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Evaluation of a column classification method using the separation of alfuzosin from its related substances

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ABSTRACT

The popularity and commercial availability of reversed-phase liquid chromatographic (RP-LC) stationary phases cause analysts to be often confronted with the problem of column selection. For this reason, general test methods to characterize RP-LC columns have been extensively studied since the 1970s. This paper focuses on correlating the column classification based on a method developed at the Katholieke Universiteit Leuven (KUL method) with the selectivity obtained for a real separation. The analysis of alfuzosin hydrochloride and related compounds was carried out according to the method prescribed in the European Pharmacopoeia (Ph. Eur.) monograph. This separation was performed on 36 new RP-LC stationary phases which had been previously characterized chromatographically. For deeper comparative analysis of KUL classification of the stationary RP-LC brands and their column performance in pharmaceutical practice two chemometric tools, such as principal component analysis (PCA) and cluster analysis (CA), have been used. It was shown that stationary phase classes closely related by KUL method gave comparable separation for alfuzosin and related compounds. Therefore, the column ranking system based on the evaluation of *F*-values can be considered as a helpful tool in the selection of a suitable column for pharmaceutical analyses.

1. Introduction

Reversed-phase liquid chromatography (RP-LC) is probably the most frequently used technique applied to separate mixtures in pharmaceutical and biomedical analyses [1]. However, owing to the large number of commercially available stationary phases in the market and the great variety of possible chromatographic systems a proper choice of a suitable column for a particular separation is a challenging task. In addition, this selection of an appropriate column complicates the fact that these RP-LC phases are often nominally identical (*e.g.*, they belong to the same chemical class) what may suggest their similar chromatographic character. However, these RP-LC columns can have a unique character because of the polar and ionic properties of RP-LC phases responsible for secondary intermolecular interaction mechanisms which may be different depending on the type of ligand, type of silica, residual silanols, end-capping, bonding density or pore size. As a consequence, chromatographic performance is often influenced. The selection of a suitable column is also a serious problem for analysts who perform analyses according to official monographs from the European Pharmacopoeia (Ph. Eur.) [2] or the United States Pharmacopeia (USP) [3]. In these monographs numerous LC methods, mainly under RP conditions, are described. However, these prescriptions only give very general information about the stationary phase to be used in terms of base-deactivation, chain length, end-capping, particle size and sometimes pore size and specific surface without mentioning the names of the RP-LC columns. Only for recently developed Ph. Eur. and USP monographs, more information about the stationary phase can be found in the databases available on their "knowledge database" [4,5]. Sometimes the brand name of the stationary phase is known, but the required column is not present in the laboratory or the prescribed one is simply not available anymore on the market. Moreover, stationary phase properties can also change during their storage time or upon usage what causes that a column does not give satisfactory results [6]. In this situation, the replacement by a suitable alternative which will provide an "equivalent" separation to the original column would be helpful. Often, trial-and-error or screening approaches based on the empirical knowledge of the analyst or on the results of a number of chromatographic tests are used. However, these investigations are often labor-intensive, cost demanding, and can produce conflicting results. Therefore, the availability of a good column characterization system based on more objective criteria, which also allows ranking of the columns, is interesting. Numerous publications were reported to describe chromatographic methods used for studying column selectivity in RP-LC [7–17]. Interesting approaches

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were the mathematical models published by the group of Snyder and Dolan [18–20], Abraham [21] and delivered by Kaliszan and co-workers [22–24] namely quantitative structure–retention relationships (QSRRs). All mentioned column classification systems were based on retention measurements of a given set of selected solutes in order to characterize columns and quantify their separation properties. Recently, a few general overviews of the application of QSRRs in comparative investigations of retention properties of RP-LC stationary phases were published [25–28]. In the same time period, a chromatographic method to characterize and classify RP-LC C18 columns was derived by Hoogmartens and co-workers from the Katholieke Universiteit Leuven called "KUL method" [29–36]. In this system, each C18 column is characterized by four parameters: the retention factor of amylbenzene, (k'_{amb}) which indicates hydrophobicity, the relative retention factor of benzylamine/phenol at pH 2.7 ($rk'_{ba/ph\ \ pH2.7}$), which gives the evaluation for silanol activity, the relative retention factor of triphenylene/o-terphenyl ($rk'_{tri/o\text{-}ter}$) reflecting the possibility for steric selectivity, and the retention factor of 2,2′-dipyridyl ($k'_{2,2'\text{-}d}$) describing silanol activity and metal impurities [33]. This approach starts with the selection of four reference parameters, corresponding to a chosen reference column or the selection of a defined reference column. Next, the *F*-value for a column *i* is calculated as the sum of squares of the differences between each parameter value of the reference stationary phase and a column *i* according to the equation:

$$F = (k'_{amb,\,ref} - k'_{amb,\,i})^2 + (rk'_{ba/ph\ \ pH2.7,\,ref} - rk'_{ba/ph\ \ pH2.7,\,i})^2 + (rk'_{tri/ter,\,ref} - rk'_{tri/ter,\,i})^2 + (k'_{2,2'-d,\,ref} - k'_{2,2'-d,\,i})^2 \quad (1)$$

The obtained *F*-value indicates the similarity of C18 brands of stationary phases of column *i* and the reference one. The lower the *F*-value, the more column *i* is similar to the reference stationary phase and the higher is the *F*-value found in the ranking (high ranked columns). The higher is *F*-value, the more significant dissimilarities between compared C18 brands of stationary phases are observed. To ensure the same weighing of each parameter they were autoscaled using formula (2) before being introduced in Eq. (1):

$$\frac{x_{ij} - x_j}{s_j} \quad (2)$$

where x_{ij} is the value of parameter *j* on column *i*, x_j is the mean value of parameter *j* on all tested stationary phases, and s_j is the standard deviation for parameter *j*.

Therefore, owing to the fact that the *F*-values are a function of four different contributions to column selectivity, all calculated *F*-values are relative to a single reference one. It is customary that the *F*-values below 2 indicate tested columns being considered as high ranked with the highest probability to find an appropriate stationary phase. Columns with *F*-values between 2 and 6 are described as intermediate, whereas columns with *F*-values above 6 are considered as low ranked. For them, the probability of the selection of a suitable stationary phase is the lowest [35,36]. Of course, it is very important to verify, whether the KUL test procedure gives reliable results by checking whether columns having similar parameters produce similar separations in pharmaceutical practice. In the literature, there are a few publications which studied the theoretical KUL classification method and which reported on different pharmaceutical applications where for each drug the separation of the main peak from its related compounds was verified [30–32,34–36]. The reported data confirmed that the ranking of the columns and the selectivity in the separations of the pharmaceuticals were significantly correlated, what indicates that KUL test procedure can be considered as an useful tool in the selection of a suitable RP-LC C18 column. However, in those comparative studies the correlation between the KUL ranking list and column performance was based on only the Ph. Eur. system suitability test (SST) or the chromatographic response function (CRF) indicating the overall selectivity [32,35]. Both SST and CRF parameters offering only general description of the pharmaceutical separations what do not provide that the stationary phases related by KUL method represent exactly the same chromatographic characteristic or that the separations are identical. In this paper, more detailed analysis of the correlation between the KUL test results to characterize or classify columns and the column performance in a real pharmaceutical analyze has been performed.

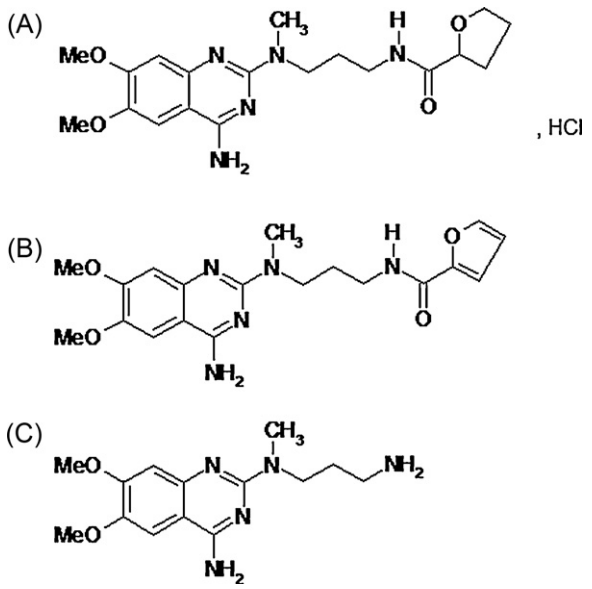

Fig. 1. Chemical structure of (A) alfuzosin hydrochloride, (B) impurity A and (C) impurity D.

As a case study, new application – the separation of alfuzosin hydrochloride and its impurities A and D (Fig. 1), as prescribed by the Ph. Eur. monograph, was chosen and carried out on 36 stationary phases previously characterized chromatographically. The Ph. Eur. system suitability test (SST) requires that peak-to-valley ratio (p/v) between height above the baseline of the peak due to impurity A and height above the baseline of the lowest point of the curve separating this peak from the peak due to alfuzosin was minimum 5.0. This parameter was calculated for all tested RP-LC columns. Next, the theoretical data set of column classification system and its practical application in pharmaceutical practice in order to check how KUL test procedure could be used to facilitate RP-LC column selection in the considered pharmaceutical analysis was examined by the evaluation whereas stationary phase classes, based on the four test parameters can be well or poorly related with alfuzosin separation described by the more detailed experimental data such as retention times (t_R) and the values of resolution (R_s) for analyzed compounds. Thus, it was established whether columns, which have closely related KUL test characteristics, show similar separation for alfuzosin and its impurities. For more clear interpretation of the theoretical results of KUL method and their column performance in real separation, the same numbers from 1 to 36 have been assigned for the stationary phases in both data sets according to the increasing *F*-values. The positions of individual stationary phases in column classes also were correlated with the SST-values. This assay was traditionally performed using principal components analysis (PCA). However, the use of other chemometric tool such as cluster analysis (CA) is also described.

2. Experimental

2.1. Reagents

Amylbenzene, benzylamine, 2,2′-dipyridyl, *o*-terphenyl, triphenylene and uracil were from Sigma–Aldrich (St. Louis, MO, USA) whereas phenol was delivered by POCH (Gliwice, Poland). *Alfuzosin for system suitability CRS* (containing alfuzosin hydrochloride, (2RS)-N-[3-[(4-amino-6,7-dimethoxyquinazolin-2-yl)methylamino]propyl]tetrahydrofuran-2-carboxamide hydr ochloride, impurity A, N-[3-[(4-amino-6,7-dimethoxyquinazolin-2-yl)methylamino]propyl]furan-2-carboxamide, and impurity D, N-[4-amino-6,7-dimethoxyquinazolin-2-yl)methylpropane-1,3-diamine), was obtained from EDQM (Strasbourg, France). Standard solution prepared in accordance with the description of the method contains alfuzosin hydrochloride at the concentration of 0.4 mg/mL in the presence of impurities A and D, both at the level about 2 μg/mL (*i.e.* 0.5% of active substance in comparison to the concentration of alfuzosin in the solution). All solvents and reagents were of Ph. Eur. quality. Acetonitrile, methanol and potassium dihydrogen phosphate of HPLC grade were supplied by J.T. Baker (Deventer, Netherlands) while phosphoric acid, perchloric acid and sodium hydroxide were delivered by Merck (Darmstadt, Germany). All solvents and reagents for the preparation of mobile phases were of analytical grade and were used as received without further purification. Water was purified (Millipore Corporation, Bedford, MA, USA) before use. Thirty-six C18 RP-LC columns examined in this study were donated by the manufacturers or the distributors. Their specifications are reported in Table 1.

2.2. Equipment and chromatographic measurements

Analyses were carried out using a Waters system (Milford, MA, USA) equipped with 2695 Separation Module, Column Heater/Cooler with three-column selector valve (Rheodyne RV500-100), 2996 Photodiode Array Detector and the Empower 2 software for data acquisition.

2.2.1. Chromatographic conditions for KUL method

General information concerning the KUL classification system, including the detail description of three isocratic chromatographic methods used for selected analytes resulting in the four final parameters, had been published earlier [33,37]. In each method, the column was maintained at 40 °C and UV detection was done at 254 nm. The flow rate was at 1 mL/min, while the injected sample volume was 20 μL.

2.2.2. Chromatographic conditions for LC analysis of alfuzosin

The separation of alfuzosin and its impurities was carried out as prescribed by the Ph. Eur. monograph. The mobile phase was composed of tertahydrofuran, acetonitrile and an aqueous solution of perchloric acid (pH 3.5) (1:20:80, v/v/v), which had been prepared by the dilution of 5.0 mL of perchloric acid in 900 mL of water, the adjustment to pH 3.5 with sodium hydroxide and dilution to 1000 mL with water. The column was thermostatted at 25 °C and the analytes of interest were monitored at 254 nm. The flow rate was at 1.5 mL/min. The injected sample volume was 10 μL.

2.3. Column examination

2.3.1. Column characterization by KUL method

Four column parameters, such as the relative retention factor of benzylamine/phenol at pH 2.7 ($rk'_{\mathrm{ba/ph\ pH2.7}}$), the retention factor of 2,2′-dipyridyl ($k'_{2,2'\text{-d}}$), the retention factor of amylbenzene (k'_{amb}) and the relative retention factor of triphenylene/*o*-terphenyl ($rk'_{\mathrm{tri/o\text{-}ter}}$) were determined for all tested columns on the basis of the retention times measured for the selected analytes using three chromatographic methods in a defined order A, B and C, as described in Section 2.2.1. In these calculations, the retention time obtained for uracil in method C was used as the dead volume. On each column, the separation was performed in triplicate and the obtained RSD values were lower than 1%. Next, column characterizations were prepared by the calculation of four parameters for all tested stationary phases. Finally, after selecting a Inertsil column (Iner GL) as the reference one due to the fact that this stationary phase in accordance to knowledge database was recommended by the Ph. Eur., the *F*-values for other tested columns were calculated according to Eqs. (1) and (2) (http://pharm.kuleuvan.be/pharmchem/Pages/ccs.html). The obtained four column parameters and data set of *F*-values for all tested stationary phases are presented in Table 2. To each column, a number from 1 to 36 was assigned according to the position in the column ranking.

2.3.2. Column performance for separation of alfuzosin

The practical test of KUL classification system was performed on 36 RP-LC C18 stationary phases during the separation of the samples containing alfuzosin hydrochloride, impurity A and impurity D prepared in accordance with the system suitability test (SST) prescribed in Ph. Eur. [2], in the chromatographic conditions described in Section 2.2.2. The sample of alfuzosin and related compounds was prepared by the dissolution of 4 mg of *alfuzosin for system suitability CRS* in 10 mL of the mobile phase. Thus, the active substance in the presence of 0.5% of its two impurities was analyzed for a verification of the SST test. For each column, three runs were carried out. The representative chromatograms from the sample performed for Iner GL, Nuc HD and Sph ODS1 are shown in Fig. 2A–C, respectively. Finally, for all C18 RP-LC stationary phases, the t_R and R_s of alfuzosin and its two impurities A and D as well as A_s the of peaks of interest obtained during LC separations were calculated. Moreover, the peak-to-valley (p/v) ratios were evaluated in accordance to the SST described in the Ph. Eur. All experimental data for the compounds of interest are reported in Table 3.

2.4. Data treatment

The comparative study of the theoretical results of column classification system for 36 stationary phases based on the KUL test procedure and its application in pharmaceutical practice for the separation of alfuzosin and related compounds was evaluated in order to check how KUL method could be used to facilitate RP-LC column selection in the considered pharmaceutical analysis.

In this assay, for a more readable interpretation of the results containing many variables and objects, multivariate data processing technique such as principal component analysis (PCA) and cluster analysis (CA) were used by employing a Statistica 9.0 software (StatSoft, Tulsa, USA). At the beginning, PCA for four column parameters: $rk'_{\mathrm{ba/ph\ pH2.7}}$, $k'_{2,2'\text{-d}}$, k'_{amb} and $rk'_{\mathrm{tri/o\text{-}ter}}$ calculated for 36 tested columns was performed. For this analysis, the numbers from 1 to 36 were assigned for the tested stationary phases in accordance to the *F*-values provided by the KUL method (Table 2). The obtained two-dimensional loading and score PCA plots are shown in Figs. 3 and 4A and B, respectively. The result of CA in form of a tree diagram for the tested stationary phases is presented in Fig. 5. This CA was performed to verify the similarities and dissimilarities of the tested RP-LC columns already obtained with the help of PCA. Finally, the experimental data set including t_R of alfuzosin and related compounds, and R_s of the peaks of interest for 36 brands of tested stationary phases (Table 3) were evaluated by both PCA and CA. In these analyses, the same numeration of the tested columns was

Table 1
List of RP-LC columns examined in this study and their properties as provided by the manufacturer.

Manufacturer/supplier	Name of the column	Length [mm]	Internal diameter [mm]	Particle size [μm]	Carbon load [%]	Pore size [Å]	Surface area [m^2/g]	Silica[a]	Endcap.	Abbreviation
ACT	ACE 5 AQ	150	4.6	5	14	100	300	EP	+	AQ
ACT	ACE 5 C18	150	4.6	5	15.5	100	300	B	+	C18
ACT	ACE 5 C18-AR	150	4.6	5	15.5	100	300	B	+	C18-AR
ACT	ACE 5 C18-HL	150	4.6	5	20	90	400	B	+	C18-HL
Agilent	Zorbax Eclipse XDB C18	150	4.6	5	10	80	180	B	+	Zor Ecl XDB
Agilent	Zorbax SB-Aq	150	4.6	5	Proprietary	80	180	EP	–	Zor SB-Aq
Agilent	Zorbax SB-C18	150	4.6	5	10	80	180	B	–	Zor SB-C18
Akzo Nobel	Kromasil 100-5 C18	150	4.6	5	19	100	340	B	+	Krom
GL Science	Inertsil ODS2	150	4.6	5	18.5	150	320	B	+	Iner GL
Hichrom	Inertsil ODS2	150	4.6	5	18.5	150	320	B	+	Iner HI
Macherey-Nagel	Nucleodur C18 Isis	150	4.6	5	20	110	340	B	+	Nuc Isis
Macherey-Nagel	Nucleodur C18 Pyramid	150	4.6	5	14	110	340	B	+	Nuc Pyr
Macherey-Nagel	Nucleodur Sphinx RP	150	4.6	5	15	110	340	B	+	Nuc Sph
Macherey-Nagel	Nucleosil 100-5 C18 AB	150	4.6	5	24	100	350	A	+	Nuc AB
Macherey-Nagel	Nucleosil 100-5 C18 HD	150	4.6	5	20	100	350	A	+	Nuc HD
Macherey-Nagel	Nucleosil 100-5 C18 Nautilus	150	4.6	5	16	100	350	EP	+	Nuc Nau
Macherey-Nagel	Nucleosil 100-5 C18	150	4.6	5	15	100	350	A	+	Nuc C18
Phenomenex	Aqua C18	150	4.6	5	15	125	320	B	+	Aqua
Phenomenex	Luna C18	150	4.6	5	17.5	100	400	B	+	Luna
Phenomenex	Prodigy ODS3	150	4.6	5	15.5	100	450	B	+	Prod
SGE	Wakosil II 5 C18 HG	150	4.6	5	15	120	300	B	+	Wak HG
Supelco	Discovery C18	150	4.6	5	12	180	200	B	+	Disc
Supelco	Supelcosil C18 DB	150	4.6	5	11	120	170	A	+	Sup DB
Thermo	Aquasil C18	150	4.6	5	12	100	310	EP	+	Aquasil
Thermo	Hypersil BDS C18	150	4.6	5	11	130	170	A	+	BDS Hyp
Thermo	Hypersil Elite C18	150	4.6	5	15	114	250	A	+	Hyp Elite
Thermo	Hypersil Gold aQ	150	4.6	5	12	175	220	EP	+	Hyp Gold aQ
Thermo	Hypersil Gold	150	4.6	5	10	175	220	B	+	Hyp Gold
Waters	Spherisorb ODS1	150	4.6	5	6.2	80	220	A	–	Sph ODS1
Waters	Spherisorb ODS2	150	4.6	5	11.5	80	220	A	+	Sph ODS2
Waters	SunFire C18	150	4.6	5	16	100	340	B	+	SunFire
Waters	Symmetry C18	150	4.6	5	19	100	335	B	+	Sym
Waters	Symmetry Shield RP18	150	4.6	5	17	100	335	EP	+	Sym Shield
Waters	Xbridge C18	150	4.6	5	18	130	185	B	+	Xbrid
Waters	Xbridge Shield RP18	150	4.6	5	17	130	185	EP	+	Xbrid Shield
YMC	YMC Pack ODS-AQ	150	4.6	5	14.1	120	300	B	+	Pack AQ

[a] A – "traditional", acidic silica gel, B – "high purity", more neutral silica gel, EP – embedded or end-capped polar group.

Table 2
The column parameters and *F*-values for thirty six tested stationary phases provided by KUL test procedure.

Analytical column	Column parameters				*F*	The position in the ranking list (column No.)
	k'_{amb}	$rk'_{tri/o\text{-}ter}$	$rk'_{ba/ph\ pH\,2.7}$	$k'_{2,2'\text{-}d}$		
Iner GL	5.170	1.445	0.051	8.667	0.000	1
Nuc HD	5.099	1.482	0.093	8.049	0.130	2
Wak HG	**4.913**	**1.353**	**0.070**	**7.422**	**0.198**	**3**
C18	4.489	1.505	0.097	6.833	0.392	4
Krom	6.199	1.491	0.091	9.038	0.401	5
Sym	6.140	1.566	0.042	8.513	0.443	6
Zor Ecl	5.762	1.284	0.082	8.179	0.495	7
Aqua C18	4.932	1.277	0.096	9.243	0.515	8
5 C18-HL	6.369	1.535	0.087	8.950	0.555	9
Hyp Elite	**4.450**	**1.502**	**0.121**	**6.807**	**0.562**	**10**
Nuc Pyr	4.682	1.259	0.060	9.879	0.568	11
Prod	**5.476**	**1.246**	**0.078**	**8.416**	**0.588**	**12**
SunFire	5.479	1.231	0.038	9.124	0.641	13
Iner HI	**4.257**	**1.639**	**0.062**	**8.431**	**0.719**	**14**
Pack AQ	4.164	1.258	0.073	9.137	0.758	15
Luna	5.509	1.172	0.050	9.012	1.014	16
Zor SB-C18	4.281	1.212	0.094	8.810	1.027	17
Xbrid	3.361	1.392	0.128	5.518	1.538	18
C18-AR	3.483	1.698	0.099	8.180	1.722	19
BDS Hyp	**3.480**	**1.569**	**0.136**	**5.123**	**1.749**	**20**
Nuc Isis	5.420	1.827	0.061	8.650	1.935	21
Disc	2.780	1.447	0.087	4.408	2.130	22
AQ	2.232	1.322	0.077	7.271	2.548	23
Nuc C18	**3.360**	**1.634**	**0.115**	**14.430**	**2.594**	**24**
Sup DB	**3.053**	**1.320**	**0.215**	**5.709**	**3.178**	**25**
Nuc Nau	2.734	1.827	0.023	5.828	3.750	26
Hyp Gold aQ	1.882	1.268	0.089	3.877	4.033	27
Nuc Sph	2.738	1.000	0.073	9.688	4.202	28
Nuc AB	**3.658**	**1.964**	**0.097**	**6.021**	**4.463**	**29**
Aquasil	**2.971**	**1.825**	**0.163**	**15.163**	**5.193**	**30**
Zorbax SB-Aq	0.863	1.192	0.109	9.989	5.920	31
Hyp Gold	**2.299**	**2.040**	**0.118**	**7.128**	**7.119**	**32**
Sym Shield	3.771	2.212	0.022	6.575	8.413	33
Xbrid Shield	2.296	2.111	0.046	4.456	8.519	34
Sph ODS2	**4.044**	**1.657**	**0.366**	**17.275**	**8.870**	**35**
Sph ODS1	**2.041**	**1.865**	**0.334**	**23.641**	**16.421**	**36**

Meaning of symbols is explained in the text. The columns non-suitable for the separation of alfuzosin are indicated in bold.

continued as described in Table 3, Figs. 4A and B and 5. The obtained loading and score PCA plots are shown in Figs. 6 and 7A and B, respectively. Graphical result of CA for this data set is illustrated in Fig. 8.

3. Results and discussion

3.1. Column characterization by KUL method

In the KUL method, each stationary phase is characterized by four finally selected parameters, k'_{amb}, $rk'_{bh/ph\ pH\,2.7}$, $rk'_{tri/o\text{-}tert}$ and $rk'_{2,2'\text{-}d}$ which can be determined using three simple, fast, repeatable and reproducible methods [33]. The values of these parameters established for 36 tested columns are summarized in Table 2. When a Iner GL column was selected as a reference one, it was found, that 20 brands of stationary phases were considered as high ranked ($F<2$), ten other columns were classified as intermediate while five others were characterized by *F*-values > 6 which means, that their chromatographic properties are significantly different in comparison to the reference one. Among them Nuc HD was found as the most similar to the reference column while Sph ODS1 was placed in the lowest ranked position. It can be also noticed that higher values of k'_{amb} and lower differences of $rk'_{bh/ph\ pH\,2.7}$, $rk'_{tri/o\text{-}tert}$ and $rk'_{2,2'\text{-}d}$ parameters were observed for high ranked stationary phases. Thus, the Iner GL column and similar ones to it belong to the stationary phases with strong hydrophobic character, an intermediate possibility for steric selectivity and silanol activity. Next, details of the interpretation of the theoretical results of KUL column classification system for 36 selected stationary phases were provided by PCA and CA. PCA allows to reduce high-dimensional data containing the *n* variables to a few latent variables or principal components (PCs) in fewer dimensions without losing significant information. It means that these PCs can be considered as new axes drawn in the original *n*-dimensional space. Thus, PCA created the projections of the objects onto the PCs called as scores. The score plot indicates relationships between the objects and allows to identify those with closely related properties, *i.e.* the stationary phases with similar column characteristics. A loading plot represents the loadings of the variables on two of the PCs what allows to give information about the original variables (*e.g.* parameters of column characterization), their influence and importance on the PCs as well as to describe the correlation between these variables. The use of PCA for the evaluation of the similarities and dissimilarities of the RP-LC stationary phases has been reported already [9,11–14,16,29,31,36]. Cluster analysis belongs to a collection of statistical methods, in which the dissimilarities (similarities) or distances between objects are used for building the hierarchy from the individual elements by progressively merging clusters. In this hierarchical clustering, a dendrogram in the form of a tree diagram is produced to the visualization of the arrangement of the clusters (observations or individuals). In a dendrogram, the *y*-axis marks the distance at which the clusters merge, while the objects are placed along the *x*-axis such that the clusters do not mix. This chemometric method was seldom used in RP-LC column selection [8,13]. In this

Table 3
Summary of retention data set of t_R and R_s for alfuzosin hydrochloride and its impurities obtained using LC method for 36 modern tested stationary phases.

Analytical column	The position in the ranking list (column No.)	Analyzed substances								SST (p/v)
		Impurity D		Alfuzosin hydrochloride			Impurity A			
		t_R	A_s	t_R	R_s	A_s	t_R	R_s	A_s	
Iner GL	1	3.59	1.13	8.21	15.21	1.08	9.73	3.62	1.05	23.0
Nuc HD	2	3.51	1.46	8.11	14.36	1.16	9.54	3.21	1.17	5.7
Wak HG	**3**	**3.42**	**2.96**	**7.24**	**9.21**	**1.12**	**8.50**	**3.11**	**1.16**	**3.0**
C18	4	3.36	1.10	7.38	15.16	1.13	8.57	3.30	1.13	12.1
Krom	5	3.87	1.15	8.94	16.74	1.06	10.72	4.21	1.01	29.0
Sym	6	3.15	1.07	7.02	15.19	1.06	8.34	3.85	1.04	19.3
Zor Ecl	7	3.39	1.05	8.11	16.80	1.18	9.89	4.52	1.06	29.5
Aqua C18	8	4.52	1.13	11.94	20.42	1.17	14.13	4.03	1.09	12.1
5 C18-HL	9	3.80	1.05	8.71	16.38	1.08	10.42	4.07	1.04	21.6
Hyp Elite	**10**	**3.46**	**1.53**	**7.79**	**10.79**	**2.60**	**7.79**	**0.00**	**n.d.**[a]	**0.0**
Nuc Pyr	11	4.20	1.16	10.78	16.67	1.21	13.21	4.09	1.17	∞
Prod	**12**	**3.77**	**1.90**	**8.53**	**15.02**	**1.17**	**10.22**	**3.96**	**1.15**	**11.1**
SunFire	13	4.04	1.09	9.65	16.84	1.11	11.63	4.13	1.05	40.4
Iner HI	**14**	**3.57**	**1.63**	**8.01**	**12.30**	**1.28**	**9.65**	**3.09**	**1.22**	**3.6**
Pack AQ	15	4.34	1.15	12.02	22.62	1.15	14.27	4.52	1.03	35.8
Luna	16	4.02	1.35	9.64	17.45	1.16	11.65	4.46	1.12	17.4
Zor SB-C18	17	3.59	1.01	8.78	17.36	1.07	10.53	4.09	1.00	25.9
Xbrid	**18**	**3.23**	**1.53**	**7.97**	**16.78**	**1.14**	**10.76**	**6.74**	**1.16**	**5.80**
C18-AR	19	4.15	1.05	9.06	16.36	1.05	10.91	4.40	1.02	31.4
BDS Hyp	**20**	**3.09**	**1.24**	**6.75**	**13.39**	**1.11**	**7.82**	**2.64**	**1.15**	**3.0**
Nuc Isis	21	3.18	1.28	6.94	11.17	1.10	8.43	3.27	1.11	10.2
Disc	22	2.84	1.03	5.86	13.22	1.02	6.70	2.83	1.04	8.8
AQ	23	3.73	1.06	8.57	16.56	1.02	10.13	3.77	1.02	14.4
Nuc C18	**24**	**4.33**	**0.96**	**13.08**	**18.95**	**0.83**	**15.34**	**2.66**	**1.12**	**2.1**
Sup DB	**25**	**3.41**	**2.51**	**8.10**	**4.42**	**1.78**	**9.68**	**0.00**	**1.36**	**1.3**
Nuc Nau	26	5.89	0.92	14.36	14.70	0.91	19.74	5.64	0.99	∞
Hyp Gold aQ	27	2.95	1.07	6.76	15.33	1.10	7.86	3.18	1.06	7.5
Nuc Sph	28	4.56	1.44	10.66	14.82	1.17	13.16	4.32	1.09	33.7
Nuc AB	**29**	**2.78**	**1.88**	**5.48**	**10.16**	**1.18**	**6.45**	**2.76**	**1.25**	**2.7**
Aquasil	**30**	**5.02**	**1.02**	**17.52**	**20.99**	**1.15**	**17.52**	**0.00**	**n.d.**	**0.0**
Zorbax SB-Aq	31	4.93	1.05	14.98	22.10	1.22	18.01	4.27	1.02	∞
Hyp Gold	**32**	**2.77**	**1.12**	**5.37**	**11.75**	**0.98**	**6.03**	**2.31**	**1.06**	**4.4**
Sym Shield	33	3.58	1.11	8.83	15.81	1.15	11.91	6.19	1.07	∞
Xbrid Shield	34	2.84	1.25	5.57	11.56	1.15	6.49	3.09	1.02	∞
Sph ODS2	**35**	**4.79**	**1.24**	**14.78**	**16.52**	**2.87**	**17.80**	**0.00**	**1.60**	**1.1**
Sph ODS1	**36**	**5.15**	**1.55**	**20.76**	**19.50**	**4.09**	**20.76**	**0.00**	**n.d.**	**0.0**

[a] n.d. – non-determined.
Meaning of other symbols is explained in the text. The columns non-suitable for the separation of alfuzosin are indicated in bold.

paper, CA was based on Ward's method which uses an analysis of variance approach to evaluate the distances between clusters, and Chebychev distance was used for the definition of two objects as "different" if they are different on any one of the dimensions.

Fig. 3 illustrates the PCA loading plot derived with four column parameters, whereas the PC1–2 score plot is shown in Fig. 4A and B, respectively. For these two-dimensional loading and score plots, two first PCs explain more than 72.44% of the data variability. The variance of the analyzed data explored by the first PC1 (44.74%) is mainly involved with the variability of $rk'_{\text{bh/ph pH2.7}}$ and $rk'_{2,2'\text{-d}}$. These two variables can be found in the same cluster on the left of the middle of the loading plot while $rk'_{\text{tri/o-tert}}$ and k'_{amb} were placed as outliers on the left of the upper side of the graph ($rk'_{\text{tri/o-tert}}$) and on the right of the bottom of the plot (k'_{amb}) (Fig. 3). Their positions can be easily explained due to the fact that these parameters give different information characterizing chromatographic properties of the tested stationary phases. As it was above mentioned, $rk'_{\text{tri/o-ter}}$ evaluates the possibility for steric selectivity while k'_{amb} reflects hydrophobicity. The observed correlation between $rk'_{\text{bh/ph pH2.7}}$ and $k'_{2,2'\text{-d}}$ is related with the same information about silanol activity provided by both parameters. Additionally, the values of $k'_{2,2'\text{-d}}$ also are involved with metal impurities. In the score PCA plot presented in Fig. 4A and B, the numbers of the tested stationary phases from 1 to 36 were assigned in accordance to the F-values calculated by KUL test procedure (Table 2). As it is shown in Fig. 4A, the columns Nos. 35 and 36 with the lowest ranked positions were found as outliers on the left of bottom of the score plot. For them significantly higher values of $rk'_{\text{bh/ph pH2.7}}$ and $rk'_{2,2'\text{-d}}$ were calculated than for other stationary phases what can be easily explained by the fact that the type-A silica characterizing by high silanol activity were used (Table 1). Moreover, these stationary phases, similar to the columns Nos. 30 and 24 contained high level of the metal contaminants (Table 2). Some columns with F-values between 1.722 and 7.119 (Table 2) were noticed within cluster I (Nos. 19, 24, 25, 29, 30, 31 and 32) which was placed in the middle side of the score plot (Fig. 4A). Their column characterizations were described by high values of $rk'_{\text{bh/ph pH2.7}}$ (>0.097). Among them there are the stationary phases with type-A silica (Nos. 24, 25, 29), the columns having polar-endcapped stationary phase (Nos. 30, 31) as well as Hyp Gold with low carbon load and wider pore size (175 Å) (Table 1). In contrary, the stationary phases Nos. 26, 33 and 34 were observed in cluster II placed on the right of the upper corner of the graph. For them embedded polar group were introduced resulting in low values of $rk'_{\text{bh/ph pH2.7}}$ parameters below 0.046. However, small distances between clusters I, II as well as cluster III containing high and intermediate ranked stationary phases means that these differences were not significant. For the easier data interpretation this part of plot in which cluster III was placed, has been enlarged and shown in Fig. 4B. It can be noticed that most of the columns Nos. 1–17 were found within

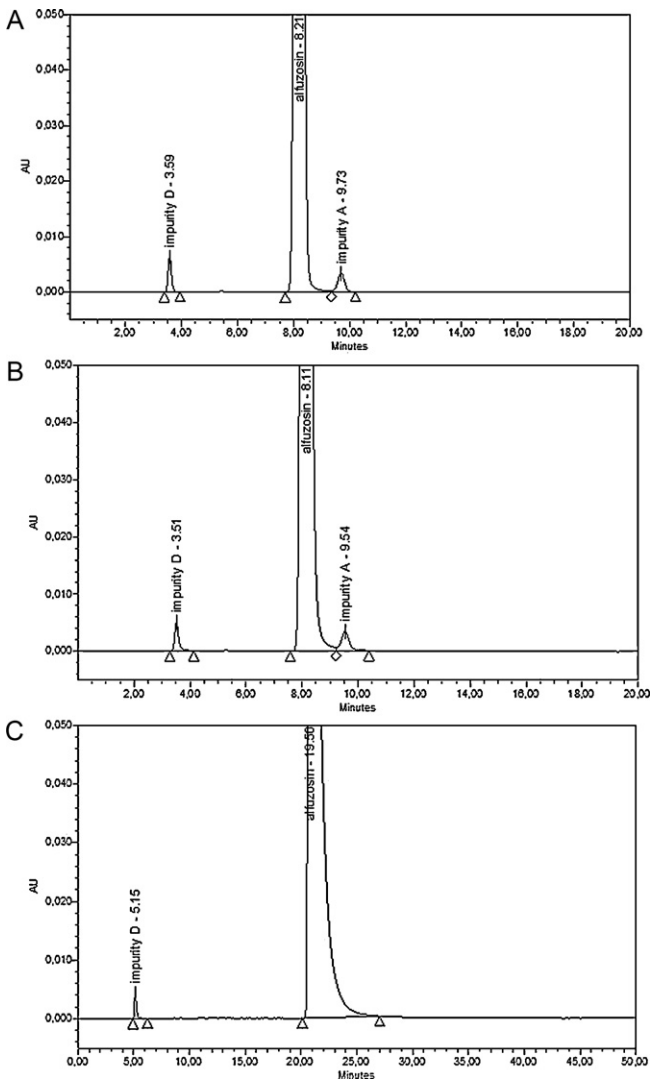

Fig. 2. Separation of alfuzosin hydrochloride and its impurities A and D performed on (A) Iner GL, (B) Nuc HD and (C) Sph ODS 1 column.

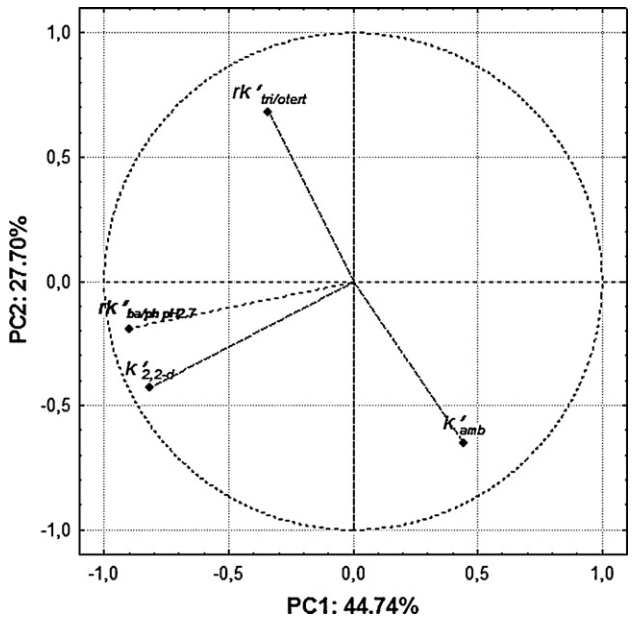

Fig. 3. Two-dimensional PCA loading plot based on the four chromatographic parameters determined in KUL method for thirty-six tested RP-LC columns.

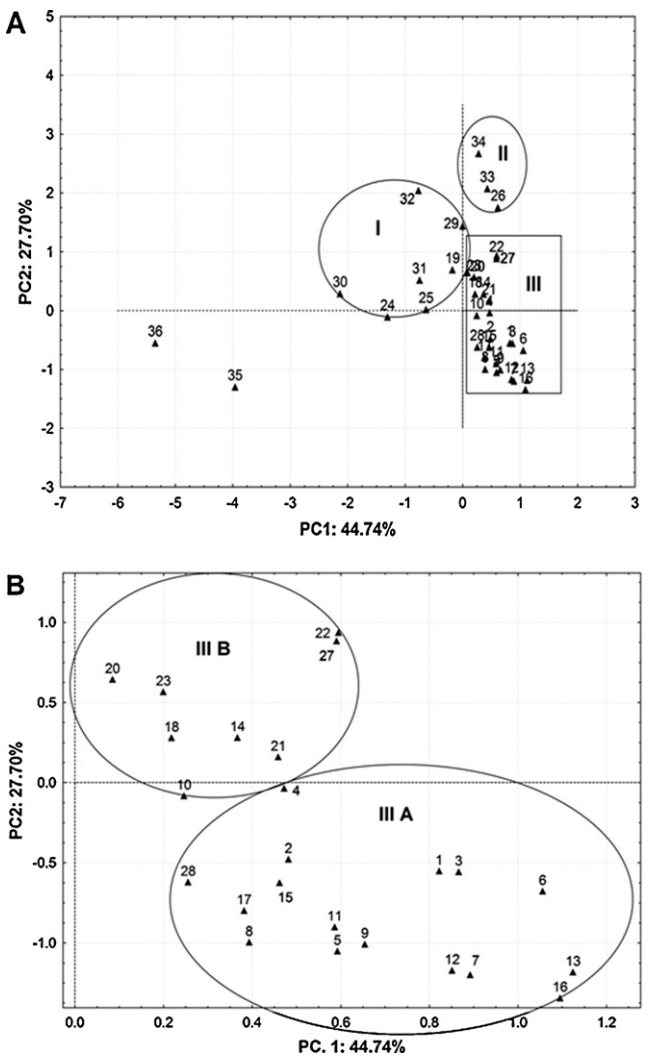

Fig. 4. (A) Projection of 36 RP-LC columns onto the space of the first two PCs (PC1 and PC2) from PCA of four column parameters provided by KUL method and (B) Cluster III, from (A), enlarged.

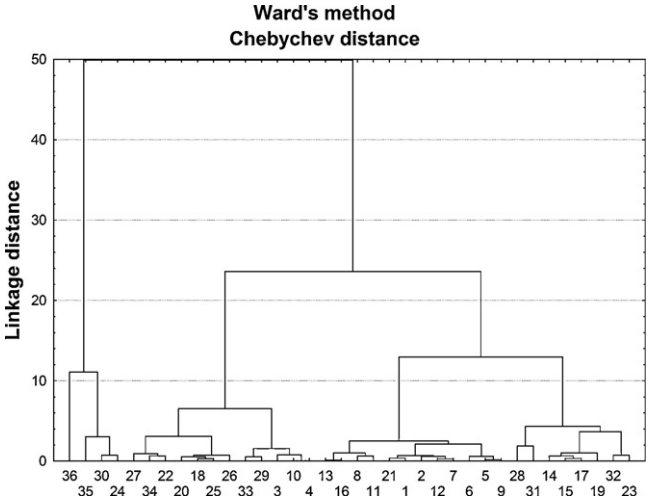

Fig. 5. Tree diagram for thirty-six tested RP-LC columns extracted from CA of four column parameters provided by KUL method.

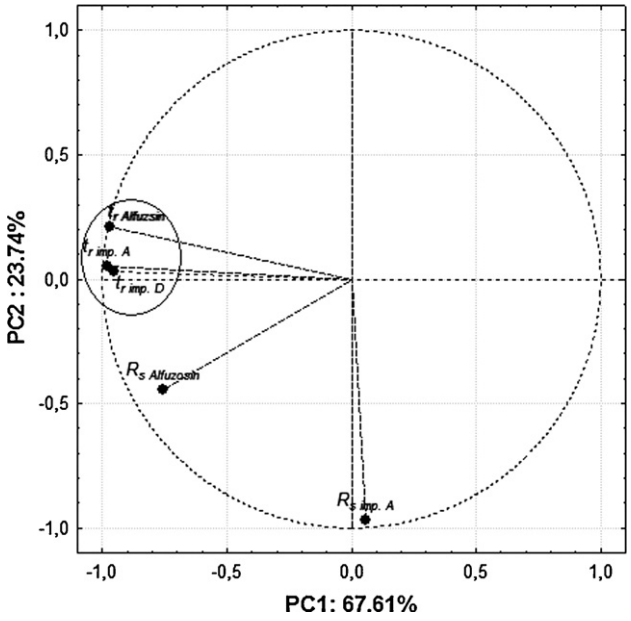

Fig. 6. Two-dimensional PCA loading plot based on based on the parameters t_R and R_s of the analytes obtained in the column performance test.

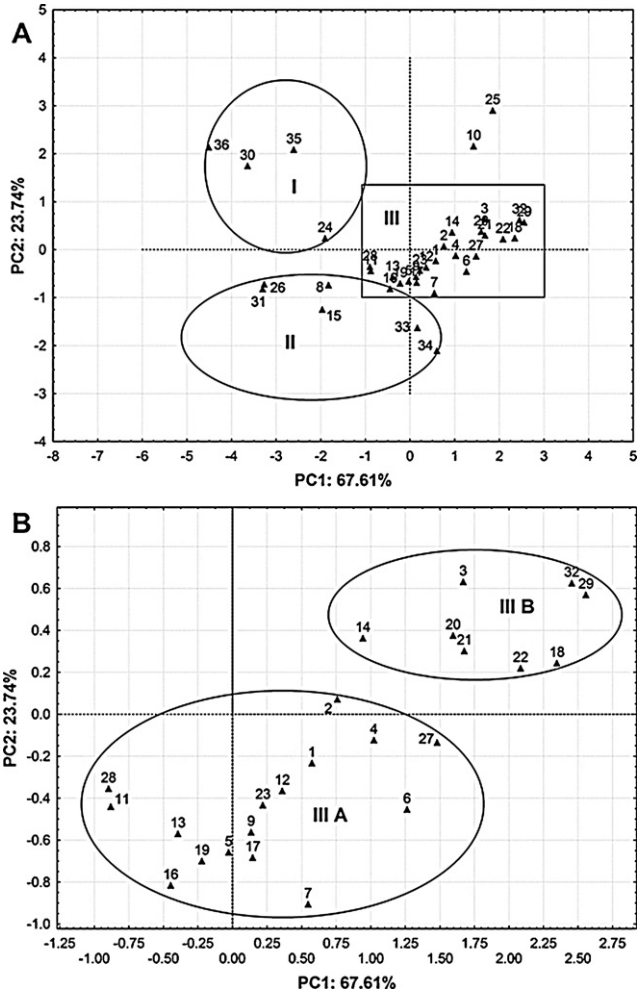

Fig. 7. (A) Projection of 36 RP-LC columns onto the space of the first two PCs (PC1 and PC2) from PCA of the retention parameters t_R and R_s of the analytes obtained in the column performance test. (B) Cluster III, from (A), enlarged.

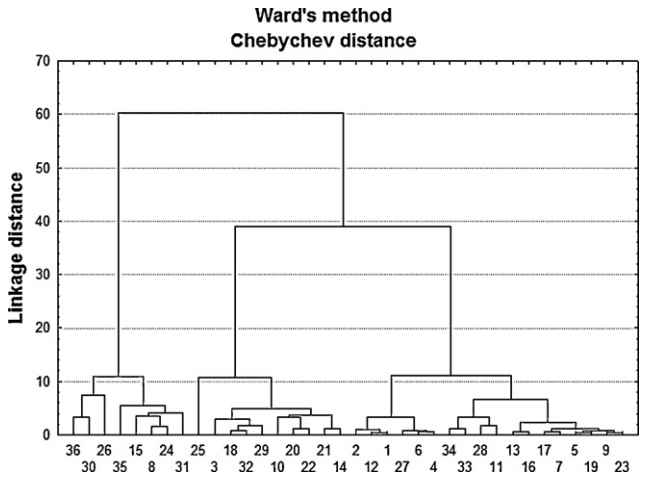

Fig. 8. Tree diagram for thirty-six tested RP-LC columns extracted from CA of the parameters t_R and R_s of the analytes obtained in the column performance test.

cluster III A in the lower part of the plot while the stationary phases Nos. 2, 3, 4 and 6 were found close to the reference column (No. 1), and other ones with *F*-values between 0.495 (No. 7) and 1.027 (No. 17) were observed at a small distance from the reference column in the bottom of the graph. The column No. 28 having *F* of 4.202 was also found between them. Other stationary phases with Nos. 20–23 as well as the columns Nos. 10, 14, 18 and 27 were present in cluster III B, on the upper side of the score plot. For them, more significant differences of $rk'_{\mathrm{bh/ph\ \ pH\,2.7}}$ parameters were observed in comparison to the columns with higher positions in the ranking list established by the KUL test. When this data set was analyzed by CA, similar results as observed for PCA were noticed (Fig. 5). The dendrogram for 36 tested stationary phases clearly confirmed that the columns Nos. 36 and 35 characterized different chromatographic properties because they were placed the furthest on the left side of the tree diagram. On the other hand, they were found near the columns Nos. 30 and 24 what indicates some similarities between them. The stationary phases Nos. 18, 20, 22, 25, 26, 27, 33 and 34 were observed in the same localization of the dendrogram on the left of the graph what is in accordance to the results of PCA (these columns with except of No. 25 were found within the clusters II and the upper side of the cluster III B – Fig. 4A and B, respectively). Moreover, the stationary phases observed in cluster III A by PCA (Fig. 4B) were together on the right of the CA plot (Fig. 5). On the other hand, the columns Nos. 19, 31, 32 and 21 were located by CA on the right of the tree diagram while their position by PCA was different (cluster I and III A – Fig. 4A and B, respectively). However, the results of both PCA and CA generally confirmed that the localization of the tested columns is in accordance to *F*-values provided by the KUL test procedure. Therefore, the *F* parameter established in the KUL method can be successfully used for column characterization [31–36]. So far, this direct way to describe the tested columns by *F*-values using chemometric analyses of the KUL results was not used.

3.2. Column selection in pharmaceutical separation

Chromatographic separation of alfuzosin and its two impurities A and D was performed on 36 new RP-LC C18 stationary phases according to the Ph. Eur. monograph [2] as a practical test of the KUL column classification system in pharmaceutical practice. In the Ph. Eur. monograph description, mobile phase composition, flow rate, injection sample and detector wavelength were exactly defined for this LC analysis, while the type of the stationary phase

only was characterized as *end-capped octadodecylsilyl silica gel for chromatography R (5 μm)*. On the other hand, for this separation an Inertsil column is recommended by the Ph. Eur., as described in the knowledge database. However, other stationary phases, which comply with the SST, are also allowed to be used. For alfuzosin, the SST requires a minimum peak-to-valley (p/v) ratio of 5.0 for the "critical pair" of impurity A and alfuzosin. The second SST requirement mentions relative retention time about 8 min for alfuzosin and about 0.4 and 1.2 for impurity D and A as compared to the main compound, respectively. Unfortunately, the use of relative retention times is problematic as discussed elsewhere [30,33]. Thus, this parameter was not considered as adequate to evaluate the quality of the separations during LC analysis. Moreover, the Ph. Eur. requires that in a related substances test or assay, the symmetry factor (A_s) of 0.8–1.5 for a peak in the chromatograph obtained with a reference solution was used for quantification, unless other prescribed requirements are introduced. Due to the fact the symmetry parameter has been not described in the SST for alfuzosin, this range of A_s is also required in LC separation of this active substance from its related compounds.

The obtained experimental data set of t_R, R_s and A_s for alfuzosin and its impurities as well as the results of the SST for 36 tested stationary phases are presented in Table 3. It can be noticed, that the t_R of the analytes of interest were shorter for sixteen tested columns in comparison to the reference one, while the longest t_R values were observed for Aquasil (No. 30) and Sph ODS 1 (No. 36). The A_s of the impurities D and A ranged from 0.92 to 2.96 and from 0.99 to 1.60 whereas for active substance were between 0.83 and 4.09, respectively. It can be also noticed that the range limit of A_s for related compounds required by the Ph. Eur. has been extended by eight stationary phases. Moreover, a p/v ratio of 23.0 was found for the reference column whereas for other tested stationary phases values were found between ∞ for completely separated peaks (Nos. 11, 26, 31, 33, 34) and 0 for non-separated ones (Nos. 10, 30, 36). Therefore, the LC assay of alfuzosin and related compounds could give appropriate results in accordance to the SST requirements for fifteen tested columns with $F < 2$ (71.4%), six stationary phases described as intermediate in the KUL list ranking (50%), and two ones having F-values > 6 (30%). These results are similar to those previously reported in publications [28,32,33,36]. For deeper analysis of the correlation between the KUL test results to characterize or classify RP-LC stationary phases and the column performance in a real pharmaceutical separation PCA and CA for a raw experimental data set of t_R and R_s of the compounds of interest were performed. The obtained loading and score PCA plots are shown in Figs. 6 and 7A and B, while the tree diagram for 36 tested stationary phases created by CA is illustrated in Fig. 8, respectively. It can be noticed that the positions of the tested columns on the PC1 axis mainly were related to the t_R of the analyzed analytes (these variables were placed in the same cluster on the left of the middle of the loading plot – Fig. 6) whereas on the PC2 axis the positions were related to the differences of R_s. These variables were placed as outliers on the bottom of the middle of the loading plot ($R_{s\ imp.\ A}$) and in case of $R_{s\ Alfuzosin}$ between the cluster described above and variable of $R_{s\ imp.\ A}$, respectively. For the loading and score plots, PC1 and PC2 explained more than 91.35% of the data variability. In the score PCA plot shown in Fig. 7A three clusters containing the columns Nos. 36, 35, 30 and 24 (cluster I); 31, 26, 15, 8, 33 and 34 (cluster II) and other stationary phases (cluster III) could be observed. Moreover, the columns Nos. 10 and 25 were found as outliers on the right of the upper side of the graph. Significant higher values of t_R for analyzed compounds, especially for active substance as well as significant higher values of R_s for alfuzosin were observed for the stationary phases placed within cluster I. However, insufficient R_s for impurity A ranging from 0 to 2.66 caused that the use of these columns to be impossible, what also has been confirmed by the SST values < 5. $R_{s\ imp.\ A}$ of 0 and $A_s > 1.5$ were also observed for the stationary phases Nos. 10 and 25 resulting in the SST below the required limits for these separations were observed. Moreover, shorter t_R for the analytes were noticed for these columns in comparison to cluster I. In the KUL method, these columns had been characterized by intermediate k'_{amb} values and high $rk'_{ba/ph\ \ pH\,2.7}$ parameters (Table 2). In the score PCA plot shown in Fig. 7A cluster II was placed in the middle of the lower part of the graph. In the KUL method, these stationary phases were characterized by low or intermediate $rk'_{ba/ph\ \ pH\,2.7}$ values as the effect of the use of embedded or end-capped polar group. It is important to emphasize here that with cluster II columns better chromatographic separation of alfuzosin and related compounds was achieved than with the reference column. Thus, low silanol activity of stationary phases can be considered as an important attribute for effective alfuzosin separation. Therefore, it should also be noticed, that the KUL method could be considered as a valuable starting point for RP-LC method development.

As illustrated in Fig. 7A, twenty-four stationary phases were found within cluster III which was placed in the middle of the score plot. This part of the score plot was enlarged and shown in Fig. 7B. Two subclusters can be noticed here where subcluster III A was placed in the lower part of the graph whereas subcluster III B was located in the upper part of the score plot. The stationary phases with except of Prod column (No. 12) included in subcluster III A were suitable for the separation of alfuzosin. Almost all mentioned columns can be also noticed in cluster III A presented in Fig. 4B. Moreover columns (Nos. 14, 18, 20, 21 and 22) were included in both clusters III B shown in Figs. 4B and 7B, respectively. The columns Nos. 3, 29 and 32 were also placed in subcluster III B (Fig. 7B). These stationary phases, similar to Nos. 14 and 20, were not suitable for the LC analysis of alfuzosin.

Thus, the results of PCA for the theoretical data set of KUL classification system based on the four test parameters and column performance for alfuzosin separation performed in accordance to Ph. Eur. monograph were well related. Additionally, the PCA results have been confirmed by CA. Fig. 8 illustrates the dendrogram for 36 tested stationary phases based on the data describing the practical application in a real pharmaceutical separation. It can be observed that the columns Nos. 36, 35, 30 and 24 were found on the left of the tree diagram together with the stationary phase Nos. 26, 15, 8 and 31. In PCA, these columns were placed in clusters I and II, respectively (Fig. 7A). In CA, the second cluster was found in the middle of the plot (Fig. 8) and included all stationary phases of cluster III B shown in Fig. 7B, and the column No. 10 placed as outlier in Fig. 7A. All stationary phases from cluster III A (Fig. 7B) were placed by CA on the right of the dendrogram (Fig. 8). Contrary to PCA, the columns Nos. 34 and 33 were found within cluster III A whereas their localization in PCA was different (cluster II). However, the position on PC1 axis in accordance to the shorter t_R of the analytes could suggest some similarities between them and the stationary phases placed within cluster III A. When the results of CA for column performance in the separation of alfuzosin were compared to the theoretical data set provided by the KUL method, significant similarities between tested columns could be noticed. In both CA plots, fourteen suitable columns for LC analysis of alfuzosin and related compounds were placed on the right of the tree diagram while ten non-suitable stationary phases for this separation were found on the left of the plot. The columns Nos. 22 and 26 which gave appropriate results for chromatographic separation of alfuzosin were found on same side of the dendrograms presented in Figs. 5 and 8. Only nine from thirty-six (Nos. 8, 14, 15, 21, 27, 31, 32, 33, and 34) were placed in different positions on the tree diagrams based on the four test parameters and column performance for alfuzosin separation. Thus, only 25% of the tested columns

were not suitably characterized and classified by the KUL test procedure.

4. Conclusions

In this paper, column classification system based on the KUL test procedure was correlated with the selectivity of the separation of alfuzosin hydrochloride and its related compounds. The suitability of tested stationary phases was evaluated by calculation of the SST in accordance to the Ph. Eur. requirement. The SST values established for 36 tested stationary phases were compared to each other and correlated with the systematic information extracted by PCA and CA from the set of four chromatographic parameters and the column performance in a real pharmaceutical separation. The obtained data confirmed that *F*-values provided by KUL method and the positions of 36 tested stationary phases on both PCA and CA plots were significantly correlated. It shows that the KUL approach provides quantitative column characteristics indicating similarities and differences in retention properties which allows, with a relatively good certainty, to classify suitably modern RP-LC columns. Thus, the KUL approach with the combination of PCA and/or CA can predict the suitability of a column required for real separation of alfuzosin and its impurities. Moreover, in this paper the list of appropriate stationary phases for LC analysis of alfuzosin and related compounds offering equivalent separation to Inertsil column was included, what from a practical point of view can be attractive for pharmaceutical industry. Moreover, the KUL method can properly evaluate the interactions between the analyte and stationary phase what allows to consider the test procedure based on *F*-values as a valuable starting point for RP-LC method development.

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