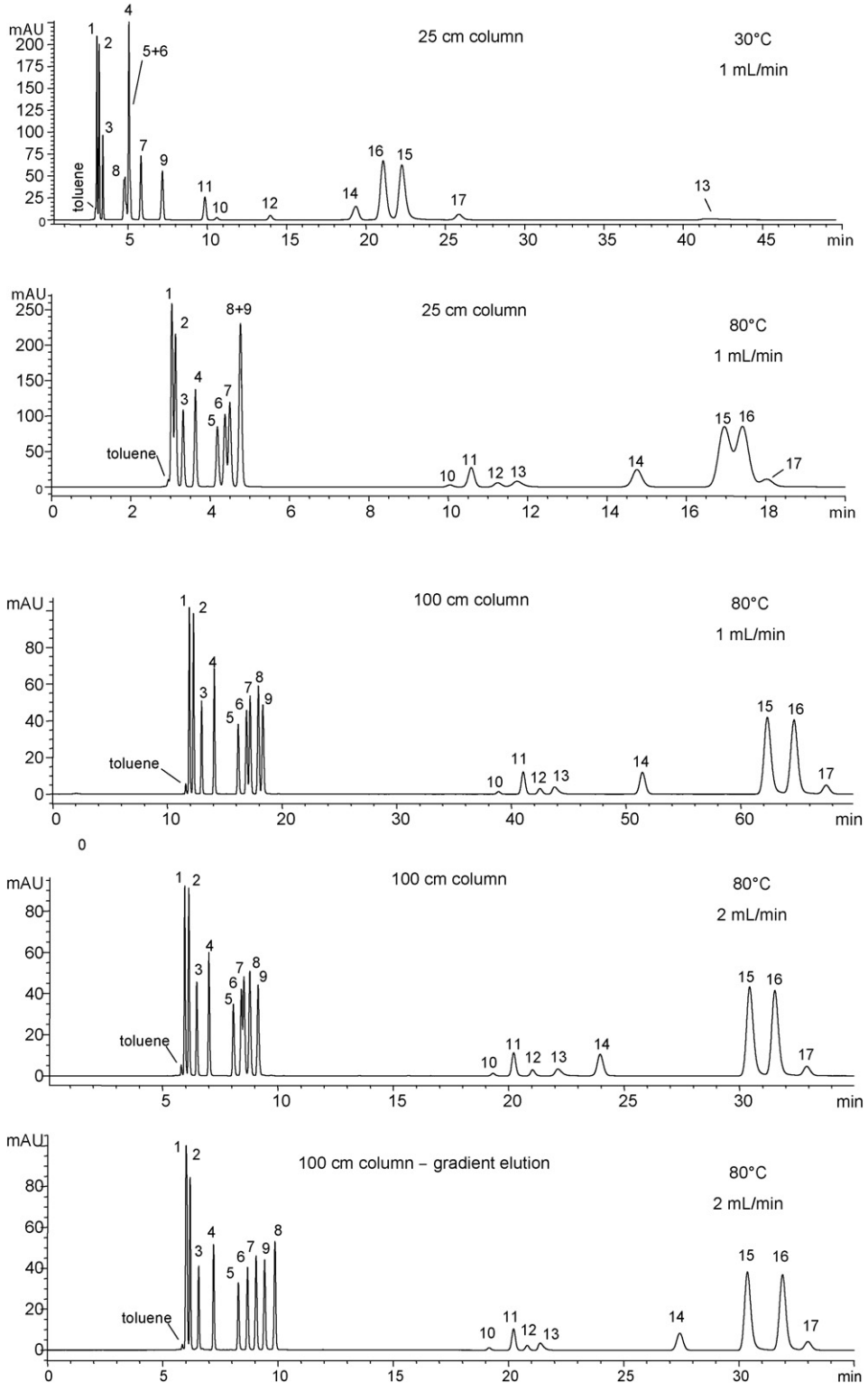

Fig. 6. Analysis of a mixture of pharmaceuticals and commercially available compounds some containing functional groups with potentially genotoxic structural alerts using a 25 cm column operated at (A) 30° and (B) 80 °C with a mobile phase flow of 1 mL/min, and a 100 cm column operated at 80 °C with a mobile phase flow rate of (C) 1 mL/min and (D) 2 mL/min under isocratic conditions and (E) 2 mL/min using a mobile phase gradient. Mobile phase isocratic: 85% acetonitrile 5 mM ammonium formate pH 5, gradient: 87–85% acetonitrile in 3 min (hold 32 min). Compounds (1) naphthylamine, (2) aniline, (3) 5-aminoindole, (4) 4-aminobenzoic acid, (5) 2-aminopyridine, (6) 3-aminopyridine, (7) 3-aminobenzoic acid, (8) 4-nitrobenzoic acid, (9) 5-amino-2-methyl-pyridine, (10) diphenhydramine, (11) nortriptyline, (12) benzylamine, (13) nicotine, (14) nitroterephthalic acid, (15) 4-aminopyridine, (16) 4-amino-2-methyl-pyridine, (17) procainamide.

4. Conclusions

Coupling columns to increase column length is a straightforward way of increasing separation efficiency in HILIC. If it is desired to use conventional LC columns and instrumentation (ΔP_{max} = 400 bar) to achieve high efficiencies, temperature can be used to reduce analysis times significantly. The kinetic plot method could be used for direct comparison of relationships between efficiency, analy-

sis time, column length and the effect of elevated temperature on these parameters.

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