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Journal of Pharmaceutical and Biomedical Analysis
32 (2003) 233–249
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
www.elsevier.com/locate/jpba

Fast separations on monolithic silica columns: method transfer, robustness and column ageing for some case studies

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Received 25 November 2002; received in revised form 28 February 2003; accepted 28 February 2003

Abstract

Six separation methods, developed on conventional silica high performance liquid chromatography (HPLC) columns were transferred to monolithic silica columns of 5 and 10 cm length. The transferred methods include the separation of an alkylbenzene mixture, the separations of drugs from their impurities (nimesulide, tetracycline, phenoxymethylpenicillin and erythromycin) and the separation of a green tea extract. The transfer of the first three methods was successful while for the latter three it was not. Increasing the flow rate up to 9 ml/min (where possible) inversely decreased the analysis time of the successfully transferred methods to 48 s (alkylbenzene mixture) 1.8 min (nimesulide mixture) and 3 min (tetracycline mixture) while still reasonable well separated peaks were obtained. The robustness and repeatability of the transferred and accelerated separations was found to be acceptable. Despite the use of flow rates up to 9 ml/min and frequent mobile phase changes with pH values varying from 3.5 to 7, the column performance was found to be rather constant and the column ageing to be minimal.

Keywords: Method development; Method transfer; Monolithic columns; Fast analysis; Robustness of separation; Column ageing

1. Introduction

Today, high performance liquid chromatography (HPLC) remains a very important technique to separate drugs from their impurities. In the European Pharmacopoeia (E.P.) [1] and the United States Pharmacopeia (USP) [2], RP-HPLC is used to separate drugs from their impurities. The separation method is described in detail in the monograph but the description of the stationary phase is often limited to column packing, dimensions and particle size [1,2]. Therefore, lots of commercially available columns fulfil these requirements although not all result in satisfactory separations due to different column selectivity and retention behaviour [3]. The transfer of separations from one column to another will thus not always be successful. The stationary phases pre-

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0731-7085/03/$ - see front matter
doi:10.1016/S0731-7085(03)00131-6

scribed in monographs usually are classical C_{18} (or C_8) silica columns. Such columns consist of silica particles and can be applied at flow rates below 3 ml/min, which might result in long analysis times. Nowadays, more and more new silica columns appear on the market with the aim to reduce analysis time, for instance by allowing higher flow rates. An example is the monolithic C_{18} silica column [4,5]. This column differs from classical silica columns since it consists of a silica rod, instead of particles and possesses a biporous structure of macropores and mesopores with diameters of approximately 2 μm and 13 nm, respectively. The biporous structure of the stationary phase provides a porosity greater than 80%, allowing chromatography with a much lower back pressure than on conventional columns. Flow rates up to 9 ml/min become possible and very fast separations can be achieved [4,5]. Monolithic silica columns are more and more commonly used and several applications already were published [6–8]. In the literature, separations within 1 min are reported for five beta-blocking drugs using a monolithic RP-18e column of 5 cm length at a flow rate of 9 ml/min [4,5].

The monolithic columns are not yet included in the official Pharmacopoeias, where classical silica particle columns are used. Therefore, it is interesting to investigate whether separations, developed on conventional C_{18} silica columns can be transferred to C_{18} monolithic ones and to examine the quality of the obtained separation. If the transfer is successful, the flow rate can be increased to decrease analysis time. The transfer from a classic C_{18} to a monolithic column results already in a gain in time even at nominal flow rate [4]. The shorter analysis times could make the monolithic columns very interesting in, for instance, routine analysis or even process analysis. However, to be applied in process analysis, the separations should be robust. This aspect has not been studied yet on monolithic columns. Therefore, in this study, six separations were transferred to monolithic silica columns and problems related to separation transfer were investigated. Then the flow rate was increased to reduce analysis times. The separations and their robustness at these new conditions were evaluated. The ageing of the column also was evaluated. The six methods for which the transfer was examined are the separation of (i) an alkylbenzene mixture [4], (ii) the anti-inflammatory drug nimesulide [1], the antibiotics (iii) tetracycline [2], (iv) phenoxymethylpenicillin [1] and (v) erythromycin [9,10] from their impurities, and (vi) the separation of caffeine and polyphenols in green tea extracts [11].

2. Experimental

2.1. Chromatographic columns

Monolithic HPLC columns from Merck (Darmstadt, Germany) were used: Chromolith SpeedROD RP-18e (50×4.6 mm) and Chromolith Performance RP-18e (100×4.6 mm). For the separation of the tetracycline mixture, a Chromolith Guard column RP-18e (5×4.6 mm) was also used.

2.2. Chromatographic conditions

2.2.1. Instruments

The HPLC system consisted of an Agilent 1100 Series degasser and Quatpump, a Hewlett–Packard series 1050 autosampler and UV detector (Agilent, Waldbronn, Germany). The system was operated with the Hewlett–Packard Chemstation interface and software. For the transfer of the tetracycline and erythromycin separations, a L-7100 pump, L-7612 solvent degasser, L-7250 autosampler, L-7400 UV detector and a D-7000 interface from Merck–Hitachi (Tokyo, Japan) were used. This system was operated with the LaChrom D-7000 HPLC MANAGER Software (Merck).

Unless specified differently, the column temperature was kept constant at 30 °C by submerging them in a thermostatted water bath of which the temperature was kept constant with a Protherm pt 5000 thermostat. The injected sample volumes and detection wavelengths were 5 μl for the alkylbenzene (254 nm), the nimesulide (230 nm), the phenoxymethylpenicillin (254 nm), the erythromycin (215 nm) and the green tea extract mixtures

(210 nm) and 20 μl for the tetracycline mixture (280 nm).

2.2.2. Chemicals/reagents

The nimesulide mixture contained 87.53 mg nimesulide, 8.69 mg impurity A, 32.78 mg impurity B, 62.41 mg impurity C, 69.95 mg impurity D and 40.00 mg impurity E, dissolved in 60.0 ml acetonitrile (ACN). Prior to injection, this solution was diluted 50 times with ACN/water 40/60 v/v. Impurities A, B, C, D and E are described in more detail in the E.P. 2002 [1].

The tetracycline sample contained 1.2% ETC (4-epi-tetracycline), 97.6% TC (tetracycline), 0.05% EATC (4-epi-anhydrotetracycline), 0.2% ATC (anhydrotetracycline) and 0.6% ADTC (2-acetyl-2-decarboxamidotetracycline). The tetracycline assay solution [2] was a 0.50 mg/ml tetracycline sample in ammonium oxalate/dimethylformamide (68/27 v/v).

The alkylbenzene mixture contained 210 mg amylbenzene (Sigma-Aldrich, Steinheim, Germany), 164 mg butylbenzene, 100 mg ethylbenzene, 4.3 mg *o*-terphenyl, 0.77 mg triphenylene, 1.8 mg uracil (all Fluka, Buchs, Switzerland) and 85 mg toluene (Merck), in 100.0 ml MeOH/water (70/30 m/m). The solution was then diluted ten times with the same solvent.

For the composition of the phenoxymethylpenicillin, erythromycin and green tea extract mixtures we refer to [1,9–11]. The nimesulide, tetracycline, erythromycin and phenoxymethylpenicillin samples were gifts from Professor J. Hoogmartens, Catholic University, Leuven, Belgium.

2.2.3. Mobile phases

The mobile phases were prepared using ACN and methanol (MeOH), both Hipersolv for HPLC (BDH Laboratory Supplies, Poole, England), ammonium dihydrogen phosphate (Fluka), dimethylformamide (DMF), ammoniumoxalate and ammonia solution 25% (Merck), all pro analysis quality.

The initial mobile phase for the nimesulide mixture contained ACN/$NH_4H_2PO_4$ 1.15 g/l, pH 7.0 (35/65 v/v) [1], the adapted mobile phase was identical except for the solvent ratio which was 30/70, v/v. Flow rates of 1.3, 5, 6, 7 and 9 ml/min were used.

The mobile phase for the tetracycline mixture contained 0.1 M ammonium oxalate, dimethylformamide and 0.2 M dibasic ammonium phosphate (68/27/5 v/v/v). The pH was adjusted to 7.6 with 3 N ammonium hydroxide [2]. Flow rates of 1–9 ml/min were used.

The initial mobile phase for the separation of the alkylbenzene mixture was MeOH/water, 76/24 m/m [4] and the adapted 70/30 m/m. Flow rates of 1, 5, 6 and 9 ml/min were used.

For the mobile phases used to separate the phenoxymethylpenicillin, erythromycin and green tea extract mixtures we refer to [1,9–11].

The pH of the buffers was adjusted using an Orion 520A (Orion Research, Boston, MA) pH-meter. Buffers were filtered through a 0.2 μm membrane filter (Schleicher & Schuell, Dassel, Germany). Buffers and mobile phases were prepared with Milli-Q water, obtained with the Milli-Q water purification system (Millipore, Molsheim, France).

3. Results and discussion

To investigate occasional problems at transfer to a monolithic column of 5 or 10 cm, the six methods were transferred using the conditions prescribed in the literature for the classic stationary phases [1,2,4,9–11].

3.1. Transfer of the nimesulide method

3.1.1. Method transfer and acceleration of the separation

The separation of the nimesulide mixture on the monolithic column was performed as prescribed in the E.P. [1]. A mobile phase containing ACN/$NH_4H_2PO_4$ 1.15 g/l, pH 7.0 (35/65 v/v) was used. The column temperature was kept at 25 °C, the flow rate at 1.3 ml/min and the detection wavelength at 230 nm. Except for the particle size, both monolithic columns fulfil the Pharmocopoeia requirements regarding column length deviations and internal diameter. The transfer to the SpeedROD column resulted in well separated peaks.

Table 1
Peak resolutions for the nimesulide mixture

Column	Mobile phase	Flow rate (ml/min)	Peak pairs					
			Impurity A–nimesulide	Nimesulide–impurity B	Impurity B–impurity C	Impurity C–impurity D	Impurity D–impurity E
SpeedROD	Initial	1.3 [a]	6.26	10.85	4.29	1.66	2.76
		1.3	5.91	10.94	4.74	1.26	2.14
		9	3.43	7.80	3.17	1.04	1.90
	Adapted	1.3	7.49	13.61	4.46	2.84	3.17
		9	5.25	8.96	3.05	2.29	2.22
Performance		1.3	9.11	15.95	4.90	3.11	4.37
		7	7.42	10.22	3.41	2.72	3.20

[a] 25 °C.

Resolutions above 1.5 were found (Table 1). The mixture was also separated at 30 °C. Thermostatted analyses above ambient temperature namely allow to work under more repeatable circumstances and permit well-defined deviations during robustness testing (see further). At 30 °C, the worst-separated peak pair (C–D) was not baseline separated anymore and resolutions of 1.3 and 1.0 were obtained at 1.3 and 9 ml/min, respectively. Resolutions above 1.5 at 30 °C were obtained when the ratio of ACN/buffer in the mobile phase was changed to 30/70 (v/v), which is in the allowable adjustment range of the Pharmacopoeia [1]. Under the latter conditions, the SpeedROD column separated the nimesulide mixture within 11 min at 1.3 ml/min and within 1.8 min at 9 ml/min (Fig. 1). The corresponding column back pressures were 20 and 144 bar, respectively, which is clearly below the 200 bar limit indicated by the manufacturer. On the Performance column, analysis times of 23 and 5 min were obtained at flow rates of 1.3 and 7 ml/min, with back pressures of 33 and 178 bar, respectively. The same separation at 1.3 ml/min on a LiChrospher 100 RP-18 (250×4.6 mm) column required 70 min.

The above shows that the analysis time could be reduced up to 40 times by using a monolithic column and increasing the flow rate, while peak resolutions remained acceptable. The number of theoretical plates for the nimesulide peak computed on the SpeedROD column was 4400 at 1.3 ml/min and 1900 at 9 ml/min, while on the Performance column it was 7200 at 1.3 ml/min and 2800 at 7 ml/min. The number of plates decreases with increasing flow rate but still was found to be sufficient for an adequate separation of the mixture.

3.1.2. Robustness of the nimesulide separation

The robustness of the nimesulide separation on the monolithic columns was evaluated. Six factors, which might influence the separation, were examined. It was the fraction organic modifier in the mobile phase, the detection wavelength, the flow rate, the temperature, the pH and the concentration $NH_4H_2PO_4$ in the mobile phase (Table 2). The detection wavelength will not immediately affect

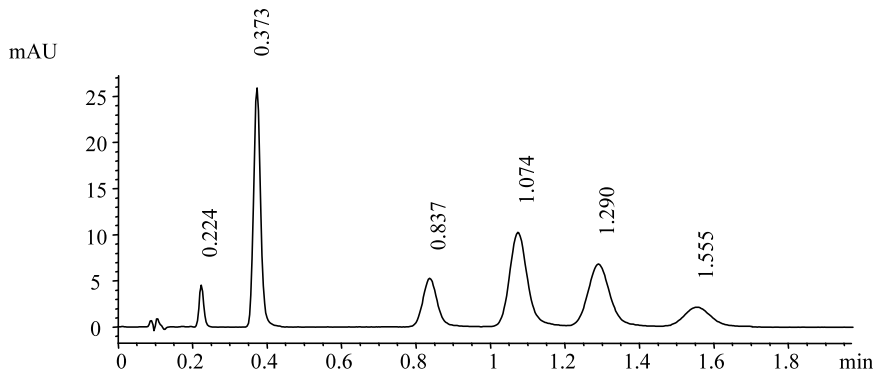

Fig. 1. Chromatogram of the nimesulide mixture on the SpeedROD column. Mobile phase ACN/$NH_4H_2PO_4$ 1.15 g/l; pH 7.0 (30/70 v/v); T = 30 °C; λ = 230 nm; flow rate, 9 ml/min. Elution order of the peaks; impurity A, nimesulide, impurity B, impurity C, impurity D, impurity E.

the separation itself but can have an influence on the measured peak shapes and on the peak integration for quantification purposes. Therefore, it was included in the robustness test. Each factor was examined at two levels; a high (+1) and a low one (−1) around the nominal level (0). A 2^{6-3} fractional factorial design (eight experiments, generators D = ABC, E = AB, F = BC) was used. The robustness tests were performed at nominal flow rates 1.3, 5 and 9 ml/min on the SpeedROD, and at 1.3, 6 and 7 ml/min on the Performance column (Table 2). The effects of the factors on the resolution were calculated as usually is done in robustness tests [12,13]. Significant effects were identified using an error estimate, based on the algorithm of Dong [13,14].

Since the impurities C–D peak pair is the worst separated one (Table 1), its separation was first focussed on. The effects of the factors on their resolution are shown in Table 3. The column temperature and the fraction ACN in the mobile phase have a significant negative effect on the resolution at all examined flow rates on both columns. Such effects also are to be expected on classical columns. The pH of the mobile phase was found to have a significant influence on the resolution of the impurity A–nimesulide and nimesulide–impurity B peak pairs (effects not shown) but not on the one between impurities C and D. The wavelength, the flow rate and the concentration $NH_4H_2PO_4$ in the mobile phase did not significantly influence the resolution.

The non-significance intervals for the factors with a significant effect on the resolution of the worst separated peak pair (impurity C–impurity D) are shown in Table 4. The non-significance intervals for the pH were computed for the nimesulide–impurity B peak pair. The different non-significance intervals found for a given factor at different flow rates can be explained by the variation in estimated effects and critical effects. The robustness test showed that to create a chromatogram with a minimal variation in separation, one should strictly control the fraction organic modifier, the column temperature and the pH of the mobile phase. Especially the levels of the fraction organic modifier in the mobile phase, as examined in the robustness test were chosen much too optimistic to represent an allowable non-significance range.

3.1.3. Injection repeatability of the nimesulide mixture

The injection repeatability was determined on both columns. Six replicate injections of the mixture were performed at low and high flow rates. The relative standard deviations (%R.S.D.) of the area under curve (AUC) and of the resolutions were calculated (Table 5).

The %R.S.D. values for the peak area are mostly within the E.P. limits, e.g. 0.85% R.S.D. for B = 2% and six replicate injections, (with B = upper specification limit, 100%). The %R.S.D. values exceeding these limits are mainly those for

Table 2
Factors and levels examined in the robustness tests for the nimesulide, tetracycline and alkylbenzene mixtures

Factors	Nimesulide mixture			Tetracycline mixture			Alkylbenzene mixture		
	(−) level	(0) level	(+) level	(−) level	(0) level	(+) level	(−) level	(0) level	(+) level
(A) Fraction organic modifier in the mobile phase	0.275 (v/v)	0.300 (v/v)	0.325 (v/v)	26 (v/v/v)	27 (v/v/v)	28 (v/v/v)	0.703 (m/m)	0.700 (m/m)	0.697 (m/m)
(B) Wavelength (nm)	227	230	233	277	280	283	251	254	257
(*C*) *Flow rate* (*ml/min*)									
SpeedROD	1.2	1.3	1.4	1.9	2.0	2.1	0.9	1.0	1.1
	4.9	5.0	5.1	5.9	6.0	6.1	4.9	5.0	5.1
	8.9	9.0	9.1	/	/	/	8.9	9.0	9.1
Performance	1.2	1.3	1.4	/	/	/	0.9	1.0	1.1
	5.9	6.0	6.1	/	/	/	5.9	6.0	6.1
	6.9	7.0	7.1	/	/	/	/	/	/
(D) Temperature (°C)	25	30	35	25	30	35	25	30	35
(E) pH	6.9	7.0	7.1	7.4	7.6	7.8	/	/	/
(F) Concentration $NH_4H_2PO_4$ (g/l)	1.04	1.15	1.26	/	/	/	/	/	/
(G) Concentration ratio (M/M) NH_4oxalate/NH_4phosphate	/	/	/	0.0975/ 0.195	0.1000/ 0.200	0.1025/ 0.205	/	/	/

/, not examined.

Table 3
Resolutions at nominal conditions (Rs_{nom}) and effects of the factors on the resolution of (a) the impurity C–impurity D peak pair of the nimesulide mixture, (b) the ETC–EATC and EATC–TC peak pairs of the tetracycline mixture and (c) the toluene–ethylbenzene peak pair of the alkylbenzene mixture

Mixture	Column	Flow rate	Rs_{nom}	E_A ($f_{ACN,\ MeOH\ or\ DMF}$)	E_B (λ)	E_C (flow rate)	E_D (T)	E_E (pH)	E_F (conc $NH_4H_2PO_4$)	E_G (M NH_4ox/NH_4phosphate)	$E_{critical}$ (5%)
Nimesulide	SpeedROD	1.3	2.84	−1.80[a]	0.01	0.05	−0.86[a]	0.06	−0.02	/	0.11
		5	2.38	−1.52[a]	−0.05	−0.04	−1.20[a]	0.01	0.33	/	0.47
		9	2.27	−1.39[a]	0.04	−0.08	−0.33[a]	0.07	0.05	/	0.17
	Performance	1.3	3.35	−1.79[a]	0.04	−0.01	−1.07[a]	0.06	−0.03	/	0.11
		6	3.32	−1.62[a]	0.03	−0.14	−1.40[a]	0.18	0.43	/	0.67
		7	3.23	−1.68[a]	−0.20	0.07	−0.80[a]	0.30	0.26	/	0.52
Tetracycline	SpeedROD	2	1.78	−0.30[a]	0.03	−0.01	−0.21[a]	−0.14	/	0.02	0.14
ETC–EATC		6	1.52	−0.27[a]	−0.04	0.13[a]	−0.38[a]	0.07	/	0.03	0.13
EATC-TC		2	3.62	−0.20	0.02	0	−0.27	1.47[a]	/	−0.28	0.28
		6	3.50	−0.16	−0.01	0.14	−0.68[a]	1.71[a]	/	−0.13	0.34
Alkylbenzene	SpeedROD	1	2.65	0.21[a]	0.02	−0.02	−0.35[a]	/	/	/	0.17
		5	2.05	0.05	−0.02	−0.06[a]	−0.14[a]	/	/	/	0.05
		9	1.64	−0.08	0.13	0.14	−0.12	/	/	/	0.43
	Performance	1	3.12	0.15[a]	−0.01	−0.20[a]	−0.31[a]	/	/	/	0.04
		6	3.03	0.04	0.03	−0.04	−0.82[a]	/	/	/	0.21

[a] Significant effect.

Table 4
Non-significance intervals for the significant factors from the robustness test of the nimesulide mixture

	SpeedROD column			Performance column		
	1.3 ml/min	5 ml/min	9 ml/min	1.3 ml/min	6 ml/min	7 ml/min
Fraction organic modifier in the mobile phase (v/v)	[0.299–0.301]	[0.293–0.307]	[0.297–0.303]	[0.298–0.302]	[0.290–0.310]	[0.292–0.308]
Temperature (°C)	[29.4–30.6]	[28.0–32.0]	[27.4–32.6]	[29.5–30.5]	[27.6–32.4]	[26.7–33.3]
pH mobile phase	/	/	[6.96–7.04]	[6.96–7.04]	[6.97–7.03]	[6.95–7.05]

/, Effect not significant.

impurities A and E, which are the substances with the lowest concentration. At nominal flow rate on the SpeedROD column all R.S.D. values clearly were below 0.85%, the variability on the Performance column was seen to be higher. The repeatability of the AUC was found to be best at a flow rate of 1.3 ml/min for both monolith columns. For most impurities the %R.S.D. tends to increase at higher flow rates. However, when comparing to the E.P. limits, we have to take into account that the %R.S.D. to be expected depends on the concentration used and that for the nimesulide impurities, we do not necessarily have appropriate concentrations to obey the E.P. limits. The impurity concentrations in the mixture are equivalent to impurity levels ranging from 0.07 to 0.7%. When we compare the %R.S.D. values with a theoretical estimate for repeatability proposed by Horwitz et al. [15], limits considerably higher than those of the E.P. were found (Table 5).

$$\%\mathrm{R.S.D.}_{\mathrm{R}} = 2^{(1-0.5\ \log\ \mathrm{C})} \quad (1)$$

Eq. (1) estimates the reproducibility $\%\mathrm{R.S.D.}_{\mathrm{R}}$, based on the concentration C of the analyte expressed as a decimal weight fraction. The within-laboratory R.S.D. is usually 1/2−2/3 of $\%\mathrm{R.S.D.}_{\mathrm{R}}$ [15]. In Table 5, it can be seen that the %R.S.D. were usually below 1/2 $\%\mathrm{R.S.D.}_{\mathrm{R}}$ although for impurities A and E still some higher %R.S.D. values were found. Nevertheless, all %R.S.D. values remained below 2/3 $\%\mathrm{R.S.D.}_{\mathrm{R}}$. The repeatability of the separation (%R.S.D. resolution) was also found to be good (Table 5).

Table 5
%R.S.D. values for (a) the AUC and (b) the resolutions for the injection of nimesulide mixture ($n = 6$)

	SpeedROD			Performance			Horwitz, 1/2 $\%\mathrm{R.S.D.}_{\mathrm{R}}$
	1.3 ml/min	5 ml/min	9 ml/min	1.3 ml/min	6 ml/min	7 ml/min	
(*a*)							
Impurity A	0.29	1.07	2.48	0.59	2.42	3.3	1.96
Nimesulide	0.15	0.14	0.17	0.32	0.53	0.82	1.38
Impurity B	0.35	0.34	0.87	0.50	1.03	0.53	1.60
Impurity C	0.26	0.31	0.69	0.58	0.77	0.33	1.45
Impurity D	0.28	0.29	0.89	0.26	1.13	0.46	1.43
Impurity E	0.70	1.16	2.02	1.56	1.31	1.74	1.56
(*b*)							
Impurity A–nimesulide	0.39	0.37	0.78	2.91	0.42	0.49	
Nimesulide–impurity B	0.28	0.43	0.60	1.62	0.47	0.47	
Impurity B–impurity C	0.37	0.63	0.26	0.97	0.35	0.42	
Impurity C–impurity D	0.51	0.35	1.01	0.96	0.25	0.25	
Impurity D–impurity E	0.45	0.51	0.53	1.16	0.56	0.46	

3.2. Transfer of the tetracycline method

3.2.1. Method transfer and acceleration of the separation

The method for tetracycline as prescribed in the USP 25 [2] was transferred to the SpeedROD column. The mobile phase contained 0.1 M ammonium oxalate, dimethylformamide and 0.2 M dibasic ammonium phosphate (68/27/5 v/v/v). The pH was adjusted to 7.6 with 3 N NH_4OH. The column temperature was 30 °C, the flow rate 2.0 ml/min and the detection wavelength 280 nm. The USP 25 prescribes a 25 cm C_8 silica column with 5–10 μm particles. Despite of the different stationary phases (C_8 vs. C_{18}), the transfer to the SpeedROD column was found to be successful.

At a flow rate of 2.0 ml/min the analysis time was 4.1 min, while at 9 ml/min it was reduced to 2.6 min. It was also seen that an increase in flow rate from 1 to 5 ml/min decreased the separation time from 7.5 to 3 min while an increase from 5 to 9 ml/min only resulted in a decrease of analysis time with 18 s (Table 6). The constant analysis time at increased flow rate would indicate an increased interaction between solute and stationary phase. An explanation for this behaviour was not immediately found. Since increasing the flow rate above 6 ml/min did not speed up the separation, fast analyses were performed at this flow rate (Fig. 2). The separation of the tetracycline mixture on a classical silica column takes about 30 min [16]. In Table 6, the resolutions between the different peak pairs are shown. It can be seen that an increase in flow rate did not decrease the peak resolutions of any peak pair, they even increased. In Fig. 2 the separation of the tetracycline resolution solution at 6 ml/min is shown.

The number of theoretical plates computed for the tetracycline peak on the SpeedROD column are 1600, 1640 and 1750 at 2, 6 and 9 ml/min, respectively. Thus, in contrast with what is expected from the Van Deemter equation [17], the number of plates remained constant or even increased slightly when the flow rate increased. For monolithic columns it is known that they give rise to very flat h/u–Van Deemter curves [4] which is confirmed by this example. It seems that the longitudinal diffusion term remains larger than or at least comparable to the mass transfer term in the Van Deemter equation leading to the constant or even increasing number of plates.

The transferred separation was also examined for system suitability as prescribed in the USP 25 [2]. The EATC–TC peak resolution should not be less than 1.3 and the relative retention time of EATC versus TC not more than 0.9. Both requirements were always fulfilled (see Table 6 for Rs). For α they were 0.6 (2 and 6 ml/min) and 0.7 (9 ml/min). In conclusion, the separation of the tetracycline mixture was successful despite the rather high pH (pH 7.61) of the mobile phase. It should be remarked that the pH of the transferred separation exceeds the maximum allowable pH for monolithic silica columns, which is 7.5.

3.2.2. Robustness of the tetracycline separation

The robustness of the tetracycline separation on the SpeedROD column was evaluated at flow rates

Table 6
Peak resolutions and analysis times for the resolution solution of tetracycline separated on the SpeedROD column

Flow rate (ml/min)	ETC–EATC	EATC–TC	TC–ADTC	ADTC–ATC	Analysis time (min)
1	1.36	3.27	4.12	2.59	7.5
2	1.37	3.19	6.14	3.17	4.1
3	1.48	3.33	6.15	3.02	3.1
4	1.51	3.27	6.35	3.02	2.9
5	1.54	3.41	6.45	3.00	2.9
6	1.58	3.45	6.33	2.95	2.7
7	1.57	3.42	6.82	3.10	2.7
8	1.54	3.56	6.44	2.94	2.7
9	1.53	3.54	5.89	2.94	2.6

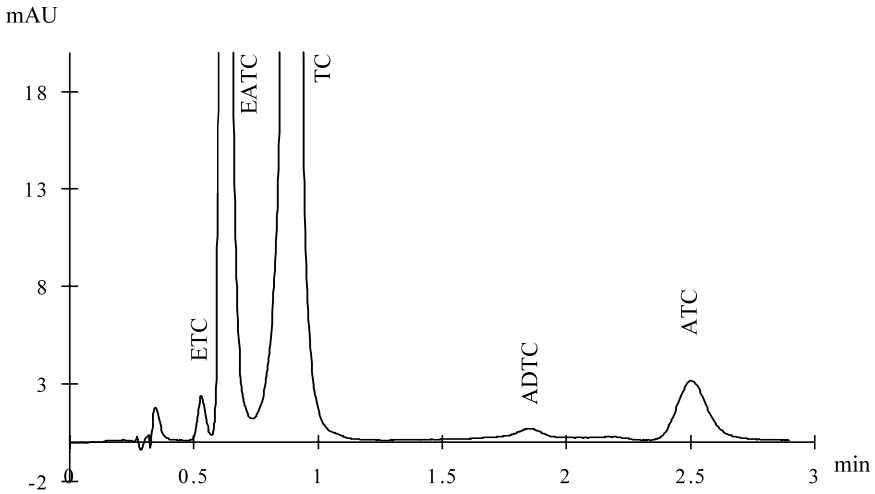

Fig. 2. Chromatogram of the tetracycline resolution solution on the SpeedROD column. Mobile phase dimethylformamide; 0.1 M ammoniumoxalate; 0.2 M dibasic ammonium phosphate (27/68/5 v/v/v) pH 7.6; T = 30 °C; λ = 280 nm; flow rate, 6 ml/min. Elution order of the peaks; ETC, EATC, TC, ADTC and ATC.

2 and 6 ml/min. In the robustness test, six factors were examined: the fraction dimethylformamide and the concentration ratio of ammonium salts in the mobile phase, the pH, the flow rate, the column temperature and the detection wavelength (Table 2). Each factor was examined at two levels around the nominal. A Plackett–Burman design for 11 factors was used [13]. Every fifth experiment, an injection at nominal levels was performed to check for time effects. The experimental set-up, computations of the effects and interpretation of the results was done using the RTS software [18]. Significant effects were identified using a standard error $(S.E.)_e$ estimated from the five dummy factor effects [18]. Fig. 2 shows that ETC–EATC and EATC–TC are the worst-separated pairs and thus their separation was focussed on. The effects on the resolution are shown in Table 3. For ETC–EATC the fraction DMF in the mobile phase and the temperature had a significant negative effect similar to what was observed for nimesulide. On the resolution of EATC–TC the temperature and pH had significant effects. For both peak pairs the influence of the temperature was larger at higher flow rates.

The non-significance intervals for the factors with a significant effect on the resolution ETC–EATC and EATC–TC were computed. For the factors fraction dimethylformamide and pH of the mobile phase, they were found equal at flow rates 2 and 6 ml/min, i.e. [26.54–27.46] and [7.56–7.64], respectively. For the temperature, the intervals were found to be narrower at high flow rate; [26.7–33.3 °C] at 2 ml/min and [27.5–32.5 °C] at 6 ml/min which is logical given the difference in effects observed. The non-significance intervals for the pH were rather narrow and thus a strict control of the pH is required to obtain robust and repeatable fast separations. Nevertheless, the robustness of the separation of the tetracycline mixture on the SpeedROD column at both flow rates can be considered comparable to the one on a classical C_8 Alltima column [16].

3.2.3. *Injection repeatability of the tetracycline separation*

Five replicate injections of the tetracycline standard preparation solution were performed on the SpeedROD column as prescribed in the USP 25 [2]. The %R.S.D. was computed for the TC peak and may not be more than 2.0% to meet the USP 25 requirements. The system suitability requirements for repeatability are satisfied since the %R.S.D. was 1.4 and 0.9 at flow rates 2 and 6 ml/min, respectively. The injection repeatability at

high flow rate was found to be at least as good as at low flow rate.

3.3. Transfer of the alkylbenzene separation

3.3.1. Method transfer and acceleration of the separation

The alkylbenzene mixture was separated on a SpeedROD column using a mobile phase of MeOH/water (76/24 m/m), UV detection was done at 254 nm and the flow rate 1 ml/min. At the prescribed conditions, the column separates the seven compounds within only 4 min. Cabrera et al. analysed a similar but less complex mixture (only four compounds) under these conditions on the Performance column within 8 min [4]. A classical LiChroCART Purospher RP-18e (125 × 4 mm) silica column needed 17 min to separate the four alkylbenzenes [4].

The separation on the SpeedROD column, resulted in baseline resolution for all peak pairs; for the worst-separated pair (toluene–ethylbenzene) a resolution of still 1.7 was found (Table 7). At a flow rate of 9 ml/min the analysis time was reduced to only 30 s but the minimal resolution decreased to 1.1 (Table 7). Since resolutions above 1.5 are desired, even at high flow rates, the mobile phase composition was changed to MeOH/water (70/30 m/m). Analysis times of 7 min (1 ml/min) and 48 s (9 ml/min) (Fig. 3) were then obtained. On the Performance column, they were 13 min (1 ml/min) and 1.7 min (8 ml/min). Higher flow rates on the Performance column resulted in back pressures above 200 bar. For the worst separated peak pair, which now was *o*-terphenyl–amylbenzene, resolutions of 2.1 (1 ml/min) and 1.3 (9 ml/min) were found on the SpeedROD and of 3.0 (1 ml/min) and 1.8 (8 ml/min) on the Performance column (Table 7). Both monolithic columns provided good separations in spite of their short length and the high flow rate applied. The number of theoretical plates on the SpeedROD column, computed for the *o*-terphenyl peak was 5200 at 1 ml/min and 1900 at 9 ml/min, while on the Performance column it was 7600 at 1 ml/min and 2700 at 8 ml/min. The relationship between flow rate, retention factor (k′) and resolution was also investigated. In Fig. 4 the retention factors and

Table 7
Peak resolutions for the alkylbenzene mixture

Column	Mobile phase	Flow rate (ml/min)	Peak pairs					
			Uracil–toluene	Toluene–ethylbenzene	Ethylbenzene–butylbenzene	Butylbenzene–*o*-terphenyl	*o*-terphenyl–amylbenzene	Amylbenzene–triphenylene
SpeedROD	Initial	1	4.39	1.74	6.02	2.11	2.00	3.20
		9	2.98	1.11	4.37	1.55	1.53	2.50
		9[a]	2.74	1.23	3.85	1.27	1.22	1.82
	Adapted	1	6.02	2.68	9.60	4.50	2.09	4.69
		9	3.61	1.66	6.29	2.84	1.32	3.10
Performance		1	7.06	3.12	11.49	5.20	2.98	4.83
		8	6.87	2.88	8.62	3.41	1.79	3.21

[a] 45 °C.

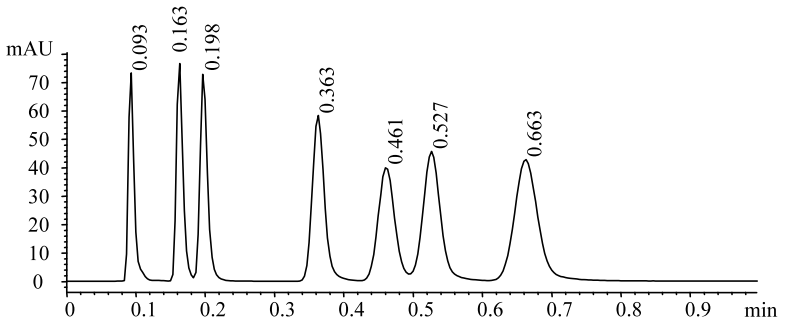

Fig. 3. Chromatogram of the alkylbenzene mixture on the SpeedROD column; T = 30 °C; λ = 254 nm; flow rate, 9 ml/min; and mobile phase MeOH/water (70/30 m/m). Elution order of the peaks; uracil, toluene, ethylbenzene, butylbenzene, *o*-terphenyl, amylbenzene, triphenylene.

resolutions obtained on the SpeedROD column are plotted as a function of the flow rate. The retention factors remained constant at all flow rates, and thus the selectivity factor (α) is not influenced by the flow rate. The resolution decreased slightly with increasing flow rate but still remained acceptable at 9 ml/min (Table 7).

The transfer of the alkylbenzene method showed that analysis times might be largely reduced by increasing the flow rate up to 9 ml/min while the performance of the column to retain and separate compounds remained fairly constant.

3.3.2. Robustness of the alkylbenzene separation

The robustness of the alkylbenzene separation on both monolithic columns was examined for four factors: the fraction of organic modifier (MeOH) in the mobile phase, the detection wavelength, the flow rate and the temperature (Table 2). Because of back pressure limitations, the robustness test on the Performance column at high flow rate was examined at 6 ml/min. A 2^{4-1} half-fraction factorial design (eight experiments, generator D = ABC) was used. Significant effects were identified using an error estimate, based on two-factor interaction effects [12,13]. Since the toluene–ethylbenzene and *o*-terphenyl/amylbenzene peaks are the worst separated (Fig. 3), their separation was first focussed on.

In Table 3, the factor effects on the resolution of toluene–ethylbenzene peaks are shown. The significant factors are the same as for the tetracycline

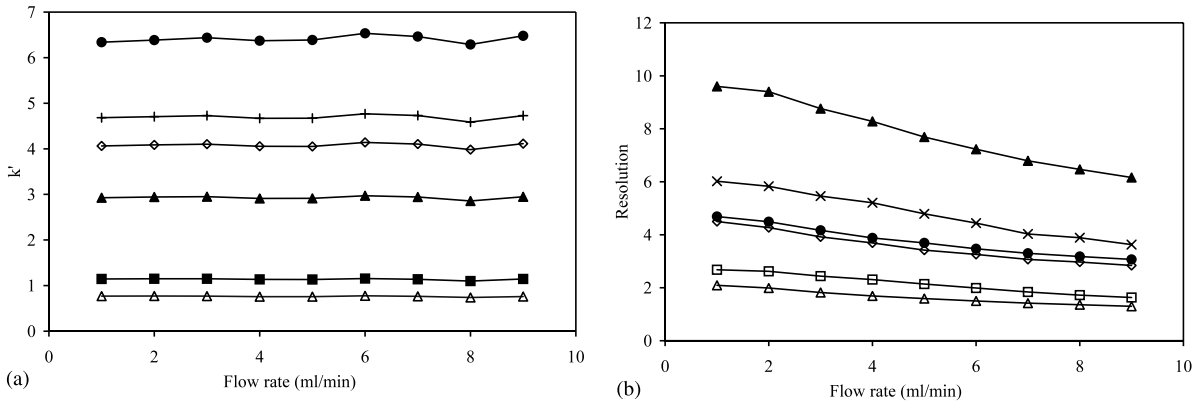

Fig. 4. (a) Retention factors and (b) resolutions of the alkylbenzene mixture compounds as a function of the flow rate. Column, SpeedROD. Legend fig a; △, toluene; ■, ethylbenzene; ▲, butylbenzene; ◇, *o*-terphenyl; +, amylbenzene; ●, triphenylene. Legend fig b; ×, Rs uracil/toluene, □, Rs toluene/ethylbenzene; ▲, Rs ethylbenzene/butylbenzene; ◇, Rs butylbenzene/*o*-terphenyl; △, Rs *o*-terphenyl/amylbenzene; ●, Rs amylbenzene/triphenylene.

and nimesulide mixtures: the fraction organic modifier and the temperature. Remarkable is that the fraction organic modifier in the mobile phase was only found to be significant at 1 ml/min. At higher flow rates, the influence of the latter factor seems to decrease. This tendency, through less pronounced, was also found in the robustness tests of the tetracycline and nimesulide mixtures. At 9 ml/min, none of the four factors influenced the resolution significantly and thus the separation is considered to be robust in the examined intervals of the factors.

Non-significance intervals were computed for the toluene–ethylbenzene resolutions. Robust separations with no significant influence of any factor would be obtained when the fraction organic modifier (m/m) and the temperature (°C) are controlled within the intervals [0.698–0.702] and [27.5–32.5] at 1 ml/min. For the modifier content this is a rather strict requirement. When a flow rate of 5 ml/min is used, the temperature should be controlled within the interval [28.1–31.9 °C] while the organic modifier, which is not significant anymore, might be varied within the interval of the design. At a flow rate of 9 ml/min no factors had a significant effect on the resolution. When the Performance column is used at a flow rate of 1 ml/min, it is advised to control the fraction organic modifier (m/m), and the temperature (°C) within the intervals [0.699–0.701] and [29.4–30.6], respectively. At a flow rate of 6 ml/min only the temperature needs to be controlled within the interval [28.7–31.3 °C]. In the above, the non-significant factors are expected to be maintained within the levels examined during the robustness test. The non-significance intervals were found to be narrower on the Performance than on the SpeedROD column. For some situations they even are predicted too small to be feasible in practice. In such situations a system suitability test might be recommended to guarantee an appropriate analysis of the method. Concerning the robustness of the separations within the examined range of the factors (Table 2), one can conclude that the effects observed on the Chromolith columns were not spectacularly different from those obtained for separations on classical columns [12,13]. However, a general tendency observed for the different separations is that the influence of organic modifier variations on the separations is reduced at higher flow rates.

3.3.3. Injection repeatability of the alkylbenzene separation

The %R.S.D. of the AUC and of the resolution was calculated for six replicate injections of the alkylbenzene mixture on both columns at low and high flow rates (Table 8). The %R.S.D. of the AUC was compared with the estimate for repeatability proposed by Horwitz et al. [15]. As can be seen in Table 8, the injection repeatability of the alkylbenzene mixture was clearly below this estimate. Relatively higher %R.S.D. values for AUC are obtained with the Performance column. The best repeatability was found on the SpeedROD column at a flow rate of 5 ml/min. Repeatability results at 9 ml/min was not considerably worse than at 1 ml/min. Also earlier we observed that intermediate flow rates might give better repeatability results than higher ones or at least comparable %R.S.D. than low flow rates.

3.4. Transfer of the phenoxymethylpenicillin, erythromycin and green tea extract methods

3.4.1. Method transfer and acceleration of the separations

The separation transfer of the phenoxymethylpenicillin, erythromycin and green tea extract mixtures to a 5 cm monolithic column was not as straightforward as for the previously discussed methods. The first transfer resulted in a chromatogram where the phenoxymethylpenicillin peak was separated from the impurities but the impurities were not baseline separated from each other. The analysis time was less than 5 min. A lack of theoretical plates might explain the unsuccessful transfer since the 5 cm column does not fulfil the minimal column length requirements of the Pharmacopoeia (25 cm $\pm$70%).

For erythromycin, two methods, i.e. an isocratic elution with ACN/phoshate buffer solution pH 7.0 (35:65 v/v) [9] and a gradient elution containing 2-methyl-2-propanol, phosphate buffer pH 7.5 and ACN were transferred [10]. To increase the number of theoretical plates, the first method for

Table 8
%R.S.D. values for (a) the AUC and (b) the resolutions for the injection of the alkylbenzene mixture ($n = 6$)

	SpeedROD			Performance, 1 ml/min	Horwitz, 1/2 %R.S.D.$_R$
	1 ml/min	5 ml/min	9 ml/min		
(*a*)					
Uracil	1.84	1.73	1.33	1.82	7.18
Toluene	3.64	0.22	0.95	2.59	4.02
Ethylbenzene	2.62	0.44	1.83	2.53	3.92
Butylbenzene	0.97	0.41	1.53	2.64	3.64
o-Terphenyl	0.55	0.26	0.99	2.54	3.51
Amylbenzene	1.11	0.58	1.51	3.16	6.30
Triphenylene	0.86	0.68	2.13	2.50	8.16
(*b*)					
Uracil–toluene	0.34	0.23	2.43	0.38	
Toluene–ethylbenzene	0.28	0.65	1.56	0.48	
Ethylbenzene–butylbenzene	0.37	0.65	1.22	0.18	
Butylbenzene–*o*-terphenyl	0.33	0.46	0.52	0.29	
o-Terphenyl–amylbenzene	0.39	0.53	< 0.005	0.27	
Amylbenzene–triphenylene	0.14	0.37	0.72	0.17	

erythromycin was also transferred to a Performance column and to a SpeedROD column coupled to a Performance one. Neither changing the mobile phase composition ratio nor increasing the column length was found to result in good enough peak separations. The transfer of the gradient elution was not successful neither.

The green tea extract mixture was analysed using gradient elution with water/MeOH and water/ACN mixtures to which trifluoroacetic acid was added [11]. No baseline separation was obtained for the polyphenols and caffeine in the extract. Here again, a change in the gradient profile did not result in acceptable separations.

Either a lack of theoretical plates or selectivity differences [3,19] between the classical and monolithic columns might be responsible for the failure of these separations. In the introduction we already mentioned that method transfer between classical silica columns neither is always successful because of selectivity differences. Therefore, it is not unexpected to obtain both successful and unsuccessful method transfers to monolithic columns. For the situation where the number of plates is too low, using a longer column or coupling several monolithic columns theoretically might overcome the problem, through we were not possible to demonstrate for one of our case studies. When selectivity differences (i.e. different elution sequence of substances as compared with the reference column) occur, additional method development becomes necessary. Since such method development was not the aim of our study, the unsuccessful transfers were not further evaluated.

3.5. Evaluation of column ageing during the case studies

The column ageing of the SpeedROD column was evaluated during its period of use. During the experiments, the column was exposed to frequent mobile phase changes (pH values varying from 3.5 to 7), varying temperatures (up to 45 °C), changing flow rates (up to 9 ml/min) and a total of more than 300 injections under very diverse conditions were made. Frequent changes in mobile phase conditions, which occurred between the different methods and during the robustness tests, are situations that especially promote column degradation [20].

The stability of the column was examined by injection of the alkylbenzene mixture twice a day at flow rates 1 and 9 ml/min during 19 and 12

days, respectively. The retention factors (k′) and resolutions were computed and plotted against the number of column volumes mobile phase pumped through the column. Fig. 5 shows that the retention factors and the resolutions remained fairly constant and only decreased slightly with increasing column age. In Table 9 the percentages decrease in retention factor and in resolution as well as the initial and end values for k′ and Rs are shown for analyses performed at flow rates of 1 and 9 ml/min. It can be seen that the percentage decrease in retention factor remains below 10% at both flow rates. The decrease in resolution also was found to be limited, considering the diversity of the conditions used on the column. Given the small decrease in resolution and retention factors, one can conclude that column degradation on the tested column was limited.

3.6. Data acquisition requirements for very fast analyses

The use of monolithic silica columns at high flow rates requires a fast data acquisition and pumping system. Fig. 3, for instance shows that the first peak (uracil, $w_{1/2} = 0.62$ s) eluted already within the first 10 s and that the entire mixture was separated within 48 s. These fast elution times require a detector and data acquisition system with a high sampling rate to be able to reconstruct the peak shape and to allow good measurements of peak parameters. Since the peak width of the uracil peak at the baseline is about 1 s, the sampling period of the data acquisition system should be chosen smaller than 100 ms, to have at least ten data points per peak. Fig. 6a shows that, particularly for early eluting peaks, sampling periods of 100 ms are not short enough to reconstruct the peak shape properly. To avoid angular peaks and to allow good measurements of peak parameters, faster detection systems are required. This can be observed in Fig. 6b were the same substance was chromatographed, but now recorded with a detector sampling time of 12.5 ms.

4. Conclusion

In this study, the transfer to monolithic C_{18} silica columns of separation methods, developed on classical C_{18} and C_8 silica columns, succeeded for three of the six selected methods. This success rate was not unexpected since the transfer of

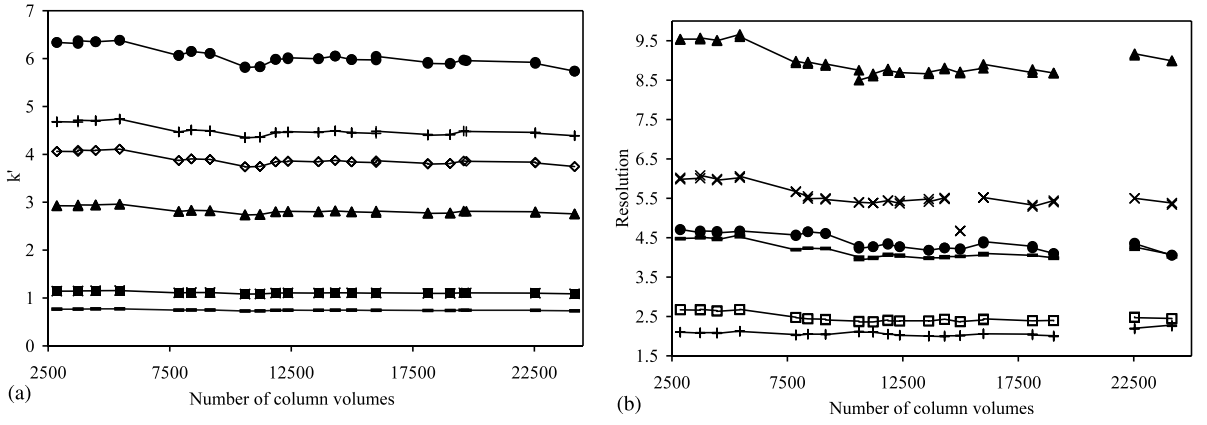

Fig. 5. (a) Retention factors and (b) resolution as a function of the number of column volumes mobile phase. Flow rate, 1 ml/min. Column, SpeedROD. Legend fig a; -, toluene; ■, ethylbenzene; ▲, butylbenzene; ◇, *o*-terphenyl; +, amylbenzene; ●, triphenylene. Legend fig b; ×, Rs uracil/toluene; ○, Rs toluene/ethylbenzene; ▲, Rs ethylbenzene/butylbenzene; -, Rs butylbenzene/*o*-terphenyl; +, Rs *o*-terphenyl/amylbenzene; ●, Rs amylbenzene/triphenylene.

Table 9
Percentage decrease in k′ and Rs and k′ and Rs values at the begin and end of the use of the SpeedROD column

Component	%Δk′ (1 ml/min)	k'_{begin}	k'_{end}	%Δk′ (9 ml/min)	k'_{begin}	k'_{end}
Toluene	5.19	0.77	0.73	7.89	0.76	0.70
Ethylbenzene	4.39	1.14	1.09	7.89	1.14	1.05
Butylbenzene	5.80	2.93	2.76	7.82	2.94	2.71
o-Terphenyl	7.64	4.06	3.75	8.54	4.10	3.75
Amylbenzene	6.20	4.68	4.39	8.25	4.73	4.34
Triphenylene	9.32	6.33	5.74	7.75	6.45	5.95
Peak pairs	%Δ Rs (1 ml/min)	Rs_{begin}	Rs_{end}	%Δ Rs (9 ml/min)	Rs_{begin}	Rs_{end}
Uracil–toluene	11.30	6.02	5.34	38.59	3.55	2.18
Toluene–ethylbenzene	8.61	2.67	2.44	32.91	1.58	1.06
Ethylbenzene–butylbenzene	5.67	9.53	8.99	1.81	6.07	5.96
Butylbenzene–*o*-terphenyl	10.29	4.47	4.01	7.02	2.85	2.65
o-Terphenyl–amylbenzene	7.14	2.10	2.25	0	1.32	1.32
Amylbenzene–triphenylene	13.62	4.70	4.06	2.63	3.04	2.96

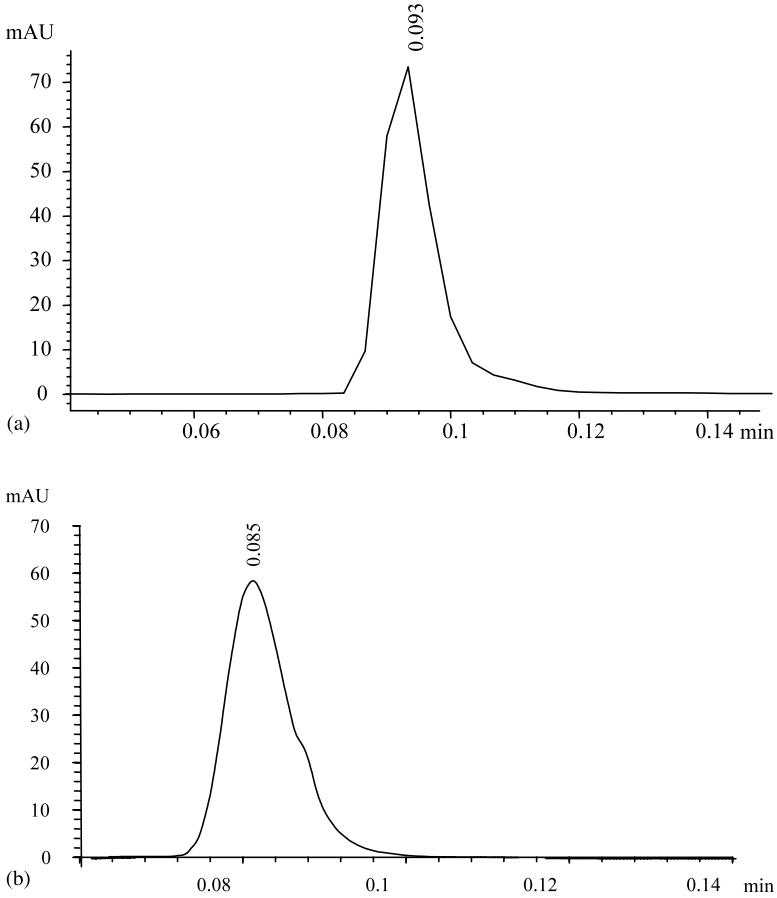

Fig. 6. Enlargement of the uracil peak from Fig. 3 recorded with a detector sampling rate of (a) 100 ms and (b) of 12.5 ms.

separations between classical C_8 and C_{18} silica columns is not always straightforward neither. By increasing the flow rate from 1 to 9 ml/min the analysis time of the successfully transferred separations could be decreased up to a factor 9. In order to obtain good separations at high flow rates, adjustments in the mobile phase solvent strength might be required. Transfer of the methods at low flow rates already results in a considerable gain in time compared with similar conditions on a classic column. The performance of the method at low or intermediate flow rate might, in a number of situations, be preferred since repeatability can be better, or the additional gain in analysis time at higher flow rates is not always pronounced. Moreover, under these conditions one does not force the equipment (both column, pump and detector) to work at its limits.

The transferred separations to the SpeedROD and Performance columns were also found to be robust. However, factors as the fraction organic modifier, the temperature and the pH might need to be controlled in rather strict intervals. The injection repeatability was found to be good, also at high flow rates, confirming the rather robust character of the separations. The SpeedROD column was also tested for time effects, and only a limited column ageing was found during the use of the column.

Acknowledgements

Anne-Marie van Nederkassel is an aspirant and Y. Vander Heyden is a postdoctoral fellow of the Fund for Scientific Research (FWO)-Flanders. The authors thank Professor J. Hoogmartens (Catholic University Leuven) for the gift of nimesulide, tetracycline, erythromycin and phenoxymethylpenicillin samples. The authors are grateful to K. Decq and A. De Schrijver for technical assistance.

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