Scientific Notes

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Characterisation of Reversed Phase Liquid Chromatographic Columns by Chromatographic Tests

Preliminary experiments and development of the protocol

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ABSTRACT

In the description of a liquid chromatographic method the European Pharmacopoeia (Ph. Eur.) and other official compendia do not mention the brand(s) of stationary phase that has to be used in order to obtain sufficient selectivity. Column selection presents difficulties since there are no column characterisation methods in the Pharmacopoeias, while more than 600 RP-LC (Reversed Phase Liquid Chromatography) columns are available on the market. Therefore this project was started to determine chromatographic parameters that would enable the selection of an appropriate column.

First a suitable test procedure is needed that allows the measurement of a number of parameters which are representative for different groups of stationary phases. Stationary phases will also be tested according to methods of Ph. Eur. monographs for a number of substances. The correlation between the general test procedure results and the separation parameters obtained in the monograph separations will be examined to define criteria for stationary phase performance, if possible. In this paper the focus is on the first step, namely the description of a suitable test procedure to characterise column properties.

Test methods described in the literature for characterisation of RP-LC columns are reviewed. It is shown that none of the described methods is sufficient to characterise all properties of stationary phases. Therefore a combination of tests is proposed in a new test procedure, taking into account literature data. The final test procedure was tried out on 4 columns.

KEYWORDS: RP-LC columns, characterization, chromatographic test, test parameters.

1. INTRODUCTION

Many HPLC methods are described in the Ph. Eur. and most use reversed phase (RP) C18 columns. In the description of a liquid chromatographic method the Ph. Eur. [1] or other official compendia such as the USP [2] give the eluent composition but they do not mention a brand of the stationary phase(s) that can be used in order to obtain sufficient selectivity. Thus monographs do not give precise information about column identity, which would give correct and reproducible results. Instead of mentioning the brand name, which is not allowed to be communicated in the official monograph, the Ph. Eur. prescribes a system suitability test and further refers to a description of the stationary phase in the reagents section with particle size, pore size, specific surface area and chain length [3]. This information is considered by the authors to be insufficient to choose a suitable column from a market offering more than 600 different brands.

Engelhardt *et al.* performed the determination of impurities of salicylic acid according to the Ph. Eur. on three different commercially available RP columns [4]. With one of the columns all acidic solutes co-eluted with the solvent, on the other columns changes in the elution sequence were observed. This example demonstrates the problem that may occur if the column properties are not sufficiently described. Steffeck *et al.* also draw the attention to the difficulties related to RP-LC column selection [5].

Many scientific papers proposed different rather simple chromatographic tests [5-45], which allow char-

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acterisation of commercial columns. However, it has never been verified sufficiently whether columns having closely related characteristics as determined by these chromatographic tests are suitable for the same chromatographic separation.

The chromatographic tests described in the literature have been reviewed and a selection of these methods was made in order to measure column efficiency, hydrophobicity, silanol activity, ion-exchange capacity, steric selectivity and presence of metal impurities. They were tested on one column and in function of the results obtained, some changes were made to the methods. Finally an adapted test procedure, including 8 different test methods, was proposed and re-tested on 4 new columns of different type. Based on these results a final test procedure is proposed.

In a future study, this test procedure will be applied to a series of columns. Since it is not feasible to examine all the stationary phases available on the market, a selection will have to be made. Columns will be examined in different laboratories in order to demonstrate the reproducibility of the results. These test results will be examined in order to classify columns based on their chromatographic performance.

In collaboration with the Ph. Eur. laboratory, representative separations will be selected from the official monographs and will be carried out on these stationary phases. This latter analytical work will also be organised in different laboratories so that reproducibility of these results will also be checked. Finally the correlation will be examined between the results of the test procedure and the chromatographic behaviour of these columns in the compendial analyses. The test procedure proposed here is complex. It is the intention to simplify it, if possible, in function of the results obtained.

The aim of this project is to formulate a chromatographic test procedure for the characterisation of stationary phases in order to facilitate selection of appropriate columns and to control the performance of a column at any time of its life cycle.

This paper focuses on reviewing the different test methods described in the literature and on the development of a protocol which allows to test the different column properties.

2. REVIEW OF RP-LC COLUMN TESTS

A number of factors influence the properties of silica based reversed phases. The nature of the silica is characterised by the particle diameter, specific surface area, pore diameter, pore volume, chemical purity and acidity. The silane bonding, e.g. length of the alkyl group, the use of mono-, di- or trichlorosilanes, the surface concentration of bonded alkyl groups and the amount of unreacted, accessible silanol groups also affect the properties of the RP stationary phases [6].

Properties of RP-LC stationary phases can be characterised by both non-chromatographic and chromatographic methods. Carbon content, amount of metal impurities, particle size, surface area, pore size, packing density and acidity can be determined by non-chromatographic methods. However, these techniques are not readily performed and cannot be carried out on the packed column without destruction.

Properties such as column efficiency, hydrophobicity, silanol activity, ion-exchange capacity, steric selectivity and the amount of metal impurities can, however, be characterised by chromatographic tests.

2.1. Column efficiency and hydrophobicity

Column efficiency is usually measured using non-polar compounds, especially aromatic hydrocarbons (benzene, toluene, ethylbenzene, butylbenzene and amylbenzene). Efficiency is generally characterised in terms of theoretical plate number, theoretical plate height or reduced theoretical plate height [7, 8] and by retention factor (k′) [9, 10]. Simple methanol - water or aqueous buffer mixtures are generally used as eluent.

Hydrophobicity is measured by the selectivity factor (α) between non-polar alkyl benzenes in a homologous series, differing by one methylene group. The selectivity factor of ethylbenzene/toluene or amylbenzene/butylbenzene pairs is typically determined for this purpose, using methanol/water or methanol/aqueous buffer mixtures as mobile phases [11-18].

Claessens *et al.* [19] compared 5 different test methods for RP-LC columns. It was found that column efficiency and hydrophobicity results from different tests are usually interchangeable and column classification by these methods will provide similar patterns.

2.2. Silanol activity

Silanol groups, remaining on the surface due to incomplete derivatization, play an important role in the retention mechanism [20]. It was an early observation that the small retention factor of nitrobenzene compared to naphthalene or benzene using *n*-hexane as eluent (normal phase mode) indicates a lack of silanol interactions [21-23]. Later, basic compounds were used to indicate silanol activity in reversed phase mode [17, 24-26]. Poor peak symmetry [5, 18, 27] and long retention times of basic compounds [24] indicate the activity and accessibility of free silanols on the silica surface. Aniline derivatives [26], pyridine [28-29] and basic drugs (propranolol, amitryptiline) [30] have been employed for these measurements. Separation of *ortho*-, *meta*- and *para*-toluidine indicates active silanol sites because of their different basicities whilst their hydrophobic properties are identical [26, 28, 31]. Large selectivity factors for aniline/phenol or caffeine/phenol are also indications of free silanol groups [32-35].

Claessens *et al.* [19] found that different silanol activity test results were generally not in mutual agreement and not interchangeable, so column classification on silanol activity depends on which test method is applied. This may be explained partly by the fact that some authors use buffers, which reduce the dissociation of silanol groups.

2.3. Ion-exchange capacity

It has been shown that ion-exchange capacity and silanol activity are not the same properties [36]. Ion-exchange capacity can be characterised by measuring the difference in selectivity coefficients of a base and a neutral compound both at low and relatively high pH values. The difference should be small. In most cases the selectivities of benzylamine and phenol are compared at pH 2.3 and 7.6 [32-34, 37-38].

2.4. Steric selectivity

Shape selectivity can be determined using two aromatic hydrocarbons one of which is twisted and the other is planar. Polyaromatic hydrocarbon pairs e.g. triphenylene (planar) and *ortho*-terphenyl (twisted) and mobile phases containing methanol/water are generally used in this test [32-34]. The selectivity factor of the compounds should be large [29, 37-38].

2.5. Presence of metal impurities

Presently manufacturers prepare RP-columns from highly purified silica. The stationary phase can be contaminated with metal ions from mobile phases and from the cartridge. Metal contamination may enhance silanol activity, polarity and the potential for chelate formation.

Metal impurities on the silica surface can be examined using chelating agents. Peak tailing of the following compounds, acetylacetone [39-40], 2,2′-dipyridyl [29] or 2,3-dihydroxynaphthalene [29, 37], is observed in the presence of metal contamination. Small theoretical plate numbers, strong retention and poor peak symmetry of these compounds are also indicators of metal ions on the silica surface [41]. Such measurements have to be carried out at the optimum pH at which these compounds can form complexes with metal ions.

A comparative study of the different methods to verify trace metal presence has not been reported. It is unclear whether different tests described in the literature provide similar information.

2.6. Other properties

Several stationary phases contain polar functional groups other than silanol groups (e.g. polar embedded columns) and their properties can be examined by polar but not basic compounds [26]. Phenol, benzoic acid [42], *o*-hydroxyhippuric acid, acetylsalicylic acid [27], parabens [30] or phthalate esters [9] are frequently used for this purpose. Long retention time and peak tailing of these compounds indicate the interaction with polar groups on the surface [41].

π-π interactions are measured with aromatic derivatives [43-45]. These effects have not been as well defined as hydrophobicity for example, but they may play an important role in the separation mechanism. These interactions have not been examined in detail.

2.7. Principles for the selection of test methods

Some recommendations and requirements for a "good column test method" are discussed below.

Generally, the tests should be able to demonstrate the differences between various stationary phases and the test conditions should be close to the chromatographic conditions generally employed.

Retention of test compounds should be in the $0.5<k'<10$ range and analysis time should be kept to a minimum. Whenever possible, easily accessible and stable drugs or chemicals should be used as probes. Probe mixtures should include acidic, basic and neutral compounds, that are easily detectable (UV 254 nm). Expensive or toxic chemicals should not be used. The results should not be dependent on the concentration of the test substances at least in a relatively small concentration range. The sample overloading effect should be avoided. Mobile phase preparation by weight is preferable for reasons of reproducibility. Temperature control is necessary, 40 °C being the lowest column temperature at which analysts can work reproducibly in a laboratory without air conditioning [42]. Measurements should be performed at least twice.

A single test method meeting all the above mentioned parameters and that also meets the selection principles cannot be found in the literature. Thus a test procedure including different tests is proposed for characterisation of RP stationary phases. Finally, after careful consideration of the literature, 8 methods were chosen [27, 29, 37, 39, 42] for testing RP columns. The selected methods are widely used in the literature, permitting several properties of RP-LC stationary phases to be tested. All are rather simple to perform (see section 4, Results and Discussion).

3. EXPERIMENTAL

Analyses were carried out using a Varian (Walnut Creek, California, USA) 9010 LC pump, a 9100 autosampler and a 9050 UV-VIS detector with ChromPerfect 4.4.0 software (Justice Laboratory Software, Fife, UK) for data acquisition.

Column temperature was maintained by immersion in a water-bath at 40 ± 0.1 °C, the laboratory was air-conditioned at 25 °C.

Solvents were of LC grade, other chemicals were AR grade. Methanol was from BDH (Poole, England),

acetonitrile was from Biosolve (Valkenswaard, The Netherlands), all other substances were obtained from Acros Organics (Beerse, Belgium).

A Consort C831 (Consort, Turnhout, Belgium) pH meter equipped with a Hamilton (Bonaduz, Switzerland) combination glass electrode was calibrated daily according to the Ph. Eur. [46] with 0.05 M potassium phthalate (4.01) and 0.05 M potassium tetraoxalate (1.78) or 0.01 M borax buffers (9.18) (entries in parentheses are buffer pH values at 25 °C). The pH of aqueous buffers of mobile phases was adjusted with concentrated H_3PO_4 or 2 M NaOH before mixing with organic solvents. Helium was used to degas mobile phases.

A Supelcosil LC-ABZ (Supelco) column (250 × 4.6 mm, particle size: 5 µm) which had been previously used for other separations was examined first. Thereafter, Hypersil ODS (ThermoQuest), Hypersil BDS (ThermoQuest), Nucleosil (Macherey-Nagel) and Kromasil (Macherey-Nagel) columns (all 250 × 4.6 mm, particle size: 5 µm) were used for checking the proposed test procedure. The stationary phases were flushed with the mobile phase for 90 minutes to ensure equilibration of the system before any sample was injected. A flow rate of 1 ml/min was used and 20 µl of sample was injected.

Samples were prepared by dissolving the chemicals in the corresponding mobile phase except in Method 6, where acetylacetone was diluted with methanol and in Method 8, where compounds were dissolved in acetonitrile. Composition of mobile phases and samples were the following:

METHOD 1 (M1) [29]

Mobile phase: methanol/water (80:20 *V/V*)

Sample: thiourea (0.2 mg), phenol (1.8 mg), toluene (7.7 mg), ethylbenzene (7.4 mg), butylbenzene (19 mg), amylbenzene (18 mg), *o*-terphenyl (0.7 mg), triphenylene (0.06 mg) dissolved in 10.0 ml of mobile phase.

METHOD 2 (M2) [42]

Mobile phase: methanol/water (50:50 *w/w*)

Sample: uracil (0.3 mg), phenol (3 mg), toluene (25 mg), ethylbenzene (25 mg), *p*-ethylaniline (3 mg) dissolved in 10.0 ml of mobile phase.

METHOD 3 (M3) [29]

Mobile phase: methanol/water (30:70 *V/V*)

Sample: thiourea (0.3 mg), caffeine (0.8 mg), theobromine (0.6 mg), theophylline (1 mg), phenol (10 mg), pyridine (1 mg), 2,2′-dipyridyl (4 mg), 2,3-dihydroxynaphthalene (16 mg) dissolved in 10.0 ml of mobile phase.

METHOD 4 (M4) [37]

Mobile phase: methanol/0.02 M potassium phosphate buffer pH 2.7 (30:70 *V/V*)

Sample: phenol (12 mg), benzylamine (12 mg) dissolved in 10.0 ml of mobile phase.

METHOD 5 (M5) [37]

Mobile phase: methanol/0.02 M potassium phosphate buffer pH 7.3 (30:70 *V/V*)

Sample: phenol (12.7 mg), benzylamine (12 mg) dissolved in 10.0 ml of mobile phase.

METHOD 6 (M6) [39]

Mobile phase: methanol/0.5 % CH_3COONa (60:40 *V/V*)

Sample: acetylacetone (3 mg) dissolved in 10.0 ml of methanol.

METHOD 7 (M7) [27]

Mobile phase: acetonitrile/0.1 M potassium phosphate buffer pH 2.3 (312:680 *w/w*)

Sample: nicotinic acid (1.5 mg), diphenhydramine (20 mg), *o*-hydroxyhippuric acid (2 mg), acetylsalicylic acid (7 mg), 5-*p*-methylphenyl-5-phenylhydantoin (MPPH) (12 mg), diazepam (1.5 mg), toluene (45 mg) dissolved in 10.0 ml of mobile phase.

METHOD 8 (M8) [37]

Mobile phase: acetonitrile/0.025 M CH_3COONH_4 (75:25 *V/V*)

Sample: 2,3-dihydroxynaphthalene (18 mg), 2,7-dihydroxynaphthalene (10 mg) dissolved in 10.0 ml of acetonitrile.

All samples were diluted 2.5, 5 and 10 times with the appropriate solvent so that 4 different concentrations were examined. The chromatograms were recorded three times at 254 nm.

Calculations of the parameters reported below were performed with ChromPerfect 4.4.0. software.

4. RESULTS AND DISCUSSION

Important parameters of the column were examined using 8 different methods (Methods 1-8). Whilst Methods 1, 2, 3 and 7 permit assessment of several parameters simultaneously, the others are specific for only one parameter. Theoretical plate numbers and retention factors of aromatic hydrocarbons (Methods 1, 2) or MPPH (Method 7) are characteristic for column efficiency. Hydrophobicity was measured by the selectivity factor of ethylbenzene/toluene (Methods 1 and 2) and amylbenzene/butylbenzene (Method 1). Peak symmetry of basic compounds, e.g. p-ethylaniline (Method 2) and diphenhydramine (Method 7) or selectivity factor of the caffeine/phenol (Method 3) are indicators of silanol activity. Ion-exchange capacity can be characterised by Methods 4 and 5. The selectivity factor of triphenylene/ortho-terphenyl is typical for steric selectivity (Method 1).

Table 1 — *Injected amounts and calculated parameters of the original tests*

METHOD 1									
Injected amount of compounds (µg)					Calculated parameters				
A	B	E	TP	oT	n (A)	k′ (A)	α A/B	α E/T	α TP/o-T
3.07	3.29	1.23	0.01	0.11	39562	2.552	1.406	1.318	2.642
5.17	5.54	2.07	0.02	0.19	41162	2.548	1.410	1.320	2.647
10.16	10.88	4.07	0.04	0.38	42279	2.544	1.408	1.320	2.639
30.60	32.78	12.27	0.11	1.14	44622	2.544	1.409	1.321	2.644

METHOD 2						
Injected amount of compounds (µg)			Calculated parameters			
T	E	pE	n (T)	k′ (T)	α E/T	PS (pE)
4.33	4.98	0.49	38075	3.647	1.667	1.510*
8.31	9.57	0.94	39668	3.649	1.668	1.462*
17.21	19.81	1.94	40654	3.652	1.669	1.516*
48.24	55.53	5.45	44253	3.661	1.675	1.470*

METHOD 3										
Injected amount of compounds (µg)					Calculated parameters					
C	P	PY	DP	$DN_{2,3}$	α C/P	PS (PY)	k′ (DP)	k′($DN_{2,3}$)	PS (DP)	PS ($DN_{2,3}$)
0.17	2.06	0.2	0.79	3.33	5.007	1.934*	3.910	–**	2.446*	–**
0.28	3.32	0.32	1.28	5.36	5.017	1.831*	3.917	18.34	2.166*	2.751*
0.55	6.47	0.63	2.49	10.45	5.008	1.804*	3.910	17.61	1.884*	4.922*
1.69	19.85	1.92	7.65	32.06	4.972	1.710*	3.889	17.09	1.702*	4.944*

METHOD 4		
Injected amount of compounds (µg)		Calculated parameter
BA	P	α BA/P
2.42	2.44	4.344
4.49	4.53	4.319
8.83	8.89	4.298
22.90	23.06	4.228

METHOD 5		
Injected amount of compound (µg)		Calculated parameter
BA	P	α BA/P
2.32	2.31	2.079
4.89	4.87	2.085
9.13	9.09	2.066
25.49	25.39	1.992

METHOD 6					
Injected amount of compound (µg)	Calculated parameters				
AA	n	PS	$PW_{50\%}$	PH/IA	PA/PH
0.61	4483	2.127	0.240	30240	0.374
1.31	5384	1.847	0.228	34501	0.360
1.97	5892	1.765	0.207	36581	0.329
5.85	7224	1.383	0.187	43475	0.304

METHOD 7							
Injected amount of compounds (µg)				Calculated parameters			
T	DIP	MPPH	DIA	n (MPPH)	k′ (T)	α DIA/MPPH	PS (DIP)
8.57	3.66	2.39	0.27	42436	6.22	1.233	1.493*
13.58	5.80	3.78	0.42	41546	6.21	1.234	1.558*
25.32	10.82	7.05	0.79	40455	6.23	1.231	1.620*
82.47	35.22	22.97	2.56	40063	6.27	1.235	1.849*

METHOD 8		
Injected amount of compounds (µg)		Calculated parameter
$DN_{2,3}$	$DN_{2,7}$	DERT
4. 29	2.51	∞**
8.86	5.19	6.25*
20.77	9.05	1.01*
36.58	18.56	0.88*

A: amylbenzene, AA: acetylacetone, B: butylbenzene, BA: benzylamine, C: caffeine, DIA: diazepam, DIP: diphenhydramine, $DN_{2,3}$: 2,3-dihydroxynaphthalene, $DN_{2,7}$: 2,7-dihydroxynaphthalene, DP: 2,2′-dipyridyl, E: ethylbenzene, pE: *p*-ethylaniline, MPPH: 5-*p*-methylphenyl-5-phenylhydantoin, P: phenol, PY: pyridine, T: toluene, oT: *o*-terphenyl, TP: triphenylene

α: selectivity factor, DERT: theoretical plate number $_{2,3\text{-dihydroxynaphthalene}}$ / theoretical plate number $_{2,7\text{-dihydroxynaphthalene}}$ (efficiency ratio test), k′: retention factor, n: theoretical plate number, PA/PH: peak area/peak height, PH/IA: peak height/injected amount, PS: peak symmetry, $PW_{50\%}$: peak width determined at 50% of peak height

* RSD > 5%

** 2,3-dihydroxynaphthalene did not give a peak when less than 5 µg was injected

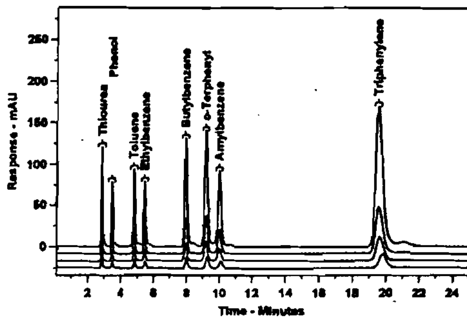

Figure 1 – *Typical chromatogram of test mixture 1*
Column: Supelcosil LC-ABZ

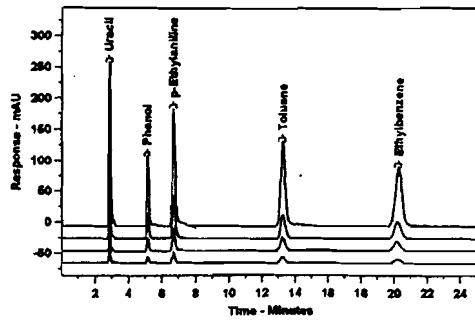

Figure 2 – *Typical chromatogram of test mixture 2*
Column: Supelcosil LC-ABZ

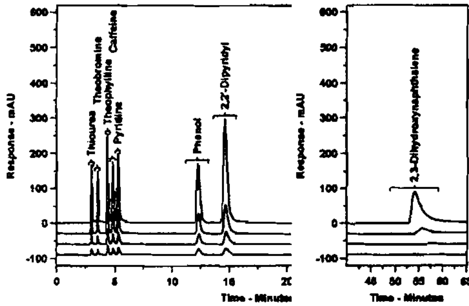

Figure 3 – *Typical chromatogram of test mixture 3*
Column: Supelcosil LC-ABZ

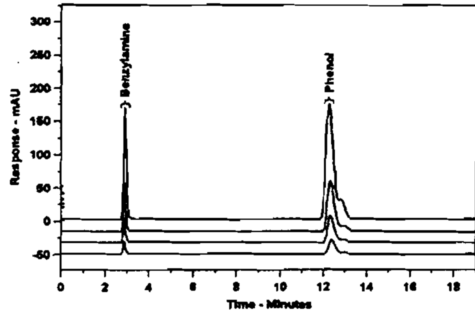

Figure 4 – *Typical chromatogram of test mixture 4*
Column: Supelcosil LC-ABZ

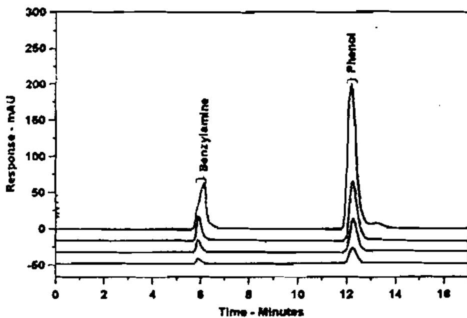

Figure 5 – *Typical chromatogram of test mixture 5*
Column: Supelcosil LC-ABZ

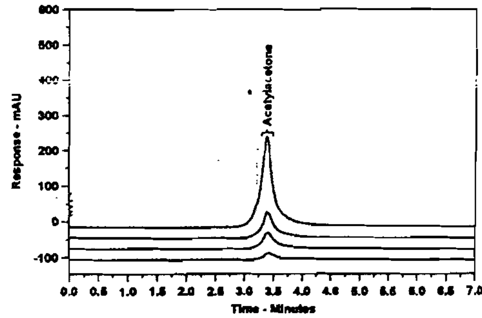

Figure 6 – *Typical chromatogram of test mixture 6*
Column: Supelcosil LC-ABZ

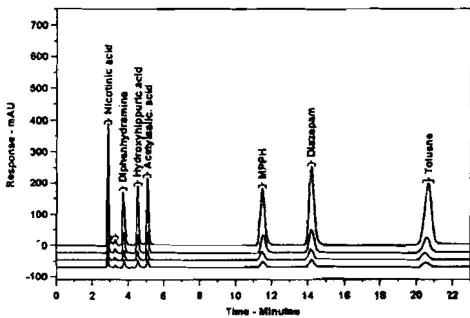

Figure 7 – *Typical chromatogram of test mixture 7*
Column: Supelcosil LC-ABZ

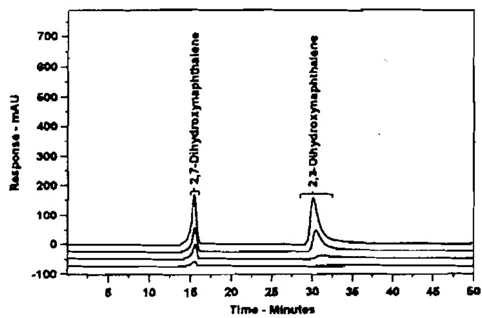

Figure 8 – *Typical chromatogram of test mixture 8*
Column: Supelcosil LC-ABZ

Retention and peak symmetry of 2,2′-dipyridyl and 2,3-dihydroxynaphthalene (Method 3) and the parameters of Methods 6 and 8 are indicative of metal contamination.

Typical chromatograms obtained with Methods 1 to 8 are shown in Figures 1 to 8, the calculated parameters on the Supelcosil LC-ABZ column are reported in Table 1. Chromatograms and parameters have been compared to the results published in the original papers.

Chromatograms obtained were similar to those reported in the literature and the repeatability was excellent. However, some small differences to the originally reported results were observed and were the following: the peak of aromatic hydrocarbons (Method 1) was slightly wider than in the original tests. The extremely long retention time and poor peak symmetry of 2,3-dihydroxynapthalene (Figures 3 and 8) and also the broad peak of acetylacetone (Figure 6) indicated a significant amount of metal ions on the surface of this column.

Concentration dependence of the calculated parameters was also studied (for data see Table 1). It was observed that selectivity factors were usually independent of the amount of compound injected, while theoretical plate numbers and retention factors were slightly dependent on this factor. However, significant variations were observed for peak symmetry factors for each compound examined with a relative standard deviation greater than 5 % in each case. In Method 6, only the peak area /peak height ratio was slightly concentration dependent. 2,3-Dihydroxynaphthalene was not detected by Methods 3 and 8 when small amounts were injected. Several parameters depended on the amount injected, therefore it is important to use the same concentration(s) to compare different columns.

5. IMPROVING THE TEST PROCEDURE

Some changes were made to improve the test procedure according to the principles reported above.

Different compounds (thiourea, uracil, nicotinic acid, nitrate) which are unretained by the stationary phase have been cited in the literature as markers by which the hold-up volume can be measured [47]. Thiourea, uracil and potassium nitrate were employed in those methods that require a hold-up volume marker. Hold-up obtained with different compounds in the different methods are reported in Table 2. It has been noted that the retention time of potassium nitrate is highly dependent on the mobile phase used and its peak shape is very poor in Methods 1 and 7. Thiourea did not dissolve completely in the mobile phase 7. Thus uracil, which is also widely used and accepted in the literature, was selected as the marker of choice since the retention time of uracil is quite independent of the mobile phase, the peak shape is excellent and this compound is readily soluble in all mobile phases.

Different phosphate buffer concentrations (e.g. 0.02 M, 0.1 M) were used in the original methods. But a 0.2 M potassium phosphate buffer has been introduced, so that buffer concentrations given in the literature are reached by dilution with water (e.g. see Method 4). The appropriate pH of 0.2 M potassium phosphate buffers is set by mixing 0.2 M H_3PO_4, 0.2 M KH_2PO_4 and 0.2 M K_2HPO_4 solutions. Phosphate concentration can be kept constant in this way. Water and organic solvents are added afterwards. Composition of mobile phase is performed by weight (*w/w*).

The two lowest concentrations were selected from the four tested, in order to minimise the number of samples and the amounts of compounds to be injected.

Phenol and other acids (ortho-hydroxyhippuric acid, acetylsalicylic acid) were left in the test mixtures to examine polar interactions between the silica surface and the analytes.

The adapted test procedure was performed twice on 4 new columns of different type. Mobile phases in the consecutive methods are of such a nature that intermediate column washing between different mobile phases is not necessary unless one is returning from mobile phase 8 to 1. After Method 8, the column was flushed with acetonitrile/water/1 M phosphoric acid (50:45:5), then with methanol/water (50:50 *w/w*) (mobile phase in Method 2) and finally with acetonitrile, each for 90 minutes. Then, Method 1 was applied again.

In general, excellent repeatability between series was observed. Exceptions were Methods 2 and 3 where the mobile phases are non-buffered and test solutions contain a basic compound. It can be seen for example

Table 2 — *Hold-up values in Methods 1, 2, 3 and 7 using different markers*

Marker	Method 1 (min)	Method 2 (min)	Method 3 (min)	Method 7 (min)
Potassium nitrate	2.87	2.73	2.74	3.85
Thiourea	2.66	2.67	2.77	2.61
Uracil	2.64	2.65	2.79	2.53

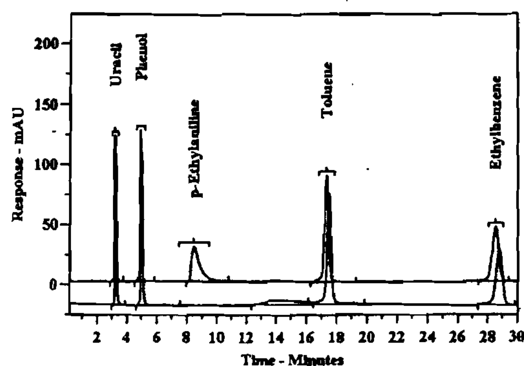

Figure 9 — *Chromatogram of Method 2 in a first (lower) and second (upper) test series, where Methods M1 to M8 were applied in ascending order.*
Column: Hypersil ODS

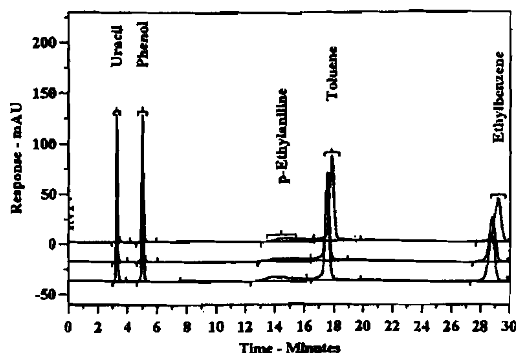

Figure 10 — *Three consecutive runs of Method 2 performed according to the final protocol (Table 3) where Methods M8 to M1 were applied in descending order*
Column: Hypersil ODS

in Figure 9 that *p*-ethylaniline gives different peaks on the Hypersil ODS column in the first (lower chromatogram) and the second (upper chromatogram) test series. The same phenomenon was observed on the other columns and also in Method 3, for pyridine. It seems that silanol groups were non-protonated initially so that *p*-ethylaniline gives a very broad peak in the first test series. However, before the second test series there is an acidic washing step, silanol groups become protonated, resulting in a sharper peak with second test series.

Therefore, an amended protocol was developed, in which the sequence of the methods was reversed for the study. The final sequence and protocol can be seen in Table 3. Neutral mobile phase (Method 5) was used before the non-buffered methods in order to approach the original conditions of the columns as closely as possible. The final protocol was tried out on the four columns twice. Excellent repeatability was observed even in the non-buffered methods. Three consecutive runs of Method 2 can be seen in Figure 10. The chromatograms are similar to the lower one in Figure 9.

6. CONCLUSION

After a study of literature data a new test protocol was developed for characterising RP-LC columns. Eight methods were selected and applied to one column. The properties of the stationary phase were characterised by the parameters that were applied in the original papers. To simplify and improve the original methods, some modifications were made. The final test protocol should allow objective comparison of all important properties of RP-columns.

In a future study this test protocol will be applied on a large number of RP-columns in order to characterise and classify them on the basis of measured parameters. It is intended to study the performance of the stationary phases to separate impurities of pharmaceutical substances using the liquid chromatographic methods described in the monographs of the Ph. Eur.

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Table 3 — *Conditions of the final test procedure, in which the sequence of the test methods M1 to M8 was reversed (descending order)*

Mobile Phase	Samples	Output Parameters	Column Property
① **M8** acetonitrile/0.025 M ammonium acetate (26.2:100 *w/w*)	Sample 1. 6.0 mg 2,3-dihydroxynaphthalene 3.0 mg 2,7-dihydroxynaphthalene dissolved in 10 ml acetonitrile Sample 2. 5 ml of Sample 1 diluted to 10 ml	$DERT = \frac{n_{2,7-dihydroxynaphthalene}}{n_{2,3-dihydroxynaphthalene}}$	metal impurity
② **M7** acetonitrile/water/0.2 M potassium phosphate pH 2.3 (312:340:340 *w/w*)	Sample 1. 0.1 mg uracil, 6.0 mg diphenhydramine 0.7 mg *o*-hydroxyhippuric acid 2.3 mg acetylsalicylic acid 4.0 mg 5-*p*-methylphenyl-5-phenylhydantoin, 0.45 mg diazepam, 14.5 mg toluene dissolved in 10 ml mobile phase Sample 2. 5 ml of Sample 1 diluted to 10 ml	n MPPH k′ toluene	efficiency
		$\alpha_{diazepam/MPPH}$	hydrophobicity
		symmetry factor diphenhydramine	silanol activity
③ **M6** methanol/0.5% CH_3COONa (118.5:100 *w/w*)	Sample 1. 1.0 mg acetylacetone dissolved in 10 ml methanol Sample 2. 5 ml of Sample 1 diluted to 10 ml	peak area/peak height acetylacetone	metal impurity
④ **M4** methanol/water/0.2 M potassium phosphate pH 2.7 (34:90:10 *w/w*)	Sample 1. 5.0 mg phenol 5.0 mg benzylamine dissolved in 10 ml mobile phase Sample 2. 5 ml of Sample 1 diluted to 10 ml	$\alpha_{phenol/benzylamine}$	ion-exchange capacity
⑤ **M5** methanol/water/0.2 M potassium phosphate pH 7.3 (34:90:10 w/w)	Sample 1. 5.0 mg phenol 5.0 mg benzylamine dissolved in 10 ml mobile phase Sample 2. 5 ml of Sample 1 diluted to 10 ml	$\alpha_{phenol/benzylamine}$	ion-exchange capacity
⑥ **M3** methanol/water (34:100 *w/w*)	Sample 1. 0.1 mg uracil 0.30 mg caffeine 0.20 mg theobromine 0.50 mg theophylline 2.8 mg 2,3-dihydroxynaphthalene dissolved in 10 ml mobile phase Sample 2. 5 ml of Sample 1 diluted to 10 ml	3.5 mg phenol 1.0 mg pyridine 3.0 mg 2,2′-dipyridyl	$\alpha_{phenol/caffeine}$ t_R theophylline and theobromine symmetry factor pyridine	silanol activity
			k′ 2,2′-dipyridyl k′ 2,3-dihydroxynaphthalene $\alpha_{2,3-dihydroxynaphthalene/2,2'-dipyridyl}$ symmetry factor 2,2′-dipyridyl symmetry factor 2,3-dihydroxynaphthalene	metal impurity
⑦ **M2** methanol/water (50:50 *w/w*)	Sample 1. 0.1 mg uracil 1.3 mg phenol 10.0 mg toluene 12.0 mg ethylbenzene 1.0 mg *p*-ethylaniline dissolved in 10 ml mobile phase Sample 2. 5 ml of Sample 1 diluted to 10 ml	n toluene, k′ toluene	efficiency
		$\alpha_{ethylbenzene/toluene}$	hydrophobicity
		$\alpha_{phenol/toluene}$	polar interactions
		symmetry factor *p*-ethylaniline	silanol activity
⑧ **M1** methanol/water (317:100 *w/w*)	Sample 1. 0.1 mg uracil, 0.6 mg phenol 2.5 mg toluene, 2.5 mg ethylbenzene 7.0 mg butylbenzene, 7.0 mg amylbenzene 0.2 mg *o*-terphenyl, 0.02 mg triphenylene dissolved in 10 ml mobile phase	n amylbenzene k′ amylbenzene	efficiency
		$\alpha_{ethylbenzene/toluene}$ $\alpha_{amylbenzene/butylbenzene}$	hydrophobicity
		$\alpha_{phenol/toluene}$	polar interactions
	Sample 2. 5 ml of Sample 1 diluted to 10 ml	$\alpha_{triphenylene/o-terphenyl}$	steric selectivity

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