Journal of Pharmaceutical and Biomedical Analysis 78–79 (2013) 161–169

Contents lists available at SciVerse ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

journal homepage: www.elsevier.com/locate/jpba

Classification of LC columns based on the QSRR method and selectivity toward moclobemide and its metabolites

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ARTICLE INFO

Article history:
Received 11 October 2012
Received in revised form 30 January 2013
Accepted 4 February 2013
Available online 21 February 2013

Keywords:
Column classification system
Quantitative structure–retention relationships
Liquid chromatographic analysis
Moclobemide and metabolites
Principal component analysis
Cluster analysis

ABSTRACT

This paper focuses on a comparative study of the column classification system based on the quantitative structure-retention relationships (QSRR method) and column performance in real biomedical analysis. The assay was carried out for the LC separation of moclobemide and its metabolites in human plasma, using a set of 24 stationary phases. The QSRR models established for the studied stationary phases were compared with the column test performance results under two chemometric techniques – the principal component analysis (PCA) and the hierarchical clustering analysis (HCA). The study confirmed that the stationary phase classes found closely related by the QSRR approach yielded comparable separation for moclobemide and its metabolites. Therefore, the QSRR method could be considered supportive in the selection of a suitable column for the biomedical analysis offering the selection of similar or dissimilar columns with a relatively higher certainty.

1. Introduction

The rapid development of the reversed-phase liquid chromatography (RP-LC) nowadays has elevated this particular separation technique to the position of one of the most powerful analytical methodologies used for drug analysis. According to the recent estimates over 600 different RP columns are commercially available and the number continues to grow [1,2]. Of course, a large variety of RP stationary phases offers better chemical stability, and the improved efficiency and reproducibility enables selection of the most appropriate column for the specific separation. On the other hand, many analysts confront the problem of selecting the appropriate column because their nominally identical structures may suggest similar chromatographic properties. However, because of the different packing material, the storage time, or usage the RP-LC columns often demonstrate a unique character which causes that chromatographic performances in pharmaceutical and biomedical analyses are different and difficult to reproduce.

Literature reports numerous tests and RP-LC phase characterization attempts by chromatographic methods, undertaken to overcome the problem of inappropriate column selection [3–12]. These include the Katholieke Universiteit Leuven method reported by the group of Hoogmartens and co-workers [13–15], and the method based on the sum of the ranking differences (SRD) published by Héberger [16,17]. Alternative approaches to the LC column selectivity based on mathematical models have been provided, to name e.g. the hydrophobic-subtraction (HS) model by the Snyder-Dolan group [18,19], or the linear solvation-energy relationship (LSER) proposed by Abraham [20], or the quantitative structure-retention relationships (QSRRs) delivered by Kaliszan and co-workers [21–23]. In the QSRR approaches, the relationships between the chromatographic parameters (y) and the structure-related variables (descriptors, x_1, x_2, . . ., x_m) corresponding to the molecular structure of a given set of solutes selected to characterize the columns and quantify their separation properties are statistically derived by the multiple linear regression model in the following form:

$$y = \beta_0 + \beta_1 x_1 + \cdots + \beta_m x_m \quad (1)$$

where β_0 is constant, while $\beta_1 \ldots \beta_m$ are the respective regression coefficients [11].

The popular QSRR model reflects $\log k_w$ as the retention factor k of the analyte, extrapolated to a virtual mobile phase composition with 0% organic modifier against the theoretically calculated logarithms of n-octanol/water partition coefficients (clog P) reflecting the hydrophobic properties of the analyte:

$$\log k_w = a_1 + a_2 \mathrm{clog}P \quad (2)$$

where a_1 and a_2 are the regression coefficients [22].

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http://dx.doi.org/10.1016/j.jpba.2013.02.020

Table 1
List of the RP-LC stationary phases studied and their characteristics as provided by manufacturer.

Name of the column	Manufacturer/supplier	Length (mm)	Internal diameter (mm)	Particle size (μm)	Abbreviation
Nucleosil 100-5 C18	Knauer	125	4.0	5	N18-125/5
Nova-Pack C18	Waters	150	3.9	4	NOP-150
Luna	Phenomenex	150	4.6	3	LUN-150
Nucleosil 100-5 C18	Knauer	125	4.0	10	N18-125/10
Aqua C18	Phenomenex	250	4.6	5	AQU-250
Inertsil C8	MZ-Analysentechnik GmbH	250	4.6	5	IN8-250
Discovery HS-C18	Supelco	150	4.6	5	DIH-150
Symmetry C8	Waters	250	4.6	5	SY8-250
Varian C18	Varian	150	4.6	5	VAR-150
BDS-Hypersil C18	Thermo	100	4.6	3	BDH-100
Synergi Polar-RP	Phenomenex	150	4.6	4	SYP-150
Nucleosil 100-7 C8	Macherey-Nagel	250	4.0	5	NU8-250
Inertsil ODS-3	Phenomenex	250	4.6	5	IO3-250
Synergi-Max-RP	Phenomenex	150	4.6	4	SYM-150
Synergi Fusion- RP	Phenomenex	250	4.6	4	SYF-250
Gemini-NX C18	Phenomenex	150	4.6	5	GEN-150
Symmetry Shield RP18	Waters	150	4.6	5	SS18-150
Symmetry Shield RP8	Waters	250	4.6	5	SS8-250
Symmetry C18	Waters	250	4.6	5	S18-250
Nucleosil 100-5 C18 AB	Macherey-Nagel	250	4.0	5	NAB-250
Inertsil ODS-2	Hichrom	150	4.6	5	IO2-150
Unison US-C18	Imtakt	125	4.6	5	UNU-125
Nucleosil 100-5 C18 HD	Macherey-Nagel	250	4.0	5	NHD-250
Fluophase PFP	Thermo	150	4.6	5	FLP-150

The log k_w-values for the analyte can be calculated from two gradient runs by solving a set of two equations proposed by Snyder and Dolan [24]. These calculations can be performed automatically using the professional DryLab software package [25].

Another QSRR model based on the molecular modeling-derived descriptors such as the total dipole moment μ, the electron excess charge of the most negatively charged atom δ_{Min}, and, the water-accessible molecular surface area A_{WAS} takes the following form:

$$\log k_w = a'_1 + a'_2\mu + a'_3\delta_{Min} + a'_4 A_{WAS} \quad (3)$$

where a'_1 to a'_4 are the regression coefficients, μ accounts for the dipole–dipole and dipole-induced dipole interactions of the analyte, δ_{Min} accounts for the polar interactions of the analyte, and A_{WAS} accounts for the evaluation of the strength of the London-type interactions of the analyte [21–23].

The literature offers a few overviews of the QSRRs in investigations of the retention properties of the RP-LC columns [26–28]. Bączek et al. [29] established stronger correlations between the theoretical column classifications based on the HS method and the QSRR models calculated for nine tested stationary phases. A poor match between the KUL test parameters and the regression coefficients calculated by the QSRR models for fifteen RP-LC stationary phases was reported for chromatographic analysis of steroids in urine samples [30]. On the other hand, it has also been found that an analyst can make a choice of an adequate stationary phase with a comparable, relatively high certainty under both the KUL test procedure, and the QSRR approach. Hence, the tested classification systems based on mathematical models and the KUL ranking system yield comparable results, even though their matching is not exactly the same. A comparative study of the RP-LC column classification based on the QSRR approach and column selectivity obtained in real pharmaceutical analysis toward lidocaine hydrochloride, tribenoside and their impurities was also reported [31]. Yet, as far as we know no application of the QSRR method in a real selectivity study involving an active substance and its metabolites in biological fluid has been reported.

Therefore, the reliability of the QSRR-model results in evaluation of the properties of the RP-LC stationary phases were examined against column performance in a real biomedical analysis. In the case study, an analysis of moclobemide (M0) and its two metabolites: Ro 12-5637 (M1) and Ro12-8095 (M2) in human plasma samples in accordance with the LC–UV method reported in Ref. [32] was performed on 24 stationary phases previously tested under the QSRR conditions. Subsequently, the raw retention parameters of the analyzed compounds such as the retention times (t_R), and resolutions (R_s) of the peaks of interest clearly distinguishing each real biomedical separation, were calculated for all examined stationary phases. Then, a verification test using two chemometric methods, namely principal component analysis (PCA) and hierarchical clustering analysis (HCA), was conducted to check whether the column classes closely related in terms of their QSRR characteristics showed similar separation for M0 and its two metabolites. The test was performed to check whether the QSRR approach could be used to facilitate the RP-LC column selection in the considered biomedical analysis. For clearer interpretation of the obtained results, the stationary phases in both data sets were assigned the same numbers from 1 to 24.

2. Material and methods

2.1. Column examination

A set of 24 RP-LC stationary phases delivered by manufacturers or distributors was investigated in this study. Their specifications are presented in Table 1.

2.2. QSRR method

2.2.1. Chemicals

The test analytes used for the construction of the QSRR models, including biphenyl, 2,2′-dinaphthyl ether, indazole, naphthalene, and 2-naphthol, were obtained from Lancaster (Newgate, UK); anisole, benzamide, benzonitrile, benzyl chloride, 4-cyanophenol, indole, 1-naphthylacetonitrile, pyrene, and phenanthrene as well as uracil were supplied by Sigma–Aldrich (St. Louis, MO, USA), while benzene was obtained from POCH (Gliwice, Poland). The HPLC grade of methanol was purchased from Merck (Darmstadt, Germany). All chemicals were used as received without further purification. Water was pre-treated in the Milli-Q Water Purification System (Millipore Corporation, Bedford, MA, USA).

Table 2
Coefficients a_1 and a_2 (±standard deviations) with their significance levels, p (underneath in parenthesis), and statistical parameters, r, s, Fisher's F-test and p (see text for explanation), of the regression equations of the forms: $\log k_w = a_1 + a_2$ clog P, provided by the QSRR for twenty four tested columns.

No.	Analytical column	a_1	a_2	r	s	Fisher's F-test	p
1	N18-125/5	0.029 (±0.089)	0.949 (±0.028) (p = 6E−14)	0.9942	0.150	1105.9	6E−14
2	FLP-150	0.210 (±0.126)	0.902 (±0.041) (p = 1E−11)	0.9870	0.214	491.6	1E−11
3	NAB-250	0.109 (±0.112)	0.891 (±0.036) (p = 3E−12)	0.9895	0.190	609.9	3E−12
4	SS18-150	0.355 (±0.158)	0.736 (±0.051) (p = 2E−09)	0.9703	0.268	209.1	2E−09
5	DIH-150	0.514 (±0.095)	0.750 (±0.030) (p = 3E−12)	0.9895	0.160	607.2	3E−12
6	NHD-250	0.307 (±0.082)	0.822 (±0.026) (p = 1E−13)	0.9934	0.1393	969.2	1E−13
7	LUN-150	0.362 (±0.122)	0.874 (±0.039) (p = 10E−12)	0.987	0.206	499.0	9E−12
8	SYP-150	0.207 (±0.110)	0.917 (±0.035) (p = 1E−12)	0.9905	0.186	674.5	1E−12
9	VAR-150	0.445 (±0.112)	0.722 (±0.036) (p = 4E−11)	0.9842	0.190	401.0	4E−11
10	**NOP-150**	**0.054 (±0.103)**	**0.941 (±0.033) (p = 4E−13)**	**0.9921**	**0.174**	**809.9**	**4E−13**
11	**N18-125/10**	**0.184 (±0.060)**	**0.912 (±0.019) (p = 6E−16)**	**0.9971**	**0.101**	**2247.5**	**6E−16**
12	**NU8-250**	**0.002 (±0.105)**	**0.786 (±0.034) (p = 5E−12)**	**0.9883**	**0.177**	**546.8**	**5E−12**
13	**SYF-250**	**0.439 (±0.076)**	**0.786 (±0.024) (p = 8E−14)**	**0.9938**	**0.128**	**1042.2**	**8E−14**
14	**SY8-250**	**0.487 (±0.104)**	**0.754 (±0.034) (p = 9E−12)**	**0.987**	**0.177**	**505.5**	**9E−12**
15	**BDH-100**	**0.095 (±0.106)**	**0.856 (±0.034) (p = 2E−12)**	**0.989**	**0.180**	**632.2**	**2E−12**
16	**AQU-250**	**0.646 (±0.235)**	**0.806 (±0.075) (p = 8E−08)**	**0.9474**	**0.398**	**113.9**	**8E−08**
17	**SS8-250**	**0.273 (±0.098)**	**0.794 (±0.031) (p = 2E−12)**	**0.9899**	**0.166**	**633.2**	**2E−12**
18	**IO2-150**	**0.240 (0.092)**	**0.867 (±0.029) (p = 3E−13)**	**0.992**	**0.157**	**852.9**	**3E−13**
19	**GEN-150**	**0.237 (±0.095)**	**0.862 (±0.030) (p = 5E−13)**	**0.9919**	**0.161**	**798.6**	**5E−13**
20	**UNU-125**	**0.670 (±0.118)**	**0.622 (±0.038) (p = 5E−10)**	**0.9765**	**0.201**	**266.9**	**5E−10**
21	**IN8-250**	**0.385 (±0.131)**	**0.901 (±0.042) (p = 2E−11)**	**0.9861**	**0.222**	**458.3**	**2E−11**
22	**IO3-250**	**0.779 (±0.212)**	**0.823 (±0.068) (p = 2E−08)**	**0.9583**	**0.359**	**146.3**	**2E−08**
23	**S18-250**	**0.453 (±0.111)**	**0.818 (±0.036) (p = 7E−12)**	**0.9876**	**0.189**	**518.5**	**7E−12**
24	**SYM-150**	**0.253 (±0.097)**	**0.855 (±0.031) (p = 7E−13)**	**0.9914**	**0.165**	**745.1**	**7E−13**

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

2.2.2. Equipment and LC conditions

All LC separations were performed on an ACME 9000 system (Younglin Instrument Corporation, Anyang, the Republic of Korea) consisting of a pump (SP 930D), thermostat (CTS30), autosampler, and a 730D UV/VIS detector. The AutoChro-3000 Chromatography Data System was used for data collection and instrument control. The gradient LC elution of the test analytes with water (solvent A) and methanol (solvent B) was performed over two times, i.e. 10 and 30 min, the content of component B was varied from 5 to 100%. Next, the following gradient program was used in both LC runs: (i) 100% B for 5 min, (ii) 5% B for 15 min to achieve column equilibration. The flow rate was 1 ml/min. All columns were thermostatted at 40 °C, and the test analytes were monitored with UV detection at 254 nm. A sample volume of 20 μl was injected into the HPLC system.

2.2.3. Column characterization

The QSRR test procedure based on 15 test solutes was performed as described in Ref. [30]. The experimental log k_w values for the test analytes used in two QSRR models were calculated by employing the DryLab software package (LC Resources, Walnut Creek, CA, USA). In these calculations, the t_R of the test analytes determined in two gradient runs and the dead volume obtained with uracil were used to solve the two equations proposed by Snyder and Dolan [24]. The *c*log *P*-values of the test analytes were calculated using the ALOGPS 2.1 program available on-line at http://www.vcclab.org. Other molecular structural descriptors like the total dipole moment (μ), electron excess charge of the most negatively charged atom (δ_{Min}), and the water-accessible Van der Waals surface area (A_{WAS}) were calculated in the HyperChem program with the extension of ChemPlus (HyperCube, Waterloo, Canada) [29]. Next, multiple regression equations were derived according to Eqs. (2) and (3) for the set of 24 RP-LC columns in the Microsoft Excel software (Microsoft, Redmond, WA, USA). Tables 2 and 3, respectively, present their regression coefficients (±standard deviations), and the parameters of the quality assay of the QSRR models for the columns, estimated using multiple correlation coefficients (r), standard errors of the equation estimates (s), significance levels of each term and the whole equation (p), and the values of Fisher's F-test of significance (Fisher's F-test).

2.3. Column performance for the determination of moclobemide

2.3.1. Chemicals

In addition to the reagents from Section 2.2.1., moclobemide (M0) was obtained from Biovena Pharma (Warsaw, Poland), Ro 12-5637 (M1) and Ro 12-8095 (M2) were kindly donated by Hoffmann-La Roche Ltd. (Basel, Switzerland), while phenacetin used as the internal standard (I.S.) was delivered by Sigma–Aldrich (St. Louis, MO, USA). The HPLC grades of acetonitrile and dichloromethane were purchased from Merck (Darmstadt, Germany). Sodium hydroxide was supplied by POCH (Gliwice, Poland), while 85% ortho-phosphoric acid was donated by Sigma–Aldrich (St. Louis, MO, USA). The control plasma was obtained from healthy volunteers.

2.3.2. Instrumentation and LC conditions

The LC equipment used was the same as that mentioned in Section 2.2.2. The chromatographic separation of M0 and its two metabolites was performed according to the method described in the Ref. [32]. First, an LC analysis of the compounds of interest was carried out on the NU18-125/5 column with a mixture of acetonitrile and water (25:75, v/v) adjusted to pH 2.7 with 85% ortho-phosphoric acid as the mobile phase. The flow rate was maintained at 1 ml/min, and the UV detection was performed at 239 nm. The LC system was operated at room temperature. Subsequently, the same chromatographic conditions as described above were applied to the other tested stationary phases.

2.3.3. Sample preparation

The quality control samples (QCs) and real plasma samples from healthy volunteers were analyzed in the same manner as the procedure described in Ref. [32]. In brief, 1 ml volume of human plasma was transferred to a polypropylene centrifuge and the I.S. solution at the concentration of 800 ng/ml was added. Next, the plasma sample was mixed with 200 μl of 1 M NaOH and 4 ml of dichloromethane and shaken mechanically for 10 min. After

Table 3
Coefficients a'_1–a'_4 (±standard deviations) with their significance levels, p (underneath in parenthesis), and statistical parameters, r, s, Fisher's F-test and p (see text for explanation), of the regression equations of the forms: $\log\ k_w = a'_1 + a'_2\mu + a'_3\delta_{Min} + a'_4 A_{WAS}$, provided by the QSRR for twenty four tested columns.

No.	Analytical column	a'_1	a'_2	a'_3	a'_4	r	s	Fisher's F-test	p
1	N18-125/5	−1.742 (±0.317)	−0.295 (±0.047) (p = 6E−05)	2.898 (±0.761) (p = 0.0029)	0.016 (±0.0008) (p = 4E−10)	0.9921	0.191	228.5	3E−10
2	FLP-150	−1.246 (±0.522)	−0.292 (±0.077) (p = 0.0031)	2.990 (±1.252) (p = 0.0359)	0.015 (±0.0013) (p = 2E−07)	0.9764	0.314	75.02	1E−07
3	NAB-250	−1.098 (±0.481)	−0.349 (0.071) (p = 0.0005)	2.659 (±1.152) (p = 0.0415)	0.014 (±0.0012) (p = 1E−08)	0.9794	0.289	86.47	6E−08
4	SS18-150	−0.445 (±0.474)	−0.230 (±0.070) (p = 0.0072)	3.527 (±1.135) (p = 0.0099)	0.012 (±0.0012) (p = 10E−07)	0.9718	0.284	62.30	3E−07
5	DIH-150	−0.397 (±0.313)	−0.302 (±0.046) (p = 4E−05)	2.465 (±0.751) (p = 0.0073)	0.012 (±0.0008) (p = 1E−08)	0.9877	0.188	146.7	4E−09
6	NHD-250	−1.091 (±0.402)	−0.295 ± (0.059) (p = 0.0004)	2.189 (±0.964) (p = 0.0443)	0.014 (±0.0010) (p = 3E−08)	0.9830	0.242	105.20	2E−08
7	LUN-150	−0.541 (±0.342)	−0.402 (±0.051) (p = 7E−06)	2.565 (±0.821) (p = 0.0097)	0.013 (±0.0009) (p = 8E−09)	0.9892	0.205	167.99	2E−09
8	SYP-150	−1.246 (±0.476)	−0.293 (±0.070) (p = 0.0015)	3.167 (±1.140) (p = 0.0179)	0.015 (±0.0012) (p = 6E−08)	0.9810	0.285	93.94	4E−08
9	VAR-150	−0.606 (±0.227)	−0.213 (±0.033) (p = 5E−05)	3.155 (±0.544) (p = 0.0001)	0.012 (±0.0006) (p = 3E−10)	0.9931	0.136	264.9	2E−10
10	**NOP-150**	**−1.615 (±0.384)**	**−0.347 (±0.057) (p = 7E−05)**	**2.326 (±0.919) (p = 0.0280)**	**0.016 (±0.0010) (p = 4E−09)**	**0.9883**	**0.230**	**153.66**	**3E−09**
11	**N18-125/10**	**−1.512 (±0.420)**	**−0.308 (±0.062) (p = 0.0004)**	**2.308 (±1.006) (p = 0.0424)**	**0.016 (±0.001) (p = 1E−08)**	**0.9849**	**0.252**	**118.3**	**1E−08**
12	**NU8-250**	**−1.724 (±0.256)**	**−0.192 (±0.038) (p = 0.0003)**	**2.539 (±0.614) (p = 0.0016)**	**0.014 (±0.0006) (p = 2E−10)**	**0.9926**	**0.154**	**244.3**	**2E−10**
13	**SYF-250**	**−0.940 (±0.353)**	**−0.260 (±0.052) (p = 0.0004)**	**2.318 (±0.845) (p = 0.0191)**	**0.013 (±0.0009) (p = 1E−08)**	**0.9857**	**0.212**	**125.92**	**8E−09**
14	**SY8-250**	**−0.776 (±0.445)**	**−0.241 (±0.066) (p = 0.0037)**	**2.414 (±1.068) (p = 0.04512)**	**0.013 (±0.0011) (p = 2E−07)**	**0.9754**	**0.268**	**71.86**	**2E−07**
15	**BDH-100**	**−1.551 (±0.377)**	**−0.270 (±0.056) (p = 0.0052)**	**2.430 (±0.905) (p = 0.0212)**	**0.015 (±0.0009) (p = 8E−09)**	**0.9863**	**0.227**	**131.38**	**7E−09**
16	**AQU-250**	**0.374 (±0.596)**	**−0.220 (±0.088) (p = 0.0295)**	**5.637 (±1.428) (p = 0.0023)**	**0.012 (±0.0015) (p = 8E−06)**	**0.9644**	**0.358**	**48.72**	**1E−06**
17	**SS8-250**	**−1.007 (±0.452)**	**−0.275 (±0.067) (p = 0.0017)**	**2.363 (±1.084) (p = 0.0518)**	**0.013 (±0.0011) (p = 2E−07)**	**0.9771**	**0.271**	**77.26**	**1E−07**
18	**IO2-150**	**−1.124 (±0.372)**	**−0.318 (±0.055) (p = 0.0001)**	**2.260 (±0.891)(p = 0.0275)**	**0.015 (±0.0009) (p = 8E−09)**	**0.9870**	**0.223**	**138.47**	**5E−09**
19	**GEN-150**	**−1.223 (±0.388)**	**−0.302 (±0.057) (p = 0.0003)**	**2.462 (±0.931) (p = 0.0228)**	**0.015 (±0.001) (p = 1E−08)**	**0.9856**	**0.233**	**125.08**	**9E−09**
20	**UNU-125**	**0.224 (±0.265)**	**−0.258 (±0.039) (p = 4E−05)**	**2.722 (±0.636) (p = 0.0013)**	**0.009 (±0.0007) (p = 2E−08)**	**0.9876**	**0.159**	**144.8**	**4E−09**
21	**IN8-250**	**−0.934 (±0.509)**	**−0.315 (±0.075) (p = 0.0015)**	**3.025 (±1.221) (p = 0.0307)**	**0.015 (±0.0012) (p = 2E−07)**	**0.9776**	**0.306**	**79.19**	**10E−08**
22	**IO3-250**	**0.481 (±0.507)**	**−0.283 (±0.075) (p = 0.0031)**	**4.998 (±1.215) (p = 0.0017)**	**0.012 (±0.0013) (p = 1E−06)**	**0.9749**	**0.304**	**70.25**	**2E−07**
23	**S18-250**	**−0.748 (±0.473)**	**−0.252 (±0.070) (p = 0.0417)**	**3.126 (±1.134) (p = 0.0187)**	**0.014 (±0.0012) (p = 2E−07)**	**0.9764**	**0.284**	**75.04**	**1E−07**
24	**SYM-150**	**−1.221 (±0.414)**	**−0.297 (±0.061) (p = 0.0005)**	**2.357 (±0.993) (p = 0.0369)**	**0.014 (±0.0010) (p = 3E−08)**	**0.9834**	**0.249**	**107.70**	**2E−08**

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

Table 4
Summary of retention data set of t_R and R_s for M0 and its two metabolites in human plasma obtained under the LC method for twenty four columns studied.

Substances		M0	M1		M2		Phenacetin (I.S.)	
No.	Analytical column	t_R	t_R	R_s	t_R	R_s	t_R	R_s
1	N18-125/5	2.51	2.95	1.61	5.15	8.39	5.53	1.64
2	FLP-150	4.86	5.86	2.88	8.16	5.28	9.06	1.68
3	NAB-250	3.95	4.56	2.56	7.31	8.44	8.75	3.25
4	SS18-150	2.03	2.23	1.93	6.31	17.27	8.11	4.32
5	DIH-150	2.26	2.58	1.82	6.90	19.31	8.30	3.98
6	NHD-250	3.45	3.95	2.60	8.13	14.24	9.48	3.41
7	LUN-150	1.61	2.28	5.54	8.15	26.91	9.56	4.09
8	SYP-150	3.07	3.46	2.74	7.40	14.81	8.43	3.31
9	VAR-150	7.95	9.35	1.82	10.33	1.58	11.95	2.02
10	**NOP-150**	3.46	4.05	1.71	7.16	5.42	7.83	**0.83**
11	**N18-125/10**	1.50	1.63	**1.13**	3.08	4.83	3.08	**0.00**
12	**NU8-250**	3.96	4.81	1.97	11.08	8.48	11.08	**0.00**
13	**SYF-250**	4.03	4.03	**0.00**	14.13	31.43	16.53	3.98
14	**SY8-250**	3.80	3.80	**0.00**	12.90	24.42	15.03	4.96
15	**BDH-100**	3.85	3.85	**0.00**	4.38	**1.39**	5.53	**1.44**
16	**AQU-250**	4.55	5.15	**0.78**	13.61	11.30	15.41	1.91
17	**SS8-250**	3.75	3.75	**0.00**	11.16	14.13	14.23	3.27
18	**IO2-150**	2.20	2.20	**0.00**	7.43	18.84	8.76	3.32
19	**GEN-150**	2.06	2.06	**0.00**	5.73	17.20	7.00	4.03
20	**UNU-125**	1.91	1.98	**0.92**	6.46	20.72	7.30	2.16
21	**IN8-250**	3.90	3.90	**0.00**	11.71	18.86	13.68	4.24
22	**IO3-250**	3.96	4.68	**1.24**	12.91	14.86	14.46	2.50
23	**S18-250**	3.33	3.33	**0.00**	11.10	29.63	13.06	4.05
24	**SYM-150**	2.21	2.21	**0.00**	6.83	23.68	8.73	5.89

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

centrifugation for 15 min (1000 × *g*) the dichloromethane layer was quantitatively transferred to another clean test tube and evaporated to dryness in a water bath at 45 °C under a gentle air stream. Finally, the residue was dissolved in 200 μl of mixed acetonitrile and water (3:2, v/v), centrifuged at 8000 × *g* for 5 min, and 20 μl of the aliquot was injected into the HPLC system.

2.3.4. Column test performance

The column test performance for the 24 stationary phases was based on LC separation of M0 and its two metabolites in the QC samples and real samples from healthy volunteers obtained after the application of a 150 mg dose of M0. The QC samples were constructed at low, middle, and high concentration levels of the compounds of interest (100, 800, and 1500 ng/ml for M0; 60, 100, and 150 ng/ml for M1, and 50, 500, and 1000 ng/ml for M2). The typical chromatograms obtained from the samples containing the compounds of interest during the LC analysis using N18-125/5 (A), SYP-150 (B), SY8-250 (C), and FLP-150 (D) are illustrated in Fig. 1A–D, respectively. The t_R of the analytes and the R_s of the peaks of interest, including the I.S, obtained during the LC analysis of M0 and its metabolites are summarized for all columns in Table 4.

2.4. Data treatment

A comparative study was conducted on the data sets, including the theoretical column classification results for the twenty four RP-LC stationary phases derived under the QSRR method, and the column test performances for the LC separation of M0 and its two metabolites, in order to check whether the QSRR approach could be considered a useful tool facilitating selection of the RP-LC column. For this assay, chemometric techniques such PCA and HCA based on squared Euclidean distance method and unweighted pair-group method using arithmetic averages (UPGMA) were applied using the Statistica 9.0 package (StatSoft, Tulsa, USA) to visualize the relationships between the theoretical and practical data sets containing many variables and objects. To begin with, PCA was performed for the autoscaled regression coefficients provided by the QSRR models (a_2 from Eq. (2) – Table 2; a'_2; a'_3; and a'_4 from Eq. (3) – Table 3), established for all columns. The loading and the score plots projecting the variables and objects (columns) in two-dimensional space, where the columns were assigned the same numbers from 1 to 24 (Tables 2 and 3), are illustrated in Fig. 2 A and B, respectively. The dendrogram produced by HCA in accordance with the above-described rules for the QSRR classification results is shown in Fig. 3. Then, the autoscaled retention parameters of t_R and R_s for M0, M1, M2 and the I.S. (Table 4), calculated for all stationary phases during the column performance test were evaluated by both chemometric methods. The two-dimensional loading and the score plots for the variables and columns, resulting from the PCA are shown in Fig. 4A and B, respectively, whereas the hierarchical tree diagram for the columns is shown in Fig. 5.

3. Results and discussion

3.1. The QSRR results

The QSRR approach involves using selected test solutes (Section 2.2.1.) which are analyzed in strictly set LC conditions (Section 2.2.2.). The QSRR models obtained for the columns studied are presented in Tables 2 and 3, respectively. It should be highlighted that all QSRR equations, including the regression coefficients, are of excellent statistical quality. It can be also noticed that the high log k_w versus *c*log *P* correlations confirmed similarity between the slow-equilibrium octanol/water partition system and the fast-equilibrium partition chromatographic process. Thus, the highest hydrophobicity according to the a_2 coefficient was recorded for N18-125/5 and NOP-150, while the lowest values were observed for VAR-150 and UNU-125 (Table 2).

The coefficients a'_2 and a'_3 reflect the specific, polar intramolecular interactions between the analytes and the stationary phase on one hand, and the eluent components on the other hand. However, the negative values of coefficients a'_2 suggest that the net effect on the retention of the dipole–dipole (and dipole-induced dipole) attractions between them is that of repulsion. The effect is due to stronger attraction between the total dipole of the analyte and the total and/or fragmental dipoles (both permanent and induced) of the polar molecules of the eluent compared to the

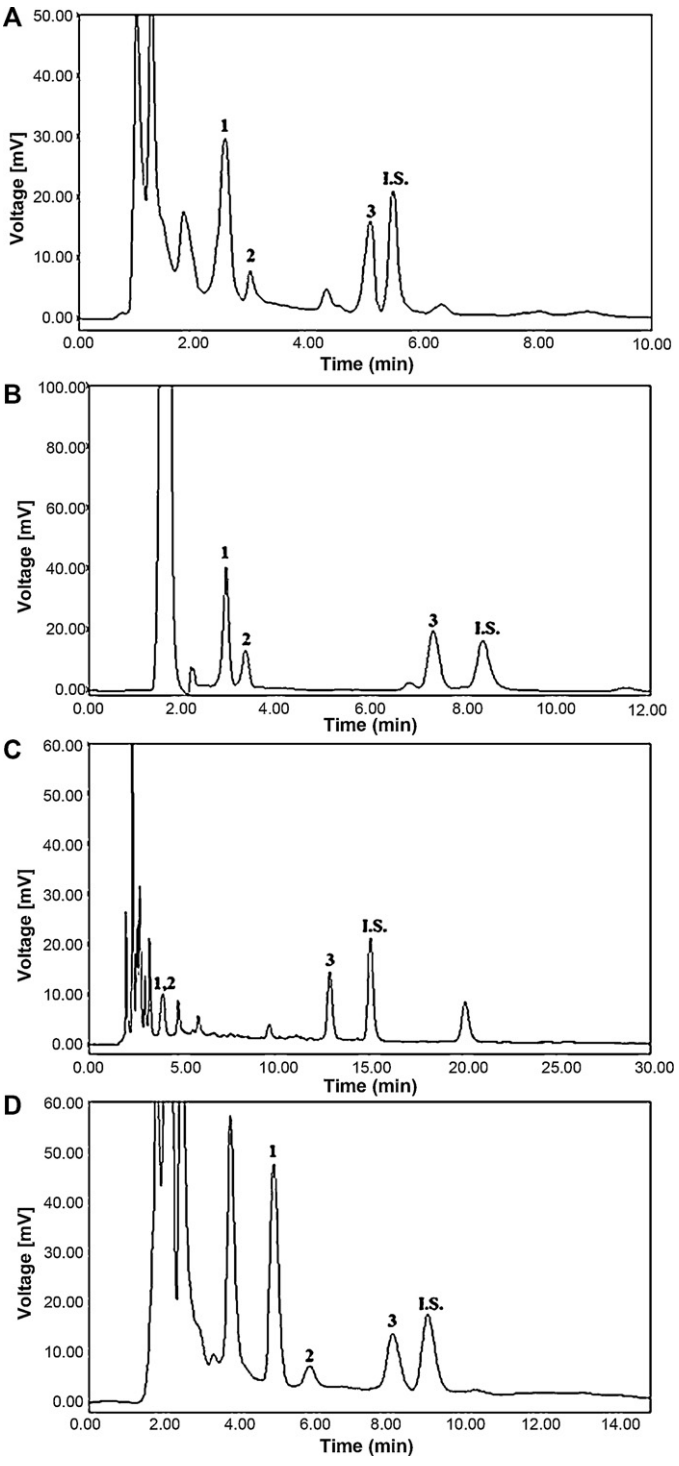

Fig. 1. LC analysis of QC sample containing M0 (1) and its two metabolites: M1 (2) and M2 (3) at the concentration levels of 1500, 150, 1000 ng mL^{-1}, and phenacetin as the I.S. (800 ng mL^{-1}) performed on: (A) N18-125/5; (B) SYP-150; (C) SY8-250 and (D) FLP-150 column, respectively.

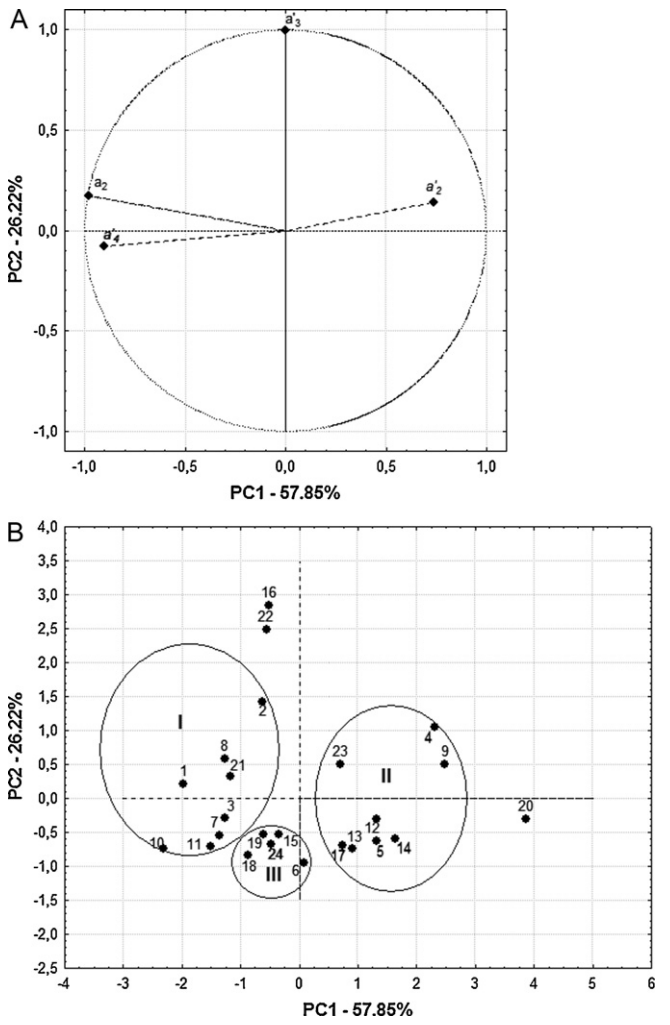

Fig. 2. Projection of the variables (A) and objects (B) onto the space of the first two PCs (PC1 and PC2) from PCA the regression coefficients provided by QSRR method for 24 columns studied.

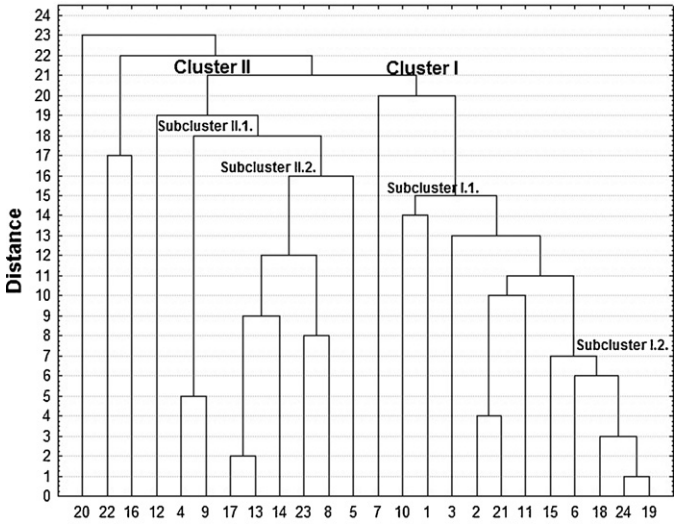

Fig. 3. The dendogram of 24 RP-LC columns extracted from HCA the regression coefficients provided by QSRR method.

respective interactions between the analytes and the non-polar alkyl chains of the stationary phases. The polarity order of the stationary phases according to the a'_2 coefficient was observed to begin with LUN-150 and end with NU8-250 (Table 3).

A rationalization similar to those described above applies to the a'_3 coefficient. However, the value of coefficient a'_3 has a positive sign because the δ_{Min} values (electron deficiencies) are negative. Thus, the higher the values of the absolute $a'_3\delta_{Min}$ term the lower

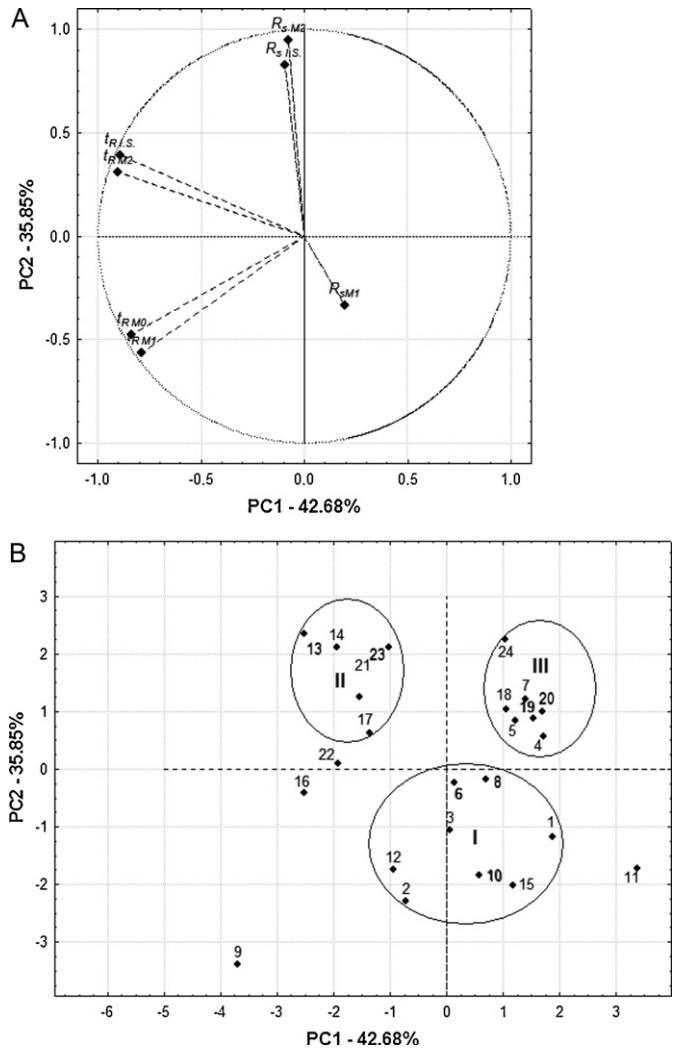

Fig. 4. Two-dimensional PCA loading plot (A) and score plot (B) based on the retention parameters (t_R and R_s) of the analytes during column performance for the determination of M0 and its two metabolites.

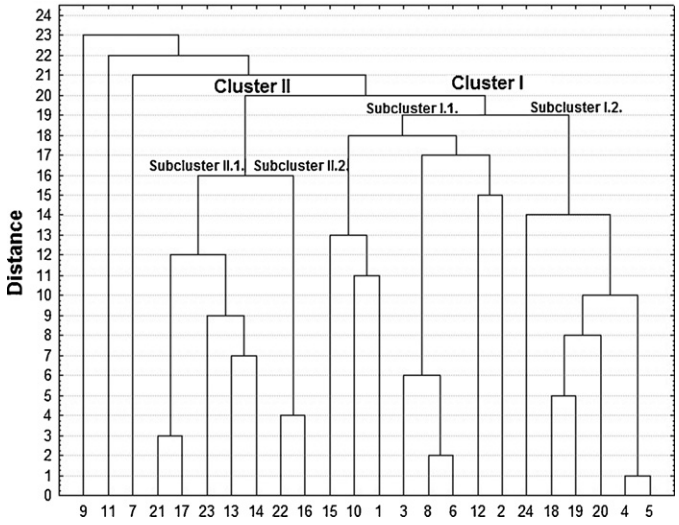

Fig. 5. The dendogram of 24 RP-LC columns extracted from HCA the retention parameters (t_R and R_s) of the analytes during column performance for the determination of M0 and its two metabolites.

retention of the analyte is indicated. This means that stronger local (fragmental) dipole–dipole interactions and/or the formation of the electron-pair-donor/electron-pair-acceptor complexes between the analyte and the stationary phase, with regard to analogous interactions with the eluent, are described by the lower values of the a'_3. Consequently, low a'_3 values suggest a stronger polarity of the stationary phase. According to a'_3, the polarity of the stationary phases decreased from AQU-250 to NHD-250. The a'_3 coefficient can also be considered a suitable parameter to reflect the analyte ability to take part in the hydrogen-bonding interactions with free silanols of the stationary phases.

Positive values of the a'_4 coefficients are observed for all columns in line with the positive contribution to the retention of the non-specific analyte-stationary phase interactions indicated by the A_{WAS} parameter. The observation can be easily explained because the interactions require close contact between the interacting molecules of the molecular fragments. Thus, higher a'_4 values can suggest that a larger surface of the stationary phase hydrocarbon moiety is accessible to the analyte molecules. Therefore, stationary phases N18-125/5, NOP-150, and N18-125/10 are characterized by the broadest surface areas of the external hydrocarbon ligands, hence their highest non-specific London interactions due to the dispersion effect. At the other end, the stationary phases UNU-125 is characterized by the lowest input to the retention of the London-type interactions.

3.2. Statistical analysis of the QSRR results

For a more detailed interpretation of the QSRR results for the 24 columns studied, two statistical techniques, namely PCA and HCA, were used. These chemometric tools offer graphic data visualization of the relationships between the variables and objects without losing any significant information. The application of statistical methods for column selectivity in the RP-LC, especially PCA, has already been discussed in the literature [4,6,15,21,29–31,33,34]. The two-dimensional loading and score plots for the variables and objects derived from the autoscaled QSRR data set, as presented in Table 2 (a_2) and 3 (a'_2, a'_3, a'_4), are shown in Fig. 2A and B, respectively. Notably, the first principal components (PC1) explained the variance of the analyzed data involved, mainly in the variability of a_2, a'_4, then a'_2. The a_2 and a'_4 variables positioned themselves close to each other, while the a'_2 was found an outlier on the PCA graph (Fig. 2A). The variability of the a'_3, also found an outlier, is mainly explained by the PC2. Thus, the first two PCs together account for 83.97% of the variance. Columns Nos. 1–3, 7, 8, 10, 11 and 21, identified by their higher a_2 and a'_4 parameters and an intermediate a'_3 (Table 3), were placed within cluster I. Columns Nos. 4, 9 and 23 described to have a higher a'_3 value, were found in the upper part of cluster II, whereas columns Nos. 5, 12–14 and 17 were placed in the bottom of this cluster. These columns were characterized by intermediate and lower values of the a_2 and a'_3 parameters. Stationary phases Nos. 6, 15, 18, 19 and 24 were placed together within cluster III, falling in the bottom part of the graph. Those columns were described as demonstrating lower a'_3 and intermediate a_2 parameters. The stationary phase No. 20, characterized by the lowest value of the a'_4 parameter, was found an outlier on the right of the plot. As concerns column No. 22, it positioned itself near column No. 16, on the upper side of the graph. Both were found to share the highest a'_3 parameter.

Similar observations can be made when the same data set is analyzed using the HCA. As shown in Fig. 3, twenty one columns studied were located within two clusters. Notably too, cluster I, which positioned itself on the right side of the dendogram, was divided into two subclusters: I.1. and I.2. The columns (Nos. 1–3, 7, 10, 11 and 21) were found positioned in subcluster I.1. The same stationary phases had also been observed in cluster I based on the

PCA (Fig. 2B). Located in subcluster I.2. at the right end of the dendogram were all columns from cluster III identified based on the PCA (Nos. 6, 15, 18, 19, 24). Moreover, all stationary phases previously found within cluster II (Fig. 2B) were located together on the left of the dendogram (cluster II – Fig. 3). As goes for the latter cluster, it is also possible to distinguish two constituent subclusters: II.1. and II.2. Columns Nos. 4, 9 and 12 were positioned in subcluster II.1., while columns Nos. 5, 8, 23, 14, 13 and 17 were found in subcluster II.2. Column No. 8 was the only one found in a different location (PCA – cluster I; HCA – cluster II). It should further be noted that the HCA indicated slightly different physicochemical properties for stationary phases Nos. 9 and 12 as well. However, no such difference was observed for column No. 12 in the PCA (Fig. 2B). Moreover, column No. 20 found an outlier, whereas Nos. 16 and 22 grouped in the same subcluster were placed at the left end of the dendogram (Fig. 3). This indicates that their characteristics provided by the QSRR method was significantly different from the other columns studied. These observations were consistent with the PCA results.

3.3. Column selectivity in separation of moclobemide

The theoretical QSSR results were examined in a real biomedical analysis for 24 stationary phases during column performance test based on the measurement of M0 and its two metabolites in the human plasma, performed in accordance with the LC method reported in Ref. [32]. For this purpose the QC samples and real samples obtained from healthy volunteers after the application of a single dose of 150 mg of M0 were analyzed using all columns studied. The experimental data of t_R and R_s for the compounds of interest are summarized in Table 4. It can be noticed, that proper LC separation of the analytes in accordance with the $R_s > 1.5$ was observed for nine columns. Insufficient separation of M0 and M1 was often observed in the LC analysis of the compounds of interest (13 columns).

3.4. Comparison based on column performance using chemometric analysis

The loading and score plots based on the autoscaled column test performance results for twenty four column studied are presented in Fig. 4A and B, respectively. The localizations of the stationary phases on the PC1 axes were related mainly to the variability of $t_{R\ M2}$ and $t_{R\ I.S.}$, and then $t_{R\ M0}$ and $t_{R\ M1}$, respectively. These variables were positioned on the left side of the middle part of the plot ($t_{R\ M2}$, $t_{R\ I.S.}$) and on the left side of the bottom part of the graph ($t_{R\ M0}$, $t_{R\ M1}$,) (Fig. 4A), respectively. The variance of the analyzed data explored by the PC2 was mainly related to the variability of $R_{s\ M2}$ and $R_{s\ I.S.}$, located in the upper part of the plot. In summary, the first two PCs explain 78.53% of the data variability. Five columns offering appropriate separation of the compounds of interest (Nos. 1–3, 6 and 8) and stationary phases Nos. 10, 12 and 15 were found within cluster I placed in the middle part of the plot (Fig. 4B). Stationary phase No. 9, also suitable for the analysis of the M0 and its metabolites, was positioned as an outlier in the bottom part of the graph. That was the phase for which the longest t_R of M0 and M1 were observed (Table 4). Only three stationary phases offering proper separation of the analytes (Nos. 4, 5 and 7) were placed within cluster III together with columns Nos. 18–20 and 24. These columns demonstrated the shortest t_R of M0 and M1, and the variable $R_{s\ M1}$-values. Stationary phase No. 11 was an outlier, and it was for that particular phase that the shortest t_R of all analytes and insufficient R_s of M1 and I.S. were observed. Stationary phases Nos. 13, 14, 17, 21 and 24, giving the LC separation of the compounds of interest with intermediate and longer t_R of the analytes, but insufficient R_s of M1 were positioned in cluster II located in the upper part of the plot.

It should also be highlighted that there are noticeable similarities between the plots presented in Fig. 2B and 4B. In both cases, the stationary phases were placed within three clusters, and the latter included most of the same columns. For example, stationary phases Nos. 13, 14, 17 and 23 were positioned in cluster II, while stationary phases Nos. 18, 19, and 24 were placed in cluster III. Moreover, in both score plots, columns Nos. 16 and 22 were found outliers. On the other hand, some columns, to name e.g. 4–7, 9, 11, 15 and 20, were found in positions different than on Fig. 2 B. Nevertheless, it is worth emphasizing that five suitable columns for the LC separation of M0 and its metabolites had been identified correctly by the QSRR models (cluster I – Fig. 2B). This indicates that the probability of selecting the proper column is relative high (5/8–62.5%) for the cluster. On the other hand, the probability for the stationary phases included in the other clusters, as well as for the ones found outliers (Fig. 2 B; cluster II–37.5% (3/8); cluster III–20% (1/5); the outliers – 0% (0/3), respectively) diminishes significantly.

Fig. 5 shows that the PCA results were confirmed by the HCA. In other words, almost all stationary phases (21) were placed within two clusters, I and II, found on the right and on the left of the dendogram, respectively. It can be also noticed that the columns in clusters I and II split into two subclusters (I.1., I.2. and II.1., II.2.). All stationary phases previously recorded in cluster III based on the PCA (Fig. 4B) were observed to place themselves in subcluster I.2, while all columns from the PCA cluster I (Fig. 4B) were found in subcluster I.1. placed in the middle part of the dendogram (Fig. 5). Moreover, all columns from cluster II (Fig. 4B) were found in subcluster II.1 identified by the HCA (Fig. 5). Notably too, stationary phases Nos. 16 and 22 were positioned in subcluster II.2. However, the distance between them and the other columns of subcluster II.1. was larger, which confirmed that they gave a slightly different separation of the analytes (longer t_R of M0 and M1 and R_s of I.S. < 2.5). Similar to what was observed on the PCA plot (Fig. 4B), columns Nos. 9 and 11 were found outliers on the dendogram obtained by the HCA (Fig. 5). Stationary phases No. 7 were the only ones found in different positions (PCA - cluster III; HCA–outlier). It was for those particular stationary phases that the highest R_s of M1 was observed.

When the dendogram based on the theoretical QSRR results (Fig. 3) was compared with the column test performance (Table 4), it was noticed that out of the eight columns located closest to the N18-125/5 column, six stationary phases were suitable for the LC analysis of M0 and its metabolites. Hence, the probability of selecting the proper column increased from 37.5% (9/24 – Table 4) to 66.6% (6/9), respectively. It confirms that the QSSR approach can be considered a helpful tool in the selection of a suitable column for biomedical analyses. Moreover, the PCA and HCA plots illustrated in Fig. 2B-5 demonstrate that graphical visualization of the data sets offers easier interpretation of the theoretical QSRR results and their correlation with the column test performance.

4. Conclusions

This paper focuses on a comparative study of column classification systems based on the QSRR method and on the column selectivity obtained in the real biomedical analysis of moclobemide and its two metabolites in human plasma, using a set of 24 stationary phases. In the QSRR approach, the models, or the statistically derived relationships between the chromatographic parameters and the descriptors characterizing the molecular structure of the analytes, are calculated in order to characterize the stationary phases and quantify their separation properties. The results based on the PCA and HCA confirmed that the use of the QSRR method increases the probability of arriving at an appropriate column selection for the specific analysis. Thus, the QSRR approach offers proper evaluation of the interactions between the

analyte and the stationary phase, which substantiates considering it a supportive tool in the selection of a suitable column for the given analysis. Moreover, the fact opens the possibility of using the QSRR method as a valuable starting point for the development of the RP-LC method. Finally, the HCA can be an attractive alternative to the PCA technique for visualization of the column selectivity data sets.

Acknowledgements

The work was supported by National Science Centre Projects no. 2011/03/VB/NZ7/02333 and no. N N405 024340.

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