RESEARCH PAPER

Chemometric evaluation of the column classification system during the pharmaceutical analysis of lamotrigine and its related substances

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Abstract This paper investigates the performance of a column classification system developed at the Katholieke Universiteit Leuven applied to pharmaceutical chromatographic analyses. The liquid chromatography assay of lamotrigine and related compounds was carried out according to the method prescribed in the European Pharmacopoeia monograph, using 28 brands of stationary phases. A ranking was built based on the F_{KUL} value calculated against the selected reference column, then compared with the column test performance established for the stationary phases studied. Therefore, the system suitability test prescribed by the European Pharmacopoeia in order to distinguish between suitable or unsuitable columns for this analysis was evaluated. Moreover, it was examined whether the classes of the stationary phases, determined using test parameter results, contain either suitable or unsuitable supports for the lamotrigine separation. This assay was performed using chemometric a technique, namely factor analysis.

Keywords Column classification systems · The Katholieke Universiteit Leuven method · High-performance liquid chromatography · Factor analysis · Lamotrigine and its related substances

Introduction

Nowadays, a very large number of different brands of reversed-phase liquid chromatography (RP-LC) stationary phases are

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commercially available on the market and new ones are being introduced regularly [1]. It means that the selection of a RP-LC column with suitable selectivity for a particular separation is difficult [2]. Many papers describing methods of characterizing columns were published to solve this issue [3–14], while others are under development [15, 16]. An interesting procedure of column selectivity of RP-LC C_{18} stationary phases, namely the Katholieke Universiteit Leuven method (KUL method), has been provided by the group of Hoogmartens and co-workers [17, 18]. In this approach, each C_{18} column is characterized by four chromatographic parameters: the retention factor of amylbenzene (k'_{amb}) reflecting hydrophobicity, the relative retention factor of benzylamine/phenol at pH 2.7 ($rk'_{ba/ph\ pH\ 2.7}$) describing possible silanol activity, the relative retention factor of triphenylene/o-terphenyl ($rk'_{tri/o-ter}$) giving an indication of steric selectivity, and the retention factor of 2,2′-dipyridyl ($k'_{2,2'-d}$) estimating silanol activity and metal impurities [19, 20]. After choosing a specific reference column or selecting four reference parameters corresponding to the selected reference phase, the F_{KUL} value for column i, being the sum of the squares of the differences between each parameter value of the reference stationary phase and that of column i is calculated:

$$
\begin{aligned}
F_{KUL} = {} & \left(k'_{amb,ref} - k'_{amb,i}\right)^2 \\
& + \left(rk'_{ba/ph\ pH\ 2.7,ref} - rk'_{ba/ph\ pH\ 2.7,i}\right)^2 \\
& + \left(rk'_{tri/ter,ref} - rk'_{tri/ter,i}\right)^2 \\
& + \left(k'_{2,2'-d,ref} - k'_{2,2'-d,i}\right)^2
\end{aligned}
\tag{1}
$$

In order to guarantee that the parameters are ascribed the same weight, each of them is auto-scaled ("normalized") before its introduction in Eq. (1) [21].

Next, a comparison is conducted between various brands of stationary phases of column i and the reference phases. The higher similarity of column i to the reference column, the lower the F_{KUL} value was observed and column i goes higher in the ranking list (high-ranked columns). Higher F_{KUL} values of column i confirm the existence of significant dissimilarities between them, and this puts the stationary phase lower on the ranking list arranged by the increasing F_{KUL} value. This convenient and useful simplification, combining four different contributors into a single parameter, has caused that all calculated F_{KUL} values for the tested stationary phases are related to a single reference phase. The stationary phases with $F_{KUL} < 2$ are commonly considered high ranking, and offering the highest probability of finding an appropriate alternative. The columns with $2 < F_{KUL} < 6$ are deemed average, whereas columns with $F_{KUL} > 6$ are treated as low ranking, offering the lowest probability of selecting the appropriate stationary phase.

Literature presents a few reports focused on the correlation between the KUL approach versus other column classification methods [22–25] and numerous reports confirming usefulness of the KUL test procedure for real pharmaceutical applications [18, 20–26]. On the other hand, most of those comparative studies were based on correlating the KUL ranking list only with real selectivity in pharmaceutical analysis described by the European Pharmacopoeia (Ph. Eur.) system suitability test (SST) values or the chromatographic response function indicating the overall selectivity [18, 20–22, 26]. Only few papers have reported more detailed correlation between the KUL test results to classify stationary phases and the column test performance in real pharmaceutical analyses. These comparative studies were based on experimental data including the retention parameters of alfuzosin and its two impurities [27] or caffeine and its four related compounds [28]. In both column test performances, the analytes were eluted in isocratic conditions, similarly to its use in the KUL. Thus, it seems to be interesting whether the KUL method can be considered as a useful tool to classify the stationary phases when gradient elution of the mobile phase is required for simultaneous LC analysis of many analytes.

Therefore, the column classification system based on the KUL procedure was compared against selectivity of the separation of lamotrigine and its seven related compounds (Fig. 1), using a set of 28 stationary phases previously tested under the KUL conditions. The LC analysis was carried out according to the Ph. Eur. monograph [29], where gradient elution of the compounds of interest was prescribed. Moreover, the SST requires that peak-to-valley ratio (p/v) between height above the baseline of the peak due to impurity G and height above the baseline of the lowest point of the curve separating this peak from the peak due to lamotrigine
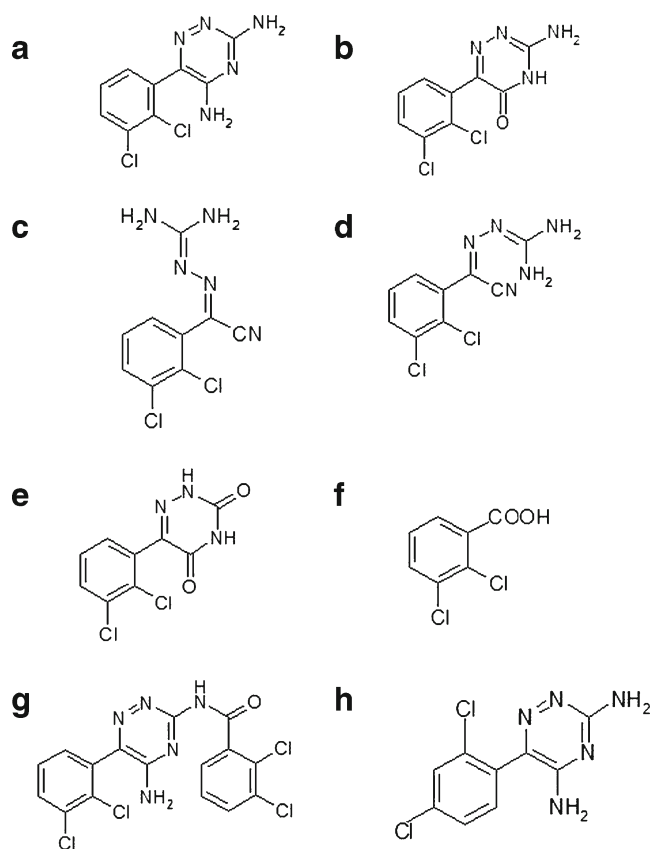
Fig. 1 Chemical structure of **a** lamotrigine, **b** impurity A, **c** impurity B, **d** impurity C, **e** impurity D, **f** impurity E, **g** impurity F, and **h** impurity G

was minimum 1.2. Therefore, the SST test was established for all RP-LC columns studied. Next, upon selection of a Hypersil BDS 100-C18 150×4.6 mm, 5 μm (HYB) column as reference, the KUL ranging list was calculated and confronted with the column selectivity towards the tested analytes described by the overall criterion (the p/v ratios). Next, the retention parameters of lamotrigine and its seven related substances were calculated for all stationary phases, including the retention time (t_R) and the resolution of the peaks of interest (R_s), providing a detailed description of the column performance in real analysis. Finally, a chemometric method, namely factor analysis (FA), was used for checking whether the column classes closely related in terms of their KUL characteristics showed similar separation for lamotrigine and its related compounds. For a more detailed interpretation of the obtained theoretical results of KUL method and their column performance in real separation, the same numbers of 1–28 were assigned for the stationary phases in both data sets. Moreover, the localizations of individual stationary phases in column classes were correlated with the SST parameters. Additionally, the second Ph. Eur. requirement involves the t_R of about 7 min for lamotrigine and the relative retention times (t_{RR}) of about 1.1, 1.3, 1.7, and 1.8 for impurities G, A, E, and F, respectively. These parameters were also taken into account when the application of the KUL method in

the selectivity study involving lamotrigine and its related impurities was examined.

Material and methods

Column examination

Twenty-eight RP-LC stationary phases examined in this study were donated by the manufacturers or the distributors. Their specifications are reported in Table 1.

KUL method

Materials

Amylbenzene, benzylamine, 2,2'-dipyridyl, *o*-terphenyl, triphenylene, and uracil were supplied by Sigma-Aldrich (St. Louis, MO, USA) whereas phenol was purchased from POCH (Gliwice, Poland). Methanol and potassium dihydrogen phosphate of HPLC grade purchased from J.T. Baker (Deventer, Netherlands) while orthophosphoric acid was delivered by Merck (Darmstadt, Germany). All chemicals

Table 1 List of C18 RP-LC tested stationary phases and their specifications as provided by the manufacturer

Number of the column	Name of the column	Manufacturer/ supplier	Carbon load [%]	Pore size [Å]	Surface Area [m²/g]	Silica	Endcap.	Abbreviation
1	Hypersil BDS C18	Thermo	11	130	170	A	+	HYB
2	Hypersil Elite C18	Thermo	15	114	250	A	+	HYE
3	Xbridge C18	Waters	18	130	185	B	+	XBC18
4	Discovery C18	Supelco	12	180	200	B	+	DISC
5	ACE 5 C18	ACT	15.5	100	300	B	+	AC18
6	ACE 5 C18-AR	ACT	15.5	100	300	B	+	AAR
7	Inertsil ODS2	GL Science	18.5	150	320	B	+	ING
8	Nucleosil 100–5 C18 HD	Macherey-Nagel	20	100	350	A	+	NUH
9	ACE 5 AQ	ACT	14	100	300	EP	+	AAQ
10	Wakosil II 5 C18 HG	SGE	15	120	300	B	+	WAH
11	Nucleosil 100–5 C18 Nautilus	Macherey-Nagel	16	100	350	EP	+	NUN
12	Inertsil ODS2	Hichrom	18.5	150	320	B	+	INH
13	YMC Pack ODS-AQ	YMC	14.1	120	300	B	+	YPAQ
14	Nucleosil 100–5 C18 AB	Macherey-Nagel	24	100	350	A	+	NUA
15	Aqua C18	Phenomenex	15	125	320	B	+	AQ18
16	Kromasil 100–5 C18	Akzo Nobel	19	100	340	B	+	KRO
17	Nucleodur C18 Pyramid	Macherey-Nagel	14	110	340	B	+	NUP
18	ACE 5 C18-HL	ACT	20	90	400	B	+	AHL
19	Nucleosil 100–5 C18	Macherey-Nagel	15	100	350	A	+	NU18
20	Prodigy ODS3	Phenomenex	15.5	100	450	B	+	PODS3
21	SunFire C18	Waters	16	100	340	B	+	SUN
22	Luna C18	Phenomenex	17.5	100	400	B	+	LUN
23	Aquasil C18	Thermo	12	100	310	EP	+	AQSIL
24	Zorbax SB-Aq	Agilent	proprietary	80	180	EP	−	ZSBA
25	Xbridge Shield RP18	Waters	17	130	185	EP	+	XBSH
26	Symmetry Shield RP18	Waters	17	100	335	EP	+	SYSH
27	Spherisorb ODS1	Waters	6.2	80	220	A	−	SODS1
28	Spherisorb ODS2	Waters	11.5	80	220	A	+	SODS2

Each column has a length of 150 mm, a diameter of 4.6 mm, and particle size of 5 um

A "traditional," acidic silica gel, *B* "high purity," more neutral silica gel, *EP* embedded or end-capped polar group

were used as received without further purification. Water was pretreated in a Milli-Q Water Purification System (Millipore Corporation, Bedford, MA, USA).

Equipment and LC conditions

All LC separations were carried out using a Waters system (Milford, MA, USA) consisting of 2,695 Separation Module, Column Heater/Cooler with three-column selector valve (Rheodyne RV500-100), 2,996 Photodiode Array Detector and the Empower 2 software for data acquisition. In each method, the column temperature was maintained at 40 °C, and the analytes were monitored at 254 nm. The flow rate was 1 mL/min, and the injected sample volume was 20 μL.

Column characterization

In order to calculate the relative retention factor of benzylamine/phenol at pH 2.7 ($rk'_{ba/ph\ pH\ 2.7}$), the retention factor of 2,2′-dipyridyl ($k'_{2,2'-d}$), the retention factor of amylbenzene (k'_{amb}), and the relative retention factor of triphenylene/o-terphenyl ($rk'_{tri/o-ter}$), three isocratic chromatographic methods in a defined order (A-B-C) were used as previously reported [22]. In these calculations, the dead volume obtained with uracil in method C was used. All measurements were repeated three times, and the RSD values were lower than 1 %. Next, four column parameter values for all examined stationary phases were calculated. Finally, upon selection of the HYB column as reference, the F_{KUL} values for the other stationary phases were calculated according to Eq. 1 using the software available online at http://pharm.kuleuven.be/pharmchem/Pages/ccs.html. These data are summarized in Table 2.

Column performance for the determination of lamotrigine

Materials

In addition to the reagents from Section "KUL method: Materials," *lamotrigine for system suitability CRS* composed of lamotrigine (6-(2,3-dichlorophenyl)-1,2,4-triazine-3,5-diamine) and the impurity G (6-(2,4-dichlorophenyl)-1,2,4-trazine-3,5-diamine) were obtained from EDQM (Strasbourg, France). Lamotrigine (100.0 %), the impurity A (3-amino-6-(2,3-dichlorophenyl)-1,2,4-triazin-5(4H)-one) (99.7 %), the impurity B ((2E)-[2-(diaminomethylidene)diazanylideno](2,3-dichlorophenyl)acetonitrile) (99.1 %), the impurity C ((2Z)-[2-(diaminomethylidene)diazanylideno](2,3-dichlorophenyl)-acetonitrile) (99.4 %), the impurity D (6-(2,3-dichlorophenyl)-1,2,4-trazine-3,5(2H,4H)-dione) (98.8 %), and the impurity F (N-[5-amino-6-(2,3-dichlorophenyl)-1,2-4-triazin-3-yl]-2,3-dichlorobenzamide) (94.6 %) were produced by Polpharma

SA (Starogard Gdański, Poland). The impurity E (2,3-dichlorobenzoic acid) was supplied by Fluka-Sigma-Aldrich (St. Louis, MO, USA). All solvents and reagents were of Ph. Eur. quality. The stock standard solution of lamotrigine and the impurity G was prepared by dissolution of 5.0 mg of lamotrigine for system suitability CRS in 2.5 mL of methanol and diluted to 50 mL with a 10.3 g/L solution of hydrochloric acid. Next, 1.0 mL of the stock test solution was diluted to 10.0 mL with a 10.3 g/L solution of hydrochloric acid resulting in lamotrigine concentration of 0.01 mg/mL in the presence of impurity G at the concentration of 50 ng/mL. Finally, this reference solution was injected into the HPLC system to check compliance with the SST requirement. The stock standard solution of the impurities A–F was prepared by dissolution of 10.0 mg of each impurity in 100 mL of methanol. Next, for preparing of the working standard solution of the impurities A–F, 1.0 mL of this stock solution was diluted to 10 mL with a 10.3 g/L solution of hydrochloric acid. The working standard solution of lamotrigine in the presence of the impurities A–G was prepared by dissolution of 8.0 mg of lamotrigine in 2.5 mL of methanol, addition of 20.0 mL of stock standard solution of lamotrigine and the impurity G prepared from lamotrigine for system suitability CRS, addition of 1.0 mL of working standard solution of the impurities A–F, and dilution to 50 mL with a 10.3 g/L solution of hydrochloric acid. Consequently, the working standard solution of lamotrigine at the concentration of 0.2 mg/mL and in the presence of its seven impurities, each at the level of 0.1 % of the parent substance, has been finally analyzed. The HPLC grades of acetonitrile, triethylamine, and hydrochloric acid used for preparing of the mobile phase and the standard solutions of the tested analytes were purchased from J.T. Baker (Deventer, Netherlands).

Equipment and LC conditions

The same LC equipment was used as that mentioned in Section "KUL method: Equipment and LC conditions." According to the Ph. Eur. monograph, the separation of lamotrigine and its related compounds was performed with gradient elution of solvent A (mix 1 volume of triethylamine and 150 volumes of a 2.7 g/L solution of potassium dihydrogen phosphate adjusted to pH 2.0 with orthophosphoric acid) and solvent B (acetonitrile). During the analysis, the following gradient program of the mobile phase was realized: (1) 0–4 min, 85 % A, 15 % B; (2) 4–14 min, gradient elution formed from 15 to 80 % B; (3) 14–14.5 min, from 80 to 15 % B; and (4) 14.5–25 min, 15 % B to achieve column equilibration. The flow rate was maintained at 1 mL/min, and the UV detection was performed at 270 nm. The LC system was operated at 35 °C. The injected sample volume was 10 μL.

Table 2 The chromatographic parameters and F_{KUL} values for 28 columns studied provided by the KUL method

Analytical column	Column parameters				F	The position in the ranking list (column no.)
	k'_{amb}	$rk'_{tri/o\text{-}ter}$	$rk'_{ba/ph\ pH2.7}$	$k'_{2,2'\text{-}d}$		
HYB	3.480	1.569	0.136	5.123	0	1
HYE	4.450	1.502	0.121	6.807	0.407	2
XBC18	3.361	1.392	0.128	5.518	0.425	3
DISC	2.780	1.447	0.087	4.408	0.473	4
AC18	4.489	1.505	0.097	6.833	0.498	5
AAR	3.483	1.698	0.099	8.180	0.587	6
ING	5.170	1.445	0.051	8.667	0.871	7
NUH	5.099	1.482	0.093	8.049	1.157	8
AAQ	2.232	1.322	0.077	7.271	1.548	9
WAH	4.913	1.353	0.070	7.422	1.567	10
NUN	**2.734**	**1.827**	**0.023**	**5.828**	**1.744**	**11**
INH	4.257	1.639	0.062	8.431	1.749	12
YPAQ	*4.164*	*1.258*	*0.073*	*9.137*	*2.124*	*13*
NUA	*3.658*	*1.964*	*0.097*	*6.021*	*2.157*	*14*
AQ18	*4.932*	*1.277*	*0.096*	*9.243*	*2.292*	*15*
KRO	6.199	1.491	0.091	9.038	2.599	16
NUP	4.682	1.259	0.060	9.879	2.674	17
AHL	6.369	1.535	0.087	8.950	2.786	18
NU18	**3.360**	**1.634**	**0.115**	**14.430**	**2.794**	**19**
PODS3	5.476	1.246	0.078	8.416	2.933	20
SUN	5.479	1.231	0.038	9.124	3.58	21
LUN	5.509	1.172	0.050	9.012	4.036	22
AQSIL	***2.971***	***1.825***	***0.163***	***15.163***	***4.124***	***23***
ZSBA	***0.863***	***1.192***	***0.109***	***9.989***	***4.437***	***24***
XBSH	**2.296**	**2.111**	**0.046**	**4.456**	**4.684**	**25**
SYSH	***3.771***	***2.212***	***0.022***	***6.575***	***6.25***	***26***
SODS2	**4.044**	**1.657**	**0.366**	**17.275**	**7.809**	**27**
SODS1	**2.041**	**1.865**	**0.334**	**23.641**	**14.641**	**28**

Meaning of symbols is explained in the text. The columns non-suitable for the LC analysis of lamotrigine according to the SST test are indicated in bold. The stationary phases non-suitable for the separation of the analytes according to the second Ph. Eur. recommendation are indicated in italics

Column test performance

Firstly, the column test performance based on the LC separation of the samples containing lamotrigine for system suitability CRS which had been prepared as described in Section "Column performance for the determination of lamotrigine: Materials," and analyzed in the chromatographic conditions presented in Section "Equipment and LC condition," was carried out on HYB column. Next, the same LC analysis was performed using other columns examined. All measurements were repeated three times. Then, the peak-to-valley (p/v) ratios were evaluated in accordance to the SST as described in the Ph. Eur. monograph, and these data are presented in Table 3. Further, the LC analysis of a sample containing lamotrigine and the seven impurities (A, B, C, D, E, F, and G) was conducted (n=3). The representative chromatograms of the sample containing the compounds of interest obtained on HYB, HYE, and SODS1 are shown in Fig. 2a–c, respectively. Then, the t_R of the analytes and the R_s of peaks of interest which were calculated from preceding peak obtained during LC separation of lamotrigine in the presence of its impurities were established for all stationary phases. Moreover, the relative retention times (t_{RR}) of the analytes expressed as the percentage of the t_{RR} values recommended by the Ph. Eur. monograph (%t_{RR}) were calculated. These experimental data describing in detail each real pharmaceutical analysis are summarized in Table 3.

Data treatment

The theoretical KUL results for the 28 columns examined and their application in pharmaceutical practice for separation of lamotrigine along with their impurities were subject to a

Table 3 Summary of retention data set of p/v and t_R, $\%t_{RR}$, and R_s for lamotrigine and related substances in column performance test for 28 columns studied

No.	Analytical column:	SST p/v	Lamotrigine t_R	Lamotrigine $\%t_{RR}$	Impurity A t_R	$\%t_{RR}$	R_s	Impurity B t_R	R_s	Impurity C t_R	R_s	Impurity D t_R	R_s	Impurity E t_R	$\%t_{RR}$	R_s	Impurity F t_R	$\%t_{RR}$	R_s	Impurity G t_R	$\%t_{RR}$	R_s
1	HYB	6.16	6.79	97	8.74	99	9.89	9.26	4.95	9.83	5.53	10.33	4.64	11.15	97	7.49	12.11	99	8.68	7.40	99	2.36
2	HYE	1.47	7.48	107	9.04	93	10.81	9.41	3.68	9.99	5.69	10.64	6.28	11.54	91	8.05	12.30	91	6.69	7.83	95	2.37
3	XBC18	4.79	6.51	93	8.61	102	9.29	9.05	3.72	9.56	4.50	10.22	5.48	11.17	101	7.03	11.77	100	4.29	7.16	100	2.32
4	DISC	8.77	6.36	91	8.82	107	14.29	9.20	4.12	9.76	6.11	10.41	6.85	11.26	104	8.16	11.98	105	6.88	7.02	100	2.71
5	AC18	7.90	7.39	106	9.07	94	12.48	9.37	3.35	9.92	6.36	10.67	7.89	11.58	92	8.91	12.22	92	6.13	7.80	96	2.61
6	AAR	4.48	7.40	106	9.16	95	12.88	9.32	1.87	9.98	7.70	10.79	8.77	11.57	92	7.84	12.42	93	8.22	7.74	95	1.99
7	ING	4.56	7.18	103	9.30	100	11.98	9.30	0.00	9.89	5.41	10.91	8.71	11.93	98	8.51	12.30	95	2.95	7.63	97	2.25
8	NUH	4.54	7.14	102	8.95	96	11.39	9.23	2.95	9.82	6.33	10.56	7.34	11.58	95	6.16	12.18	95	3.62	7.59	97	2.31
9	AAQ	1.27	7.03	100	9.16	100	12.25	9.37	2.31	9.86	5.39	10.71	8.86	11.72	98	9.28	12.35	98	5.71	7.36	95	1.58
10	WAH	6.13	6.65	95	8.90	103	12.17	9.17	2.70	9.71	5.73	10.51	7.88	11.45	101	8.58	12.07	101	5.54	7.35	100	2.66
11	**NUN**	**1.00**	**7.68**	**110**	**10.32**	**103**	**0.00**	**9.52**	**11.48**	**10.32**	**5.91**	**11.53**	**8.41**	**13.32**	**102**	**2.73**	**12.94**	**94**	**10.50**	**7.71**	**91**	**0.00**
12	INH	1.30	7.10	101	9.22	100	10.01	9.22	0.00	9.84	4.53	10.76	6.84	11.73	97	6.77	12.31	96	3.95	7.53	96	1.92
13	*YPAQ*	*4.91*	*7.93*	*113*	*9.51*	*92*	*14.30*	*9.65*	*1.53*	*10.21*	*6.59*	*11.11*	*9.87*	*12.04*	*89*	*9.57*	*12.75*	*89*	*7.23*	*8.22*	*94*	*2.17*
14	*NUA*	*1.47*	*5.48*	*78*	*8.29*	*116*	*15.50*	*8.66*	*3.49*	*9.20*	*5.67*	*9.93*	*7.26*	*10.80*	*116*	*7.81*	*11.53*	*117*	*6.68*	*6.05*	*100*	*2.29*
15	*AQ18*	*3.02*	*7.99*	*114*	*9.48*	*91*	*12.50*	*9.66*	*1.81*	*10.23*	*5.90*	*11.07*	*8.09*	*12.00*	*88*	*8.42*	*12.73*	*89*	*6.53*	*8.25*	*94*	*2.08*
16	KRO	7.94	7.47	107	9.08	94	13.89	9.23	1.82	9.80	6.89	10.71	10.34	11.75	93	10.14	12.24	91	4.73	7.83	95	2.84
17	NUP	2.58	7.08	101	9.08	99	14.13	9.13	1.01	9.72	5.57	10.68	9.60	11.67	97	9.00	12.28	96	5.30	7.48	96	1.70
18	AHL	10.83	7.40	106	9.09	94	13.32	9.24	1.79	9.81	6.40	10.72	9.87	11.74	93	9.85	12.23	92	4.49	7.77	95	2.74
19	**NU18**	**1.08**	**7.38**	**105**	**9.24**	**96**	**10.02**	**9.43**	**1.54**	**10.03**	**5.17**	**10.74**	**5.86**	**11.61**	**93**	**6.76**	**12.48**	**94**	**6.91**	**7.63**	**94**	**1.50**
20	PODS3	8.36	7.32	105	9.09	96	12.70	9.28	2.02	9.87	6.42	10.71	8.61	11.66	94	8.54	12.29	93	5.55	7.72	96	2.68
21	SUN	5.19	7.56	108	9.32	95	14.06	9.32	0.00	9.91	6.43	10.96	10.87	11.96	93	9.59	12.41	91	4.22	7.95	96	2.25
22	LUN	6.04	7.33	105	9.23	97	13.16	9.23	0.00	9.82	6.26	10.83	11.04	11.82	95	9.54	12.33	93	4.86	7.74	96	2.47
23	*AQSIL*	*1.00*	*7.90*	*113*	*9.68*	*94*	*11.22*	*9.69*	*0.83*	*10.35*	*7.30*	*10.97*	*5.09*	*11.73*	*87*	*5.78*	*12.68*	*89*	*7.09*	*7.90*	*91*	*0.00*
24	*ZSBA*	*1.00*	*5.18*	*74*	*8.54*	*127*	*20.87*	*8.87*	*3.09*	*9.16*	*2.92*	*10.17*	*10.06*	*10.85*	*123*	*6.51*	*11.96*	*128*	*10.28*	*5.20*	*91*	*0.00*
25	**XBSH**	**1.00**	**6.75**	**96**	**8.84**	**101**	**11.26**	**8.92**	**1.08**	**9.53**	**6.88**	**10.53**	**9.94**	**11.75**	**102**	**10.69**	**12.01**	**99**	**2.24**	**7.09**	**95**	**0.00**
26	***SYSH***	*1.00*	*5.91*	*84*	*8.98*	*117*	*3.80*	*8.61*	*15.25*	*9.28*	*3.03*	*10.71*	*1.13*	*12.04*	*120*	*11.94*	*12.04*	*113*	*0.00*	*6.36*	*98*	*0.00*
27	SODS2	1.11	6.92	99	8.70	97	9.61	9.18	4.42	9.73	5.00	10.22	4.38	11.31	96	7.28	12.36	99	6.71	7.31	96	1.93
28	SODS1	1.00	6.95	99	9.02	100	12.00	9.25	2.35	9.85	6.34	10.34	5.02	11.15	94	8.16	12.43	99	12.05	6.97	91	0.00

Meaning of symbols is explained in the text. The columns non-suitable for the LC analysis of lamotrigine according to the SST test are indicated in bold. The stationary phases non-suitable for the separation of the analytes according to the second Ph. Eur. recommendation are indicated in italics

Fig. 2 LC analysis of the sample containing lamotrigine and impurities A–G at the concentration levels of 0.2 mg mL^{-1} for parent substance and 0.1 % for the related compounds performed on **a** HYP, **b** HYE, and **c** SODS1 column, respectively
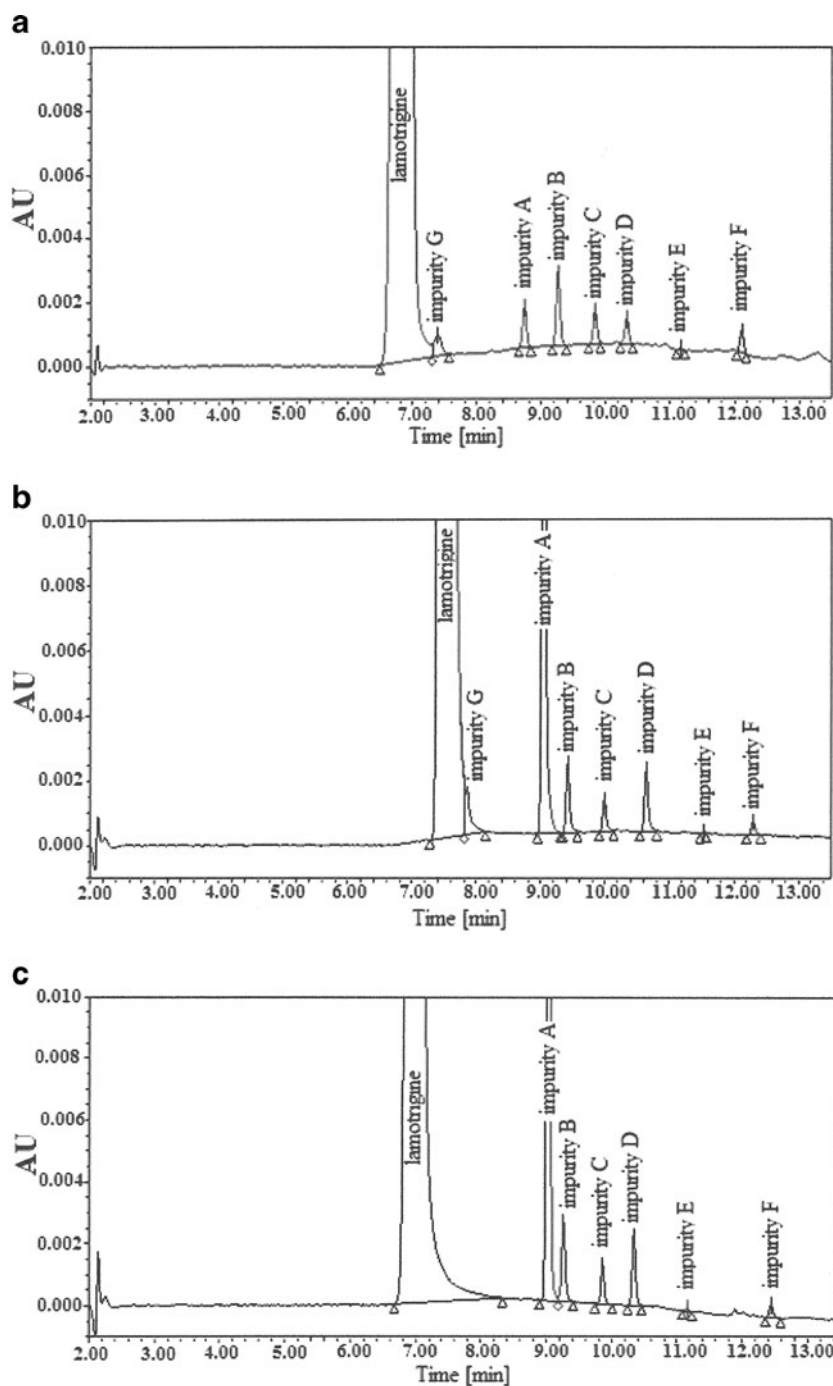
comparative study in order to check whether the KUL test procedure could be used to facilitate RP-LC column selection in the considered pharmaceutical analysis. To visualize the relationships between the data sets containing many variables and objects, a chemometric technique, namely FA based on the algorithm without rotation mode was applied using the Statistica 9.0 package (StatSoft, Tulsa, USA). The stationary phase 1–28 numbering in accordance with the F_{KUL} values provided by the KUL method (Table 2) was retained unchanged in these calculations. Firstly, FA extracted from the standardized four chromatographic parameters provided by the KUL method, was calculated for all stationary phases. The obtained FA plots projecting the variables and the objects (columns) in two-dimensional space are shown in Fig. 3a and b, respectively. Then, FA based on the standardized retention parameters of t_R and R_s for lamotrigine and its seven impurities (A, B, C, D, E, F, and G) as well as the p/v values established according to the SST test (Table 3) for all stationary phases was performed. Two-dimensional plots for the variables and

Fig. 3 Projection of the variables (**a**) and objects (**b**) onto the space of the first two PFs (PF1 and PF2) extracted from FA four column parameters provided by KUL test procedure for the 28 stationary phases studied
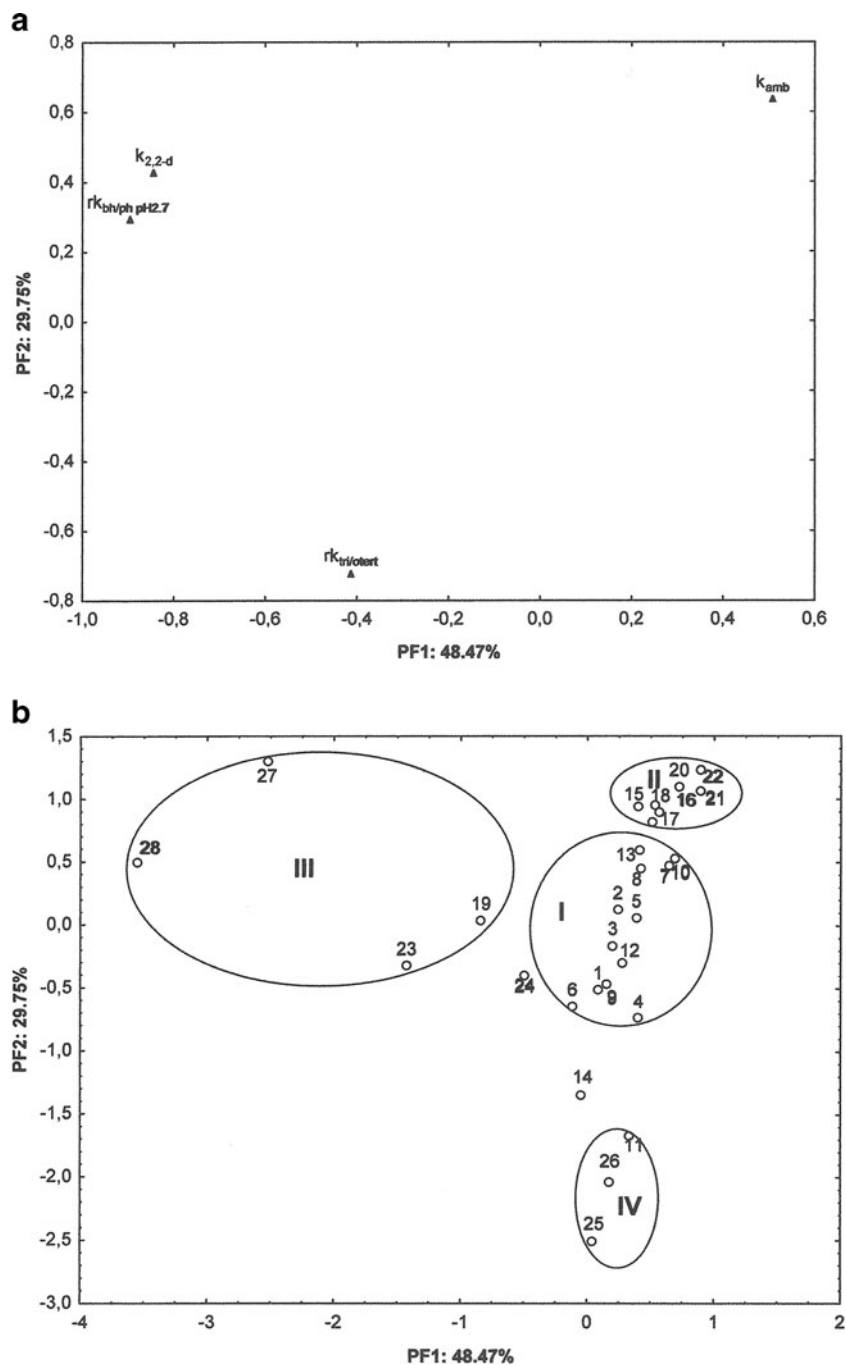
the columns resulting from the FA are illustrated in Fig. 4a and b, respectively.

Results and discussion

Column characterization

The KUL approach involves using selected test solutes which are analyzed in strictly set LC conditions. Thus, each column in the KUL ranking system is characterized using sets of four

column parameters, which enables establishment of the F_{KUL} values. The column parameter values established for 28 stationary phases are summarized in Table 2. In accordance with the k'_{amb} values, the hydrophobicity order was observed ascending from ZSBA to AHL. Silanol activity described by $rk'_{ba/ph\ pH2.7}$ is due to the silanol groups, which remain on the surface because of incomplete end-capping. Their presence plays an important role in the analyte retention mechanism. The highest values of the parameter were calculated for SODS2 and SODS1, while the lowest were found for SYSH and NUN. The values of $rk'_{tri/o-ter}$ and hence, the steric selectivity of the

Fig. 4 Two-dimensional FA plots of the variables (**a**) and objects (**b**) based on the retention parameters (t_R and R_s) of the analytes during column performance for the LC analysis of lamotrigine and its related substances
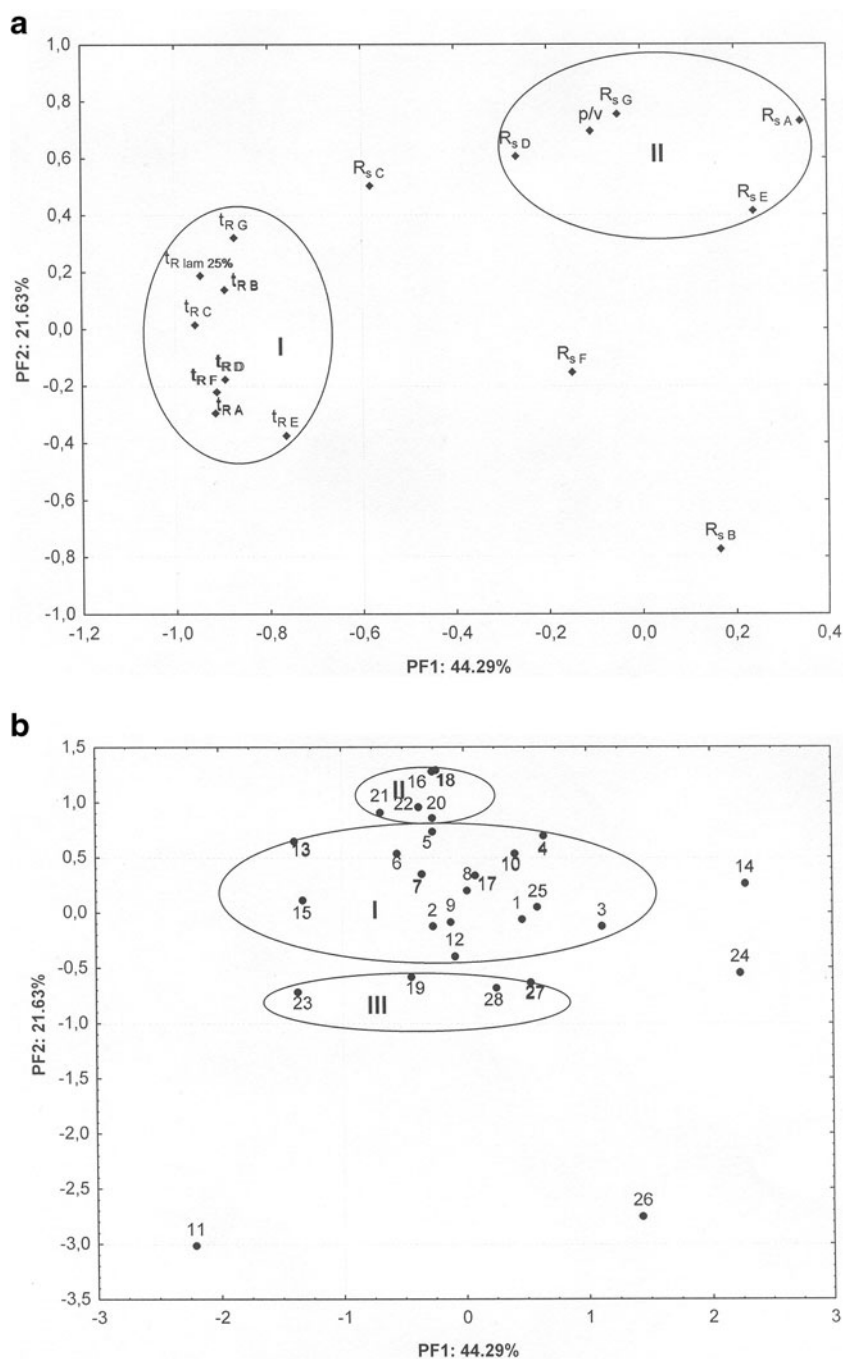
stationary phase were observed to rise beginning with LUN and ending with SYSH. Metal contamination may enhance silanol acidity polarity and the chelate formation potential. Based on the numerical data from Table 2, the highest value of the $k'_{2,2'd}$ parameter indicating silanol activity and metal impurities were calculated for SODS1, whereas the lowest was obtained for DISC. In the study, the reference stationary phase (HYB column) was characterized as having a low hydrophobic nature, an intermediate potential for steric selectivity, and revealing lower silanol activity and metal contamination despite the fact that type-A silica was used (Table 1). When KUL ranking list was

taken into account, 11 columns were found to be high-ranked ($F_{KUL} < 2$), where HYE also based on the type-A silica with lower $k'_{2,2'd}$ parameter was considered as most similar to the reference column ($F_{KUL} = 0.407$). Thirteen stationary phases were identified as intermediate, while columns SYSH to SODS1 were described as low-ranked ones ($F_{KUL} > 6$), which indicates that their chromatographic properties were significantly different from the reference column. They were characterized by higher $rk'_{tri/o-ter}$ parameters.

Next, for clearer interpretation of the theoretical KUL results for the 28 columns studied, FA based on the

standardized four chromatographic parameters was performed. The two-dimensional plots for the variables and objects derived from KUL data set (Table 2) are shown in Figs. 3a and 4b, respectively. The use of chemometric techniques for the evaluation of the similarities and dissimilarities of the RP-LC stationary phases has been reported [5, 9, 15, 23, 30–33], but only few papers have presented the possibility of the multivariate methods for the column selectivity study in detail so far [27, 28, 33]. It can be highlighted that the variability of $rk'_{ba/ph\,pH2.7}$ and $k'_{2,2'd}$ was mainly explained by the first principal factor (PF1). These variables positioned themselves close to each other on the left on the upper side of the plot (Fig. 3a). The localization of the variables and objects on the PF2 axes mainly resulted from the variability of $rk'_{tri/o-ter}$ and k'_{amb}. These variables were found outliers on the bottom part and on the upper side of the graph, respectively. In summary, 78.22 % of the variance of the analyzed data set can be explained by the first two PFs. Columns with high-ranked positions ($F_{KUL}<2$) (nos. 1–10, 12) as well as the stationary phase no. 13 ($F_{KUL}=2.124$) were found in cluster I located on the right in the middle part of the graph (Fig. 3b). Their characteristics include lower and intermediate $k'_{2,2'd}$ and $rk'_{tri/o-ter}$ parameters (Table 2). For them, except of nos. 1, 2, 8, and 9, the type-B silica was used. The columns positioned as intermediate in the KUL ranking list (nos. 15–18, 20–22), identified by higher k'_{amb} against lower $rk'_{tri/o-ter}$ values, were placed on the right on the upper side of the FA plot. These stationary phases were based on the type-B silica but the surface areas calculated for them were higher than observed in cluster I (Table 1). On the other hand, low-ranked stationary phases with the type-A silica (nos. 27 and 28) were found together with column nos. 19 and 23 within cluster III located on the left on the upper part of the plot (Fig. 3b). For them, high silanol activity probably related with high level of the metal contaminant which leads to significantly higher $k'_{2,2'd}$. At first, the position of the column no. 11 seems to be surprising because this column was positioned together with the stationary phase nos. 25 and 26 within cluster IV falling in the bottom side of the graph. On the other hand, each of these stationary phases has EP group resulting in lowest values of $rk'_{bh/ph\,pH2.7}$ parameters, while $rk'_{tri/o-ter}$ values were the highest. Only column no. 14 was itself near column no. 24 between clusters I, III, and IV. For these stationary phases, intermediate $rk'_{ba/ph\,pH\,2.7}$ parameters were calculated by the KUL test procedure. These columns have different type of the silica (A and EP), but their common feature was probably high carbon load. Unfortunately, this parameter was proprietary for stationary phase no. 24.

Comparison based on the overall criterion of column selectivity in separation of lamotrigine

A test of the reliability of KUL column classification system in pharmaceutical practice was based on LC separation of lamotrigine and its impurities, which was performed with gradient elution according to the Ph. Eur. monograph [29], using set of 28 new RP-LC C18 stationary phases. For this LC analysis, the Ph. Eur. defined the stationary phase as *end-capped octadecylsilyl silica gel for chromatography R (5 μm)* having size of 150×4.6 mm. On the other hand, according to the knowledge database [34], a HYB column is recommended by the Ph. Eur., but other stationary phases in compliance with the SST can be also used for this analysis. As mentioned, the SST requires a minimum peak-to-valley (p/v) ratio of 1.2 for the impurity G and lamotrigine. Thus, for each stationary phase, this parameter was calculated. The obtained results confirmed that the SST value of 6.16 was calculated for the HYB column whereas the p/v ratios ranged from 1.00 to 10.83 for other stationary phases studied (Table 3). Thus, an appropriate LC separation of lamotrigine and related compounds in accordance to the SST requirement was observed for 11 high-ranked stationary phases (11/12; 91.67 %), nine columns described by the F_{KUL} values between 2 and 6 (9/13; 69.23 %), and none with low position in the KUL list ranking (0/3; 0 %). These results are similar to those previously found in the literature [18, 20–22, 24–28]. Moreover, the separation of lamotrigine at the concentration of 0.2 mg/mL in the presence of seven related compounds, each at the level of 0.1 % of parent substance was performed using the stationary phases examined (Fig. 2). Next, for each column, the t_R and R_s for lamotrigine and its impurities were calculated, and these data are summarized in Table 3. Moreover, according to the Ph. Eur. monograph, the $\%t_{RR}$ parameters for parent substance and the related compounds A, E, F, and G were established. Notably, the t_R of the compounds of interest were longer for most stationary phases examined comparing to the reference column, while the shortest t_R values were noticed for NUA (no. 14) and ZSBA (no. 24). It should be also noticed that column nos. 13–15, 23, 24, and 26 were not in compliance with the second Ph. Eur. recommendation (at least one analyte gave the $\%t_{RR}$ values higher than 10 %). Thus, both p/v parameters and the Ph. Eur. recommendation related with the t_R of the analytes were realized by all columns classified as high-ranked in the KUL ranking list, while the probability of the appropriate LC separation of the compounds of interest significantly decreased according to increasing of the F_{KUL} parameter. It confirms that the column classification results provided by the KUL method and the column selectivity in separation of lamotrigine based on both the Ph. Eur. requirements for the stationary phases examined were significantly correlated.

Comparison based on the column selectivity in separation of lamotrigine and its related compounds using chemometric method

The evaluation of the KUL test procedure as a useful tool for predicting the appropriate stationary phases for the LC separation of lamotrigine in the presence of its impurities based on detailed description of the column test performance results was conducted using FA. For this assay, the %t_{RR} variables were omitted because the information provided by this variable is 100 % redundant when the t_R of the analytes are taken into account. For this reason, the inverse of the matrix cannot be computed and the FA can basically not be performed or the FA is less reliable. Therefore, FA extracted from a standardized experimental data set of t_R and R_s of lamotrigine and the impurities of A, B, C, D, E, and G, as well as the SST test parameters established for the columns was calculated. The FA plots of variables and objects are presented in Fig. 4a and b, respectively. It should be noticed that here, the variance of the analyzed data involved in the variability of t_R of the analytes was mainly explained by the first PF1. These variables were found in cluster I, on the left side of the central part of the FA plot (Fig. 4a). The positions of the columns on the PF2 axes turned out to be related mainly to the variability of the $R_{s\ B}$, $R_{s\ G}$, and $R_{s\ A}$ and then, the p/v and $R_{s\ D}$ parameters. The variable of $R_{s\ B}$ was found as an outlier on the bottom side of the graph, while the above-mentioned variables were located in cluster II on the right of the upper part of the plot, respectively. In summary, the first two PFs account for 65.92 % of the data variability. Notably, most of the stationary phases positioned themselves within three clusters including columns 1–10, 12, 13, 15, and 17 (cluster I); 16, 18, and 20–22 (cluster II); and 19, 23, 27, and 28 (cluster III), respectively (Fig. 4b). Fourteen columns forming cluster I were suitable for the LC determination of lamotrigine along with their impurities A–G according to the SST test. The second Ph. Eur. recommendation was not complied for stationary phases 13 and 15, which were found separately on the left side of cluster I. All columns located in cluster II offered also appropriate separations of the compounds of interest according to both Ph. Eur. requirements. For them, medium and higher SST values were calculated, whereas the intermediate values of t_R and R_s of the analytes were observed. Contrary to them, insufficient p/v parameters were established for the columns positioned within cluster III. Two stationary phases, nos. 14 and 24, were found close to each other on the right of the plot, while columns 11 and 26 were located on the bottom side of the graph. Among them, the column nos. 14 and 24 gave shorter t_R of the analyzed substances, while these values for the NUN (no. 11) were the longest, respectively. On the other hand, only NUA column gave an appropriate separation of lamotrigine and the impurity G, but the Ph. Eur. recommendation related

with the t_{RR} of the analytes was not realized. Nevertheless, it is worth emphasizing that there are visible similarities between the graphs illustrated in Figs. 3b and 4b. Thus, most of the stationary phases included within cluster I and cluster II extracted from FA based on four chromatographic parameters (Fig. 3b), were also found in cluster I and II based on column test performance, respectively (Fig. 4b). Only positions of column nos. 15 and 17 were different (cluster II—Fig. 3b; cluster I—Fig. 4b, respectively). The column nos. 19, 23, 27, and 28 offering insufficient LC separation of the compounds of interest were positioned in cluster III, while the stationary phases, nos. 14 and 24, were found close to each other in both FA plots. On the other hand, different localization of XBSH column was also noticed (cluster IV—Fig. 3b; cluster I—Fig. 4b). Some inconsistencies were also observed for column nos. 11 and 26. These stationary phases were found close to each other in Fig. 3b, while they were positioned separately in the bottom part of the FA plot in Fig. 4b. Despite this, the localizations of 23 columns were consistent in both FA plots. It confirmed that the KUL method identified correctly most of the stationary phases examined. This fact allows to conclude that the usage of column classification system based on the KUL test procedure increases the probability of selecting columns that will be similar to and dissimilar from a reference stationary phase. Moreover, according to FA plots illustrated in Figs. 3a–4b, it can be noticed that the graphical visualization of the variables and the objects in the space created by the first PFs gives the possibility to perform the comparative study based on clearer and more in-depth interpretation of the obtained results. The results also confirmed that the stationary phases based on the type-B silica were the most suitable for the LC analysis of lamotrigine in the presence of its impurities when the lowest probability of an appropriate separation of the tested analytes was calculated for the columns containing embedded polar functional groups or polar end-capping.

Conclusions

This paper focuses on an application of the column classification system based on the KUL method in a selectivity study involving lamotrigine and its related impurities, using a set of 28 stationary phases. In the method proposed by Hoogmartens et al., each column is characterized by four chromatographic parameters which allow the calculation of the F_{KUL} value versus the reference stationary phase, following which the ranking list of the columns is built. The suitability of the columns examined was verified by the calculation of the SST according to the Ph. Eur. monograph and the chemometric assay of the column test performance. In summary, the F_{KUL} values provided by the KUL method

and the positions of the 28 stationary phases studied, as specified by the KUL characteristics on FA plot were significantly correlated with the localization of stationary phases on the FA graph extracted from the retention parameters of the analytes indicating in detail each real pharmaceutical analysis. Thus, the results confirmed that the probability of appropriate column selection for the given analysis is higher after using the KUL test procedure. Moreover, an application of multivariate data processing, i.e., FA can be considered as valuable starting point for improving the reliable evaluation of the column classification system in pharmaceutical practice.

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