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Ruggedness tests on the high performance liquid chromatography assay of the United States Pharmacopeia 23 for tetracycline·HCl: comparison of different columns in an interlaboratory approach[1]

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Abstract

Ruggedness tests were performed on the United States Pharmacopeia assay for tetracycline·HCl to examine problems previously reported in the literature. The experiments were performed on nine different columns. The effects of four factors selected from the procedure were examined on qualitative responses by performing half-fraction factorial designs at three different ages on each column. The influence of column ageing was separately evaluated by injections under nominal method conditions at different ages. The C-8 columns gave a separation that was as good as, or better than, the C-18 ones and were less influenced by ageing. The normalized effects of each of the factors on a response were compared and found to be more or less equal for most of the columns and to remain constant with time. The responses were most affected by the pH of the mobile phase and by the content of the organic modifier. In general it was found that C-8 columns were preferred for this assay.

Keywords: Chemometrics; Fractional factorial designs; High performance liquid chromatography; Ruggedness tests

1. Introduction

The high performance liquid chromatography (HPLC) assay of the United States Pharmacopeia 23 (USP) for tetracycline·HCl [1] cannot always be performed without problems. Problems were described for the separation of tetracycline (TC) and 4-epianhydrotetracycline (EATC) [2] with the pressure caused by the flow rate given in the USP [2,3] and with degradation of the prescribed C-8 stationary phase [3]. It was claimed that C-18 columns perform better for this assay than C-8 columns [2,3].

To obtain a more general perception of the above-mentioned problems it was decided to perform ruggedness tests on nine different columns.

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The experiments were carried out in three different laboratories. This study is however not an interlaboratory comparison of columns. It is only a study of method ruggedness performed in different laboratories.

The aim of this study was to examine the influence of four factors selected from the method description on a number of responses. The influence of these factors was examined on each of the columns as a function of column age.

2. Theory

The four factors that were selected (Table 1) were examined in a half-fraction factorial design with resolution IV, 2^{4-1} (IV) [4]. The generator for the design was $D = ABC$ which gives the defining relation $I = ABCD$. The resulting design is shown in Table 2. For each factor three levels were considered: a nominal one and two extremes. The nominal level (level 0) is the one specified in the procedure of the USP. The extreme levels deviate from the nominal one. Only the extreme levels were examined in the fractional factorial design.

A number of responses (Y) were measured, where possible, for 4-epitetracycline (ETC), 4-epianhydrotetracycline (EATC), tetracycline (TC), anhydrotetracycline (ATC), chlortetracycline (CTC) and 2-acetyl-2-decarboxamidotetracycline (ADTC). The responses retention time, capacity factor, relative retention (selectivity factor) and resolution were determined. The resolution was calculated for all experiments and for all peaks as

$$R_s = \frac{1}{4}\cdot\left(\frac{k'_2}{k'_1} - 1\right)\cdot\sqrt{N_p}\cdot\left(\frac{k'_1}{k'_1 + 1}\right) \quad (1)$$

where R_s is the resolution between two peaks, k'_1 and k'_2 are the capacity factors of the first and second peaks respectively and N_p is the number of theoretical plates of the column. The value for N_p was calculated from the peak of TC. The formula usually applied for resolution

$$R_s = \frac{2(t_2 - t_1)}{W_1 + W_2} = \frac{1.177(t_2 - t_1)}{[(W1/2)_1 + (W1/2)_2]} \quad (2)$$

was not used because in previous experiments [3] it was seen that the peak width at half height ($W1/2$) could not always be determined for all peaks while N_p for the TC peak could always be calculated.

The effect of each factor on a response was calculated as

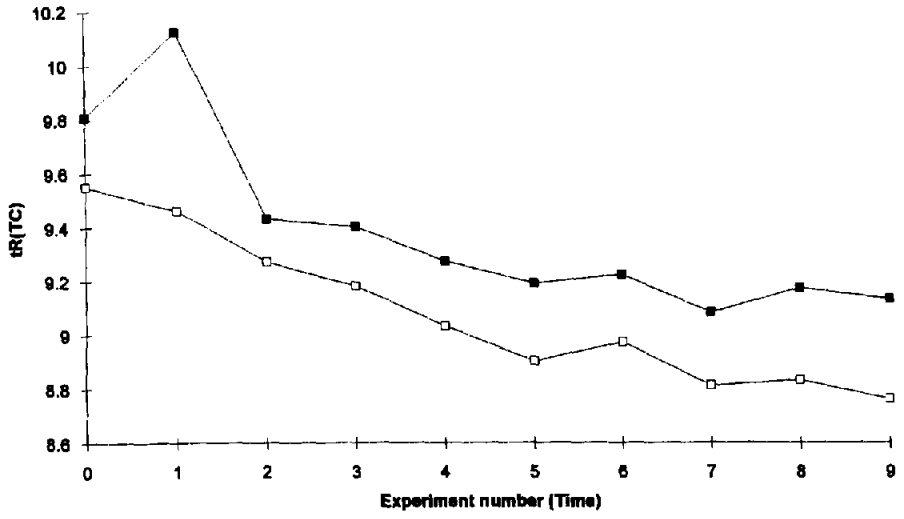
$$E_X = \frac{\sum Y(+)}{N/2} - \frac{\sum Y(-)}{N/2} \quad (3)$$

Table 1
Specifications for the factor levels. Level 0 is the nominal level; levels −1 and +1 are the extreme levels

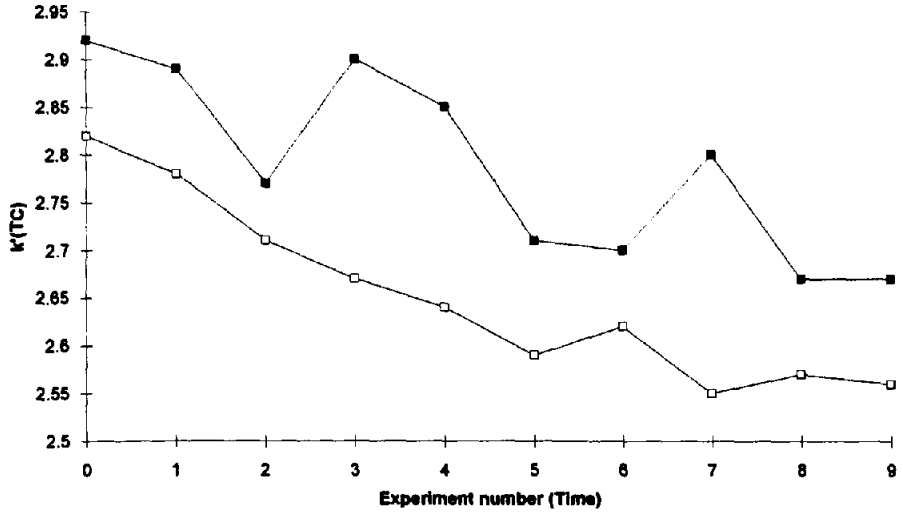
Factor	Level	Value of the factor	Amount of water added (ml)
(a) M(ammonium oxalate) / M(ammonium phosphate)	−1	0.19 / 0.38	
	0	0.20 / 0.40	
	1	0.21 / 0.42	
(b) DMF (ml)	−1	260	375
	0	270	365
	1	280	355
(c) pH	−1	7.45	
	0	7.65	
	1	7.85	
(d) Flow (ml min^{-1})	−1	0.9	
	0	1.0	
	1	1.1	

Table 2
Half-fraction factorial design for four factors: 2^{4-1} (IV) with generator $D = ABC$. The columns of contrast coefficients for the two-factor interactions are also shown

Exp.	Factor				Interaction		
	A	B	C	D	$AB+CD$	$AC+BD$	$AD+BC$
1	−	−	−	−	+	+	+
2	+	−	−	+	−	−	+
3	−	+	−	+	−	+	−
4	+	+	−	−	+	−	−
5	−	−	+	+	+	−	−
6	+	−	+	−	−	+	−
7	−	+	+	−	−	−	+
8	+	+	+	+	+	+	+



(a)



(b)

Fig. 1. Effect of column ageing on (a) nominal retention time; (b) nominal capacity factor of TC. Columns: Alltima C-8 of the same batch. Time interval between experiments 0 and 9:4 weeks. Instrument 1 (□): Set-up in which the columns are rinsed with water. Instrument 2 (■): Set-up in which mobile phase was pumped over the column overnight at a flow rate of 0.2 ml min^{-1}.

where E_X is the effect of factor X on response Y when factor X is changed from level (+) to level (−), $\Sigma Y(+)$ and $\Sigma Y(-)$ are the sums of the responses when factor X is at level (+) or (−) respectively and N is the number of experiments in the design. The effects were normalized by dividing the effect by the mean nominal response and multiplying by 100:

$$\%E_X = \frac{E_X}{\bar{Y}_n} \cdot 100 \quad (4)$$

where $\bar{Y}_n$ is the mean nominal response obtained at the same age of the column for which E_X was also determined.

To distinguish significant effects a statistical method was used. The statistical method was a t-test method [5–7]. A factor is statistically signifi-

cant if the t-test statistic is above a critical value

$$t = \frac{|E_X|}{(SE)_e} \geq t_{critical} \quad (\alpha = 0.05) \tag{5}$$

or if the effect of the factor is larger than a critical effect value, $|E_X| \geq E_{critical}$ with $E_{critical} = t_{critical} \cdot (SE)_e$ where $(SE)_e$ is the standard error on E_X. The standard error $(SE)_e$ was estimated from the effects of the two-factor interactions ($E_{X_iX_j}$) [4]:

$$(SE)_e = \sqrt{\frac{\sum E^2_{X_iX_j}}{n_{X_iX_j}}} \tag{6}$$

where $n_{X_iX_j}$ is the number of two-factor interaction effects used and $t_{critical} = t_{n_{X_iX_j}}$ is the tabulated t value with $n_{X_iX_j}$ degrees of freedom. The two-factor interaction effects, $E_{X_iX_j}$, were calculated analogously to the main factor effects, E_X, by using the columns of contrast coefficients (see Table 2). This interpretation method was demonstrated to give reliable results ([3]; unpublished information).

3. Experimental

3.1. Test solutions

The assay solution of the USP is a solution containing 500 mg l^{-1} TC·HCl in diluting solvent (ammonium oxalate (0.1 M)–dimethylformamide (680:270, v/v)). The design was performed on three test solutions. They were prepared from three solid samples. The samples consisted of: (a) a mixture of 16% ETC, 12% EATC, 20% TC and 52% ATC; (b) a mixture of TC containing 3% ETC, 2% EATC, 0.9% ADTC and 3% ATC and (c) a sample containing chlortetracycline (CTC). The test solutions (a) and (b) were prepared by dissolving mixtures (a) and (b) respectively in diluting solvent to a concentration of 500 mg l^{-1}. For test solution (c) the concentration was 400 mg l^{-1}. The test solutions were prepared daily in volumetric flasks of brown glass or were wrapped in an aluminium sheet to avoid deterioration. For the same reason the flasks were kept in the dark, at room temperature, between the injections.

3.2. Nominal chromatographic conditions [1]

The mobile phase consists of ammonium oxalate (0.1 M)–dimethylformamide–dibasic ammonium phosphate (0.2 M) (680:270:50, v/v/v) adjusted to pH 7.6–7.7 with ammonium hydroxide (3 N) or phosphoric acid (3 N), filtered through a membrane filter of 0.5 μm or finer porosity and degasified. The column consists of a 4.6 mm i.d. × 3 cm guard column (10 μm packing C-8) and a 4.6 mm i.d. × 25 cm analytical column (5–10 μm packing C-8). The flow rate of the mobile phase is 2 ml min^{-1} and the loop/injection volume is 20 μl. The column effluent is measured with a UV detector at a wavelength of 280 nm. Experiments are carried out at room temperature.

The flow rate was reduced to 1 ml min^{-1} because problems with too high a pressure occurred in previous experiments [3].

3.3. Materials

TC, ETC, EATC, ATC and CTC were obtained from Acros Chimica (Beerse, Belgium). The three laboratories will be referred to as labs A, B and C. ADTC and the sample mixtures were provided by lab. A. Ammonium oxalate monohydrate (Riedel-de Haën, Seelze, Germany (lab. A); Merck, Darmstadt, Germany (labs. B and C)), dimethylformamide (Acros Chimica (lab. A); Riedel-de Haën (lab. B); Merck (lab. C)), dibasic ammonium phosphate (Merck), ammonium hydroxide (28–30%: Acros Chimica (lab. A); 25%: Merck (labs. B and C)), and phosphoric acid 85% (Acros Chimica (lab. A); Merck (labs. B and C)) were all of pro analysi (GR) quality. Water was obtained with the milliQ system (Millipore, Bedford, MA). The pH of solutions was measured with a Consort P514 pH meter (Turnhout, Belgium) (lab. A), a ψ43 Beckman pH meter (Beckman, San Ramon, CA) (lab. B) and a WTW Microprocessor pH meter pH537 (WTW, Weilheim, Germany) (lab. C). The mobile phase was filtered through a membrane filter and degasified in an ultrasonic bath. The HPLC pumps were a L-6200 (Merck-Hitachi) (lab. A) and a Model 625 Multisolvent LC System (Millipore-Waters, Milford, MA) (lab. B), both equipped with a Marathon autosampler (Spark Holland, Emmen, The Netherlands), and a Varian

Model 5000 Liquid Chromatograph (Varian, Palo Alto, CA) equipped with a Rheodyne injector (Cotati, CA) (lab. C).

The columns used were (1) C-8 columns: (a) Rosil 5 μm (Bio-Rad, Eke, Belgium) (lab. A), (b) Alltima 5 μm (Alltech, Deerfield, IL) (lab. B), (c) Adsorbosphere 5 μm (Alltech) (lab. C); (2) C-18 columns: (a) Partisil ODS-3 10 μm (Whatman, Clifton, NJ) (lab. A), (b) Ultrasphere ODS 5 μm (Beckman) (lab. C), (c) Hypersil ODS 10 μm (Alltech) (lab. B), (d) LiChrosorb 5 μm (Merck) (lab. C); and (3) protected columns: (a) Chromspher 5B 5 μm (Chrompack, Middelburg, The Netherlands) (lab. A) and (b) Spherisorb (pH stable) S5 PC18 (PhaseSep, Queensbury, UK) (lab. C). The protected columns are claimed to be pH stable at high pH. The dimensions of the columns were 250 mm × 4.6 mm i.d. A guard column was placed just in front of the analytical column. Within each lab. an identical type of guard column was used for all three analytical columns. The guard columns used in the different labs. were 50 mm × 4.6 mm i.d. Rosil (Bio-Rad) C8 8 μm (lab. A), 30 mm × 4.6 mm i.d. Hypersil (Shandon, Runcorn, UK) MOS-1 C8 10 μm (lab. B) and 30 mm × 4.6 mm i.d. LiChrosorb (Merck) RP8 10 μm (lab. C).

The effluent of the columns was measured with a Merck-Hitachi L-4000 UV detector (lab. A), a 990 Diode Array Detector (Millipore-Waters) (lab. B) and a Merck-Hitachi L-4200 UV–Vis detector (lab. C). The peaks were integrated using a HP3396A (Hewlett-Packard, Avondale, PA) integrator (lab. A), a Waters 5200 Printer Plotter plus a NEC Powermate 386/33i Data Station (lab. B) and a Merck-Hitachi D-2000 Chromato-Integrator (lab. C).

4. Results and discussion

4.1. The ruggedness experiments

The ruggedness tests were performed on three types of column: three C-8 columns; four C-18 columns and two protected ones. The C-8 columns were studied because this is the type of column prescribed by the USP; the C-18 columns because better results than with the C-8 columns were claimed [2,3] and the protected columns because it was hoped that they would degrade less than the other columns because they were claimed to be stable against the rather high pH of the mobile phase.

Four factors were selected from the analytical procedure to be examined with the different columns. The factors were investigated in the 2^{4-1} (IV) half-fraction factorial design shown in Table 2. The factors selected to be examined in the design were (a) the amounts of inorganic salts in the mobile phase (± ionic strength); they were changed at different levels in such a way that the ratio M(ammonium oxalate)/M(dibasic ammonium phosphate) remains as 0.5, the ratio of the moles remains 6.8; (b) the amount of dimethylformamide (DMF) in the mobile phase; (c) the pH of the mobile phase; and (d) the flow of the mobile phase. The levels for the factors used in the design are given in Table 1. For practical reasons the nominal mobile phase was prepared by mixing ammonium oxalate (0.20 M)–dimethylformamide –dibasic ammonium phosphate (0.40 M)–water (340:270:25:365, v/v/v/v) to yield the same mobile phase as the one described by the USP. This way of preparing the mobile phase allowed one to keep the concentrations of the inorganic salts and the final volume of the mobile phase constant even when the volume of DMF needs to be

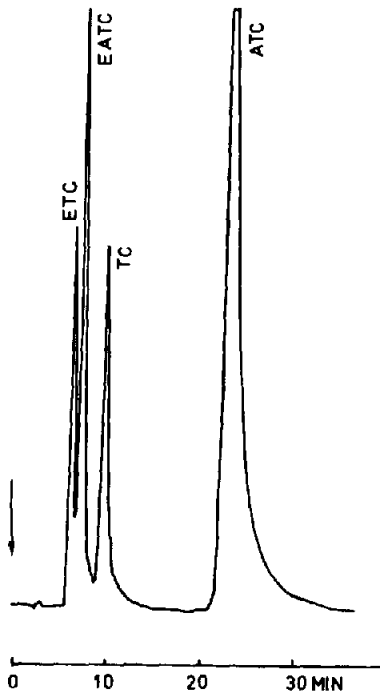
Table 3
The ages of the columns, expressed as the number of column volumes

Type	Column make	Age (column volumes)		
		t_0	t_1	t_2
C-8	Alltima	412	3471	6488
	Adsorbosphere	326	3469	6529
	Rosil	278	4670	11087
C-18	Ultrasphere ODS	580	4329	8078
	Hypersil ODS	428	3559	6690
	LiChrosorb	481	3995	7315
	Partisil ODS-3	265	6256	11232
Protected	Spherisorb	409	4472	8495
	Chromspher	199	4900	10048

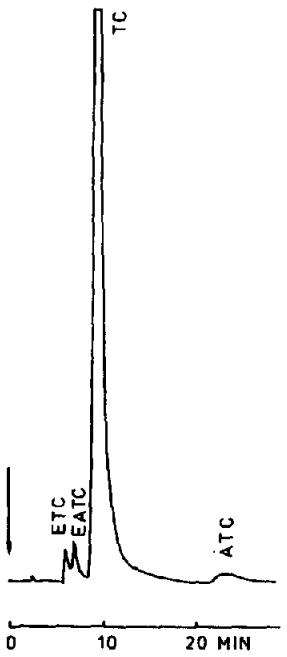
changed during the performance of the design (see Table 1). The preparation of the mobile phases, the pH measurements and the adjusting of the pH were standardised for the three laboratories. The pH meter was calibrated before adjusting the pH of a solution. Within one lab. all experiments were performed by the same operator.

On each column the design was performed at three different ages of the column (represented by t_0, t_1 and t_2). The design was executed in the shortest time possible. When changing the chromatographic conditions between two experiments the system was stabilized for at least 20 min prior to the first injection. If the experiments of one design took more than 1 day the flow was stopped overnight after rinsing the column with milliQ water for at least 20 min at nominal flow rate. This was done to minimize the ageing of the colum within the duration of a design. However, in later experiments, it was observed that the ageing was similar when working in this way as when pumping mobile phase at low flow rate (0.2 ml min^{-1} overnight through the column (see Fig. 1). Between the execution of the different designs on one column it was intended to age the column by pumping mobile phase under nominal conditions through it for a period of about 110 h (about $4\frac{1}{2}$ consecutive days) without recycling the mobile phase. The age of a column (t_0, t_1 or t_2) was expressed as the average number of column volumes of mobile phase that have run through the column between the start and end of the experiments at that specific age plus the number of column volumes already run through it before the beginning of the experiments at that age. The designs at the different ages were performed for each of the three test solutions. The test solutions were also injected under nominal conditions three times before and after the design. All columns were new at the beginning of the experiments. The different ages of the columns, expressed as column volumes, are shown in Table 3.

The effect of the ageing of the column was not examined as a factor of the design, as was done earlier [3], to avoid possible masking of the effect of other factors by a potentially large degradation of the used columns. The influence of ageing on the different responses can be evaluated from the



(a)



(b)

Fig. 2. (a) Chromatogram of test solution (a) injected under nominal conditions on the new C-8 Adsorbosphere column. (b) Chromatogram of test solution (b) injected under nominal conditions on the new C-8 Adsorbosphere column.

Table 4

(a) Average results and relative standard deviations under nominal conditions and at time t_0 for the different qualitative responses. (b) Repeatability results (expressed as %RSD) for each response for the different columns. (c) Reproducibility results obtained with one type of column.

(a)

Column	Average nominal results at time t_0 ($n = 6$)												
	k'					α			R_s			Asf	
	ETC	EATC	TC	ATC	CTC	EATC	TC	ATC	ETC–EATC	EATC–TC	TC–ATC	TC	CTC
C8-Alltima	1.62	2.30	3.37	9.97	7.24	1.42	2.08	6.16	4.02	4.94	23.12	1.10	0.94
C8-Adsorbosphere	1.27	1.56	2.38	6.81	5.33	1.23	1.89	5.39	1.39	3.40	13.92	1.86	1.45
C8-Rosil	1.69	2.18	3.18	11.24	7.64	1.29	1.88	6.65	2.04	3.56	21.73	1.80	1.45
C18-Ultrasphere	1.82	2.52	4.13	16.26	10.78	1.39	2.28	8.94	3.23	5.75	30.98	1.20	0.90
C18-Hypersil	1.53	2.17	4.17	12.30	9.06	1.42	2.72	8.03	2.97	7.47	18.60	1.33	1.21
C18-LiChrosorb	1.41	1.80	2.95	7.96	6.42	1.28	2.09	5.59	2.02	5.26	15.74	1.61	1.75
C18-Partisil	2.09	2.58	4.09	11.16	9.06	1.23	1.95	5.31	1.43	3.82	12.58	1.35	1.10
Prot-Spherisorb	0.77	0.77	1.22	2.99	2.59	1.00	1.58	3.87	0.00	1.73	5.35	3.61	1.94
Prot-Chromspher	1.78	1.78	2.87	6.37	6.13	1.00	1.62	3.59	0.00	3.95	9.05	1.85	1.45
%RSD	24.46	28.78	30.49	41.21	33.75	12.77	17.23	29.46	72.37	37.13	46.48	43.20	25.98

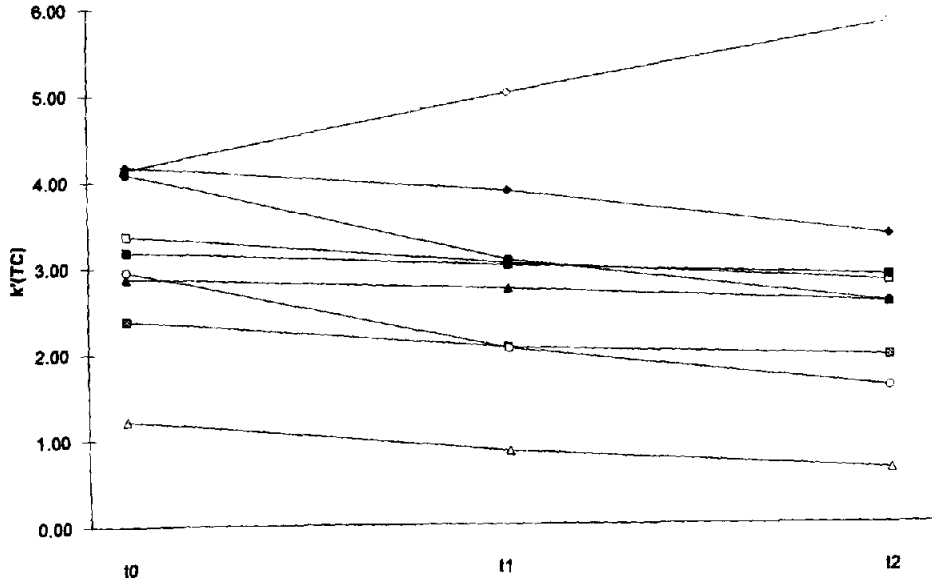
(b)

Column	Relative standard deviaiton (%RSD) under repeatability conditions ($n = 2 \times 3$)										
	k'					α			R_s		
	ETC	EATC	TC	ATC	CTC	EATC	TC	ATC	ETC–EATC	EATC–TC	TC–ATC
C8-Alltima	0.89	1.22	0.97	1.52	0.91	0.36	0.17	0.84	1.16	2.28	1.74
C8-Adsorbosphere	1.21	1.43	1.53	2.16	1.69	0.47	0.38	0.95	3.47	2.84	3.09
C8-Rosil	0.28	0.16	0.21	0.47	0.32	0.25	0.34	0.69	1.88	1.62	1.83
C18-Ultrasphere	0.66	1.11	0.58	1.30	0.48	0.66	0.25	0.85	3.32	0.66	1.83
C18-Hypersil	0.65	0.86	0.78	1.29	0.70	0.26	0.23	0.62	1.08	1.16	0.96
C18-LiChrosorb	2.01	2.85	2.29	7.99	3.03	0.90	1.11	5.47	3.74	5.61	2.99
C18-Partisil	0.37	0.45	0.67	1.68	1.11	0.13	0.43	1.54	1.28	1.41	2.27
Prot-Spherisorb	1.76	1.76	1.34	2.89	1.77	0.00	0.52	0.97	[a]–	3.07	1.49
Prot-Chromspher	0.33	0.33	0.17	0.28	0.15	0.00	0.18	0.50	[a]–	0.87	0.82

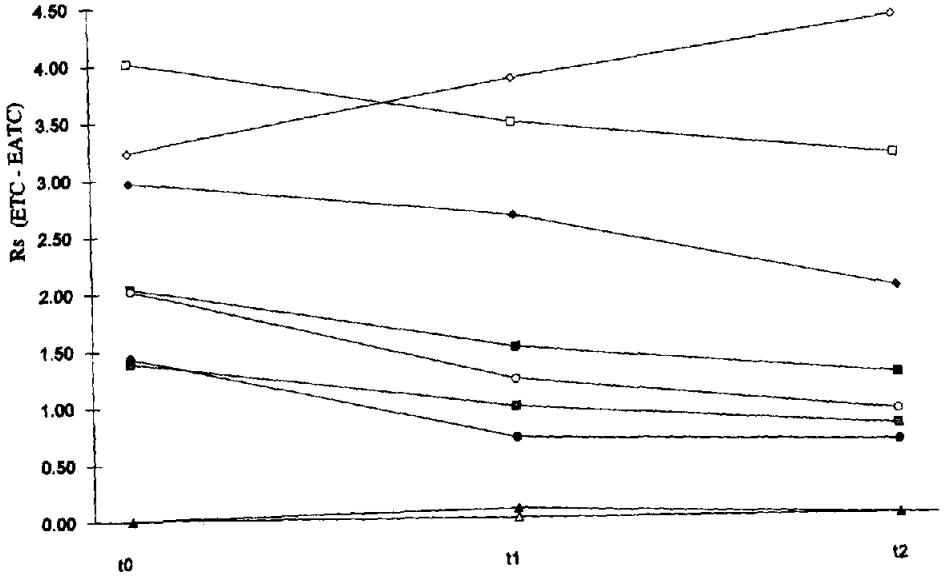
(c)

Column	Relative standard deviation (%RSD) under reproducibility conditions ($n = 3 \times 6$)										
	k'					α			R_s		
	ETC	EATC	TC	ATC	CTC	EATC	TC	ATC	ETC–EATC	EATC–TC	TC–ATC
C8-Alltima	[a]–	6.76	6.69	[a]–	[a]–	2.78	3.68	[a]–	[a]–	15.76	[a]–

[a] Could not be or was not determined



(a)



(b)

Fig. 3. (a) Capacity factor of TC. (b) Resolution between ETC and EATC under nominal conditions and at different ages. The results presented are the averages of the nominal injections performed before and after each design: □, C8-Alltima; ■, C8-Adsorbosphere; ■, C8-Rosil; ◇, C18-Ultrasphere; ◆, C18-Hypersil; ○, C18-LiChrosorb; ●, C18-Partisil; △, Prot-Spherisorb; ▲, Prot-Chromspher.

Table 5
The average nominal results and the average design results for the capacity factors of TC and EATC at the different column ages: %Δ(0–1) = relative change between t_0 and t_1; %Δ(0–2) = relative change between t_0 and t_2

Column	Average nominal results			Relative decrease		Average design results			Relative decrease	
	t_0	t_1	t_2	%Δ(0–1)	%Δ(0–2)	t_0	t_1	t_2	%Δ(0–1)	%Δ(0–2)
Effect of ageing on k'(TC)										
C8-Alltima	3.37	3.04	2.82	−9.87	−16.16	3.29	3.04	2.99	−7.84	−9.37
C8-Adsorbosphere	2.38	2.06	1.95	−13.65	−18.19	2.24	2.03	1.91	−9.27	−14.58
C8-Rosil	3.18	3.01	2.88	−5.22	−9.34	3.05	3.02	2.81	−0.98	−7.97
C18-Ultrasphere	4.13	5.02	5.83	21.38	40.89	4.59	4.47	4.38	−2.65	−4.44
C18-Hypersil	4.17	3.87	3.36	−7.02	−19.35	3.80	3.50	3.54	−7.82	−6.87
C18-LiChrosorb	2.95	2.05	1.59	−30.58	−46.13	2.93	2.00	1.53	−31.79	−48.02
C18-Partisil	4.09	3.07	2.57	−24.79	−37.09	3.77	2.90	2.34	−23.12	−37.78
Prot-Spherisorb	1.22	0.86	0.64	−29.59	−47.67	1.23	0.86	0.64	−29.69	−47.55
Protect-Chromspher	2.87	2.74	2.57	−4.40	−10.32	2.87	2.71	2.50	−5.46	−13.09
Effect of ageing on k'(EATC)										
C8-Alltima	2.30	2.09	1.99	−9.31	−13.47	2.24	2.09	2.09	−6.59	−6.99
C8-Adsorbosphere	1.56	1.38	1.25	−11.86	−19.87	1.50	1.37	1.28	−8.92	−14.58
C8-Rosil	2.18	1.99	1.93	−8.39	−11.38	2.10	2.05	1.94	−2.48	−7.82
C18-Ultrasphere	2.52	2.58	2.97	2.30	17.66	2.57	2.48	2.65	−3.26	3.41
C18-Hypersil	2.17	2.02	1.83	−6.71	−15.40	2.08	1.91	1.96	−7.82	−5.62
C18-LiChrosorb	1.80	1.33	1.02	−25.86	−43.37	1.83	1.33	1.04	−27.40	−43.56
C18-Partisil	2.58	1.98	1.69	−23.15	−34.40	2.39	1.88	1.59	−21.05	−33.55
Prot-Spherisorb	0.77	0.53	0.40	−30.80	−48.37	0.78	0.55	0.41	−29.03	−46.94
Protect-Chromspher	1.78	1.71	1.58	−3.65	−11.20	1.76	1.70	1.57	−3.13	−10.59

measurements at the nominal level at the different ages of the column.

4.2. Results at nominal level

Injection of test solution (a) yielded, on each new column, a resolution between EATC and TC larger than 1.2 as required by the USP. A chromatogram obtained from solutions (a) and (b) on the C-8 Adsorbosphere column is shown in Fig. 2 Since only qualitative responses describing the quality of the separation of the substances were examined and since solutions (a) and (b) had similar results for these responses, only the results for the solutions (a) and (c) will be discussed in more detail later.

It could be argued that in the interpretation of the results at the nominal level the influence of the different columns is not always separated from the influence of the different laboratories. Basically this is true but since only qualitative responses (capacity factors, resolutions, relative retentions) were considered it is assumed that observed differences are mainly due to differences in columns and much less to the influence of the laboratories. This hypothesis is also confirmed by the results shown in Table 4(c). It can be observed that the variance or the %RSD caused under reproducibility conditions (three columns of one batch were tested in the three labs. at three different days with two replicates) is considerably smaller than that obtained from the comparison of the columns (see Table 4(a)). Even without testing statistically it can be assumed that the conclusion drawn by comparing the results of different columns is due to the columns and not to inter-laboratory differences.

The capacity factors (k') obtained on the C-18 columns for the TC (see Fig. 3a) and CTC peaks were in general higher than those on the C-8

columns (see Table 4). However, within each type of column there was a rather large variation in the results (see Table 4). For the other substances (ETC, EATC and ATC) no clear difference could be observed in the capacity factors obtained on the C-8 and C-18 columns (see Table 4). The protected Spherisorb column had noticeably smaller k' values than all other columns.

With ageing of the columns the capacity factors decreased, as one would normally expect [8], with the excepton of those on the C-18 Ultrasphere column (see Fig. 3(a)). The decrease in the capacity factors is caused by deterioration (degradation) of the stationary phases [8]. This degradation can be expected to increase even more when one is not working in the pH interval between 2.0 and 7.5 [8,9], as is the case in this assay. Therefore the results of the C-18 Ultrasphere column (see Fig. 3(a)) were bizarre; moreover because the average result at the nominal level and the average result of the design were not similar for this column. For all other columns both results were comparable, as expected, and had the same tendency, i.e. decreasing, with ageing (see Table 5). For the C-18 Ultrasphere column, however, nominal results were measured that increased with ageing while the design averages remained constant. The relative decrease was in general larger on the C-18 columns (see Table 5). This is the opposite to what was observed in Ref. [3], but there only one column of each type was examined.

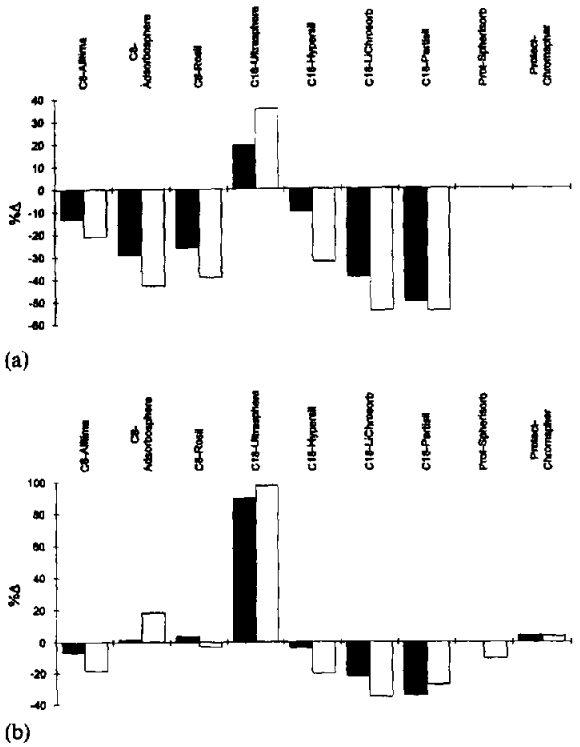


Fig. 4. The relative change in resolution with ageing of the columns: (a) for R_s(ETC–EATC); (b) for R_s(EATC–TC). ■, % Difference $(t_0 - t_1)$; □, % Difference $(t_0 - t_2)$.

The protected columns, applied because it was thought that they would degrade less, showed a similar or even larger (Spherisorb column) degradation than the other columns.

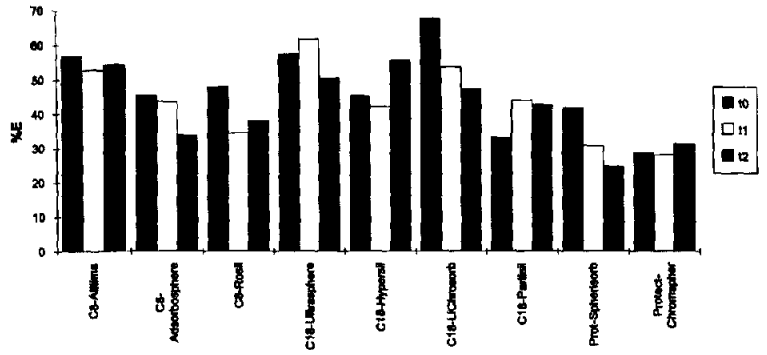
A large variation in the resolution of two adjacent peaks was observed on the different columns (see Fig. 3(b)). The protected columns did not allow separation of ETC and EATC and this is also the case on the new columns. Therefore they are not useful for this application. Between C-8 and C-18 columns no real difference was found for resolution. The C-8 Alltima column was the one that allowed the best separation between ETC and EATC. It also performed well for the separation of the other substances.

With ageing of the columns the resolution between all peak pairs decreased on the C-18 columns, with the exception of the C-18 Ultrasphere. On the C-8 columns the resolution decreased for ETC–EATC (Fig. 4(a)) and for TC–ATC (not shown), but somewhat less than on the C-18 columns, while for EATC–TC (Fig. 4(b)) it remained almost constant. The relative decrease was in general again larger on the C-18 columns. The relative retention (selectivity factor) was, for all peaks, calculated with the ETC peak as reference. The results observed were completely analogous to those for resolution.

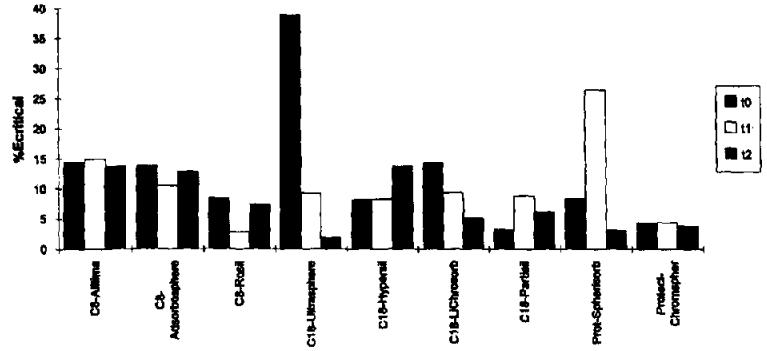
The asymmetry factor (tailing factor) was determined under nominal conditions for the TC and CTC peaks according to the USP 23,

$$\text{Asf} = \frac{W_{0.05}}{2f} \tag{7}$$

where $W_{0.05}$ is the width of the peak at 5% height and f is the distance from the peak maximum to



(a)



(b)

Fig. 5. (a) The normalized effects of the pH on the resolution between EATC and TC at the different column ages and on the different columns. (b) Normalized critical effects ($\alpha = 5\%$) on the resolution between EATC and TC at the different column ages and on the different columns.

the leading edge of the peak measured at 5% of the peak height from the baseline. The Asf for both substances was very large on the Spherisorb column. In general the Asf of TC was better on the C-18 columns than on the C-8 columns, except for the C-8 Alltima column. The latter gave very good Asf values for both TC and CTC peaks. With ageing of the columns the Asf remained quite constant, both for TC and CTC on all columns with the exception of the C-18 LiChrosorb and the protected Spherisorb columns for which the Asf decreased, i.e. became better.

4.3. Calculation and comparison of effects

The effect of the factors on the retention time, the capacity factor, the relative retention with reference to ETC and the resolution between consecutive peaks was calculated. The responses were determined for ETC, EATC, TC, ATC and CTC, whenever possible. On none of the columns was ADTC clearly separated from the other substances. On the Ultrasphere column, and also during experiments on other columns not described here, a small peak was sometimes observed between EATC and TC. This peak is thought to correspond to demethyltetracycline (DMTC). Because DMTC and ADTC are mostly co-eluting with other substances it is not interesting to consider the effects on quantitative responses (contents) or on peak areas.

The effects were normalized with reference to the mean nominal result measured on the specific column and at the column age at which the effects were calculated. A normalized effect of a factor equal to 30% means that by changing the factor from (+) level to (−) level the response changes

by 30% compared to the nominal response. The normalized effects were plotted in graphs such as Fig. 5(a). Similar plots were also made for the normalized critical effects (Fig. 5(b)). These plots allowed one to observe the occasional change of an effect as a function of time as well as to compare the effect of a factor on the different columns. From these plots it could be observed that the effect of a factor remained more or less constant in time even when the response (e.g. retention time, resolution) changed with ageing of the column (see Figs. 1, 3 and 4). It was also seen that the effect of a factor was more or less the same for most columns. However, some columns showed a different effect for certain responses. These exceptions will be discussed further.

The normalized critical effects at a given level of confidence (e.g. 5%) could also be considered to be more or less the same for the majority of the columns. Again there were some exceptions. From Fig. 5(b) it can be observed that some critical effects were very high while others were very low, sometimes even for the same column. This was due to the fact that the critical effect was estimated from only three two-factor interactions, i.e. with a relatively low number of degrees of freedom. Considering the fact that for most of the responses the critical effects remained constant at the different ages, for the statistical interpretation one critical effect per column was calculated from the nine two-factor interactions determined at the different ages.

4.4. Interpretation of the results from the designs

For the capacity factor (k') the (normalized) critical effect was 3% or less with the exception of C-8 Rosil, C-8 Alltima and C-18 Hypersil. The latter two columns had an $\%E_{critical}$ value which was 15–25% depending on the substance, while C-8 Rosil had an intermediate $\%E_{critical}$ value of about 10%. The content of inorganic salts had nowhere a significant effect. This means that the absolute value of the effect estimated for this factor was always smaller than the critical effect. The (normalized) effect of the inorganic salts content was $< 2\%$, while for C-18 Hypersil and C-8 Alltima it was somewhat larger (4–6%).

A decrease in the DMF content caused a larger capacity factor for all substances and on all columns as expected since this leads to a decrease in the elution strength of the mobile phase. The effect was on all columns about 10–15%, while it was somewhat smaller on C-8 Hypersil (4–6%).

For ETC, EATC and ATC a lower pH gave a higher k' value, i.e. the effect was negative and amounted to 7–9%. The C-18 Ultrasphere and Hypersil columns were exceptions. Here the effect was the inverse or negligible. For TC however the effect, about 10%, is opposite (positive) to that of the other peaks, i.e. the lower pH value gave a lower k' value. For C-18 Ultrasphere and Hypersil a positive effect of 25% was observed. When it is said that an effect is positive or negative no quality label is given to that effect but only an indication in which direction the effect acts and how the response changes as a function of the change in a factor. In this context, a positive effect only means that the average response measured at the high level of the factor is higher than the one at the low level and a negative effect means that the opposite is observed.

The opposite effects of pH observed for ETC, EATC and ATC on the one hand and TC on the other hand were also observed on the columns eexamined in Ref. [3]. These differences are most probably due to the pK_a values of the substances. TC and related substances have three pK_a values. The second pK_a value (pK_{a_2}) of TC is approximately 7.70 [10–12]. Those of substances related to TC and that were reported in the literature are situated in the interval between 7.30 and 8.60 [11–13]. For some substances different values are reported, e.g. for chlortetracycline pK_{a_2} values of 7.44 and 7.36 were found. However, these pK_a values are all situated around the nominal pH of the mobile phase. The different effects observed for the substances are most probably due to the fact that their pK_{a_2} value is either within, below or above the pH interval examined by the design.

For CTC no pH effect was observed except on C-18 Ultrasphere and Hypersil where it was positive and about 10%.

The flow rate had no effect on k'. For the C-8 Alltima an effect of 6–10% was calculated, but this was still considerably smaller than the critical

effect on that column. For the resolution between consecutive peaks a critical effect of about 10–15% was found on all columns and for the different peak pairs. The exceptions here were the C-8 Alltima and C-18 Hypersil columns where for the resolution between ETC and EATC critical effects of 60% and 30% respectively were found. The effects of the inorganic salts and the flow rate were not significant on whatever response. The amount of DMF had a significant negative effect (20–25%) on R_s(ETC–EATC), which was smaller for C-18 Ultrasphere (15–20%) and Hypersil (< 5%). The negative effect means that the lower DMF value increased the resolution between ETC and EATC. The amount of DMF did not significantly affect the R_s(EATC–TC) and R_s(TC–ATC) values. The pH in contrast did not influence R_s(ETC–EATC) but significantly changes R_s(EATC–TC) and R_s(TC–ATC). The effect on the resolution between EATC and TC was positive (45–50%) and between TC and ATC it was negative (20–30%) on all columns. These latter effects could be expected considering the effects of the pH on the capacity factors of the substances involved.

For the relative retention (selectivity factor, α) the critical effect for all substances was 2–3% except for C-8 Alltima where it was 13%. The amounts of inorganic salts and the flow rate had a negligible effect. The DMF content had a negative effect (about 4%, with the exceptions of C-8 Alltima (7%) and C-18 Hypersil (1–2%)) on all substances. A lower DMF content gave a higher α value, i.e. the k' value of EATC, TC and ATC increased relative to that of ETC.

The effect of the pH on α(EATC) was negligible (< 2%). On C-8 Alltima and C-18 Hypersil it was about 8% which for the former column was still much below the critical level. The effect on α(TC) was significant (15% with the exceptions of C-8 Alltima, C-18 Ultrasphere and Hypersil (25–30%)) and positive. The lower pH value gave a lower α(TC) value, which could be expected from the effects of pH on the k' values of ETC and TC. The effect of the pH on α(ATC) and α(EATC) was negligible (exceptions were C-8 Alltima and C-18 Hypersil (10%).

To summarize it can be said that the qualitative responses examined in this study were mainly affected by the pH. The TC peak especially was rather sensitive to changes in pH. The DMF content also influenced the responses significantly though to a smaller extent. The effect of the amounts of inorganic salts (ionic strength variations) and of the flow rate can be considered to be negligible. Effects measured on the different columns were in general quite similar.

5. Conclusions

Exceptions to the general observations were found for C-18 Ultrasphere, C-18 Hypersil and C-8 Alltima. Since these columns were all tested in one laboratory one may wonder whether the deviating results were entirely due to the columns involved or also to the general experimental conditions which seemed to be different from the other two laboratories and resulted in, for instance, higher experimental error values estimated from the designs.

In general C-8 columns are preferred for the determination of degradation and related products of TC since most of them gave shorter analysis times than the C-18 columns and they were less influenced by ageing of the column. However, in previous studies [3] it was shown that exceptions exist. The C-18 columns can be considered to be a valuable alternative. The protected columns however were not suitable for this application.

This study also showed that the separation of the different substances is not optimal under the conditions of the USP. With none of the columns is the ADTC peak separated while the separation between ETC and EATC is not complete. Moreover, the pH of the mobile phase is prescribed in a region where the responses are not rugged and behave differently for the different peaks.

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