Ionic Liquids as Novel Solvent Additives to Separate Peptides

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A novel analytical approach involving the addition of an ionic liquid into the mobile phase of the thin-layer chromatography (TLC) system during the optimization of chromatographic separation of peptides was demonstrated. Different behavior of peptides in the TLC sytem was observed after the addition of 1,3-dimethylimidazolium methyl sulfate to the eluent in comparison to the system without the ionic liquid. The objective of the work was to study the effect of the addition of different contents of ionic liquid to the mobile phase comprising mostly water and to observe the behavior of peptides' retention. The potential usefulness of environmentally friendly ionic liquids for the optimization of separation of peptides was demonstrated. An increase of R_f values was observed with increasing the ionic liquid content in the mobile phase. The benefits of the used approach were related to the separation achieved. Finally, quantitative structure-retention relationships (QSRR) were used for the studies on the predictions of peptides' retention in the TLC systems with the addition of ionic liquid in terms of the predictions performed recently in HPLC systems.

Key words: Ionic Liquids, Peptide Separation, Quantitative Structure-Retention Relationships (QSRR)

Introduction

Thin-layer chromatography (TLC) in protein chemistry involves the recovery of peptides for further structural analysis, their identification, peptide mapping, protein and peptide fractionation as well as determination of molecular weights of peptides and proteins (Bhushan and Martens, 1996). Still, TLC can offer specific advantages in peptides' separation. New approaches recommend even the imaging of thin-layer chromatograms with the use of matrix-assisted laser desorption/ionization-mass spectrometry (MALDI-MS) (Gusev *et al*., 1995a, b) or direct TLC-MALDI-MS coupling (Mehl and Hercules, 2000; Creselius *et al.*, 2004)

Like in HPLC, the favorable physical characteristics of silica make the silica-based stationary phases the most popular also in TLC. However, an undesirable property of silica is a surface acidity due to the free or isolated (non-hydrogen-bound) silanols (Kaliszan *et al.*, 2004; Marszałł *et al*., 2005). Those silanols have a strong deleterious effect on the chromatographic behavior of basic and amphoteric analytes. Because of the acidic nature of free silanols, a binding of organic bases takes place which results in increased retention (up to analyte immobilization at the beginning of the chromatographic bed) and broad, tailing analyte peaks (Snyder *et al*., 1997; Vervoort *et al*., 2001, 2002). It was previously found (Kaliszan *et al*., 2004; Marszałł *et al*., 2005) that ionic liquids of the imidazolium class, added to mobile phases at contents of 0.5–1.5% (v/v), efficiently block silanols and provide excellent thin-layer chromatographic separations of strongly basic drugs which were otherwise not eluted, even with neat acetonitrile as the mobile phase. The silanol suppressing potency of imidazolium ionic liquids was demonstrated to markedly exceed that of the standard mobile phase additives, like triethylamine, dimethyloctylamine and ammonia. Other authors (He *et al*., 2003; Zhang *et al*., 2003) applied ionic liquids also successfully in HPLC. In view of proteomics and applications of TLC for peptide separations it is worth noting that ionic liquids were employed with promising results as matrices for matrix-assisted laser desorption/ionization mass spectrometry (Armstrong *et al*., 2001) and were analyzed in electrospray ionization mass spec-

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trometry (Alfassi *et al*., 2003). The study of the retention behavior of the homologous series of peptides in normal-phase TLC on a silica support was previously undertaken, also to test a possibility to combine on-line the proposed TLC-ionic liquid separation of peptides with MALDI-MS during the identification of peptides (Bączek *et al*., 2005a). Potential compatibility of ionic liquids and TLC with MALDI-MS was recently presented in the literature (Ivleva *et al*., 2004, 2005).

The objective of this work was to study the effect of addition of different concentrations of 1,3-dimethylimidazolium methyl sulfate to a mostly water composing mobile phase on peptides' retention. Corresponding relationships were analyzed between the ionic liquid composition and TLC retention. Moreover, the preliminary experiments were undertaken to study the peptides' retention behavior in the TLC systems with the addition of ionic liquid in terms of the predictions of their retention with the use of quantitative structure-retention relationships (QSRR) approach.

Experimental

Materials

The ionic liquid studied, 1,3-dimethylimidazolium methyl sulfate, was from Fluka (Buchs, Switzerland) and was used as obtained, without any additional pretreatment. Angiotensin II (DRVYIHPF) was purchased from Fluka. The following peptides: AA, AG, AF, YL, DD, and ML were from Sigma-Aldrich (St. Louis, MO, USA). All other peptides have been synthesized at the Department of Organic Chemistry, University of Gdańsk according to a general procedure reported elsewhere (Atherton and Sheppard, 1989).

Acetonitrile of HPLC-grade was from Merck (Darmstadt, Germany). Isopropanol, chloroform, methanol, ethanol, 25% aqueous ammonium solution were from POCh (Gliwice, Poland). Trifluoroacetic acid was from Sigma-Aldrich. Water was prepared with Milli-Q system (Millipore, Bedford, MA, USA).

Thin-layer chromatography (TLC)

TLC experiments were carried out on aluminium-backed 5 cm × 7.5 cm × 0.2 cm plates, covered with silica gel $60F_{254}$. The ready-made TLC plates were from Sigma-Aldrich. Chromatograms were developed to a distance of 6.0 cm in a horizontal chamber (Modlin, Lublin, Poland), using the eluent water with the addition of 0.1% trifluoroacetic acid, 0% or 5% of acetonitrile (in the case of angiotensin II or all other peptides, respectively) and the appropriate amount of ionic liquid. The concentration of ionic liquid was between 0 and 10% (v/v): 0%; 0.1%; 0.5%; 1%; 5%; 10%. Fluorescamine was used as a label. It reacts with primary amine groups of proteins; unbound dye was nonfluorescent. The dried plate was dipped into the solution of 0.05% fluorescamine in acetone (Sigma-Aldrich); label's sensitivity depended on the number of amine groups present in the analyte. The developed plates were dried in the air at room temperature. After about 0.5 h, the plates were placed under UV light of multiband UV 254/365 nm of a Spectroline hand lamp (Spectronics, Westburg, NY, USA) and visualized at $\lambda = 365$ nm. Each determination was performed at ambient temperature [(21 ± 20) °C]. The retention data reported a mean values of five independent experiments. Because of the need to solve all the peptides tested, the addition of 5% acetonitrile was required in the mobile phase used.

QSRR analysis

Rational optimization of chromatographic separations in gradient elution is a big challenge for analytical chemists. Accurate predictions of retention could be achieved theoretically, if thermodynamics of all processes involved in the separation as well as the nature of intermolecular interactions determining molecular recognition of the analytes by the counterparts forming the chromatographic systems are properly understood and subsequently quantified. However, that situation is rather unrealistic. Therefore, in chemical practice approximate predictions, however useful, can be realized which are valid in statistical extrathermodynamic (chemometric) terms rather than in strict thermodynamic categories. That approach is recognized under the acronym QSRR: quantitative structure-retention relationships. QSRR are therefore statistically derived relationships between a chromatographic parameter (dependent variable) and the descriptors characterizing the molecular structure of analytes (independent variables). QSRR are applied to: (i) get insight into the molecular mechanism of separation operating in a given chromatographic system; (ii) identify the most informative structural descriptors of analytes; (iii)

evaluate complex physicochemical properties of analytes, *e.g.*, lipophilicity; (iv) evaluate properties of stationary phases; (v) predict relative differences in biological activities within a set of congeneric drugs or other xenobiotics; (vi) predict retention for a new analyte (Kaliszan, 1987, 1993).

In the current study, as independent variables molecular structural descriptors of the test peptides were calculated by the molecular modeling programs: HyperChem for personal computers with the extension ChemPlus (HyperCube, Waterloo, Canada) and ACD (Advanced Chemistry Development, Toronto, Canada). As a dependent variable the value of R_M [$R_M = \log (1/R_f - 1)$] for 30 peptides was used.

QSRR equation was derived by means of multiple regression analysis employing the Statistica computer program (StatSoft Inc., Tulsa, OK, USA) run on a personal computer. Regression coefficients, multiple correlation coefficients, *R*, standard errors of estimate, *s*, significance levels of each term and of the whole equation, *p*, and the values of the *F*-test of significance, *F*, were calculated.

Results and Discussion

In a preliminary experiment, angiotensin II (DRVYIHPF) was chromatographed in a TLC system with the mobile phase comprised of water with the addition of 0, 1, 3 and 10% of 1,3-dimethylimidazolium methyl sulfate. The relationship between the percentage of ionic liquid used and the retention factor (R_f) is presented in Fig. 1. It can be noted that regular dependence was observed indicating the stable influence of the ionic liquid added to the water. Moreover, the ability of 1,3-dimethylimidazolium methyl sulfate addition to modify effectively the retention of DRVYIHPF in the TLC system used was proven. From "green chemistry" point of view, the worth is the lack of a standard organic solvent (*e.g.*, acetonitrile, methanol) applied in that experiment. On the other hand, that experiment could be considered as a novel possibility for peptides separations in proteomic research, also in the aspect of the combination of TLC and MALDI-MS (Ivleva *et al.*, 2004, 2005).

The influence of 1,3-dimethylimidazolium methyl sulfate on TLC retention of 11 dipeptides and 38 peptides was further considered. In the first experiment, it was revealed that at 25% of ionic liquid in the mobile phase, the disturbance of the chromatogram was observed and all spots have moved just in the front. Moreover, the poor differentiation in peptides' retention was demonstrated with the mobile phase comprising of 0.1% and 0.25% ionic liquid applied. It must be noticed that

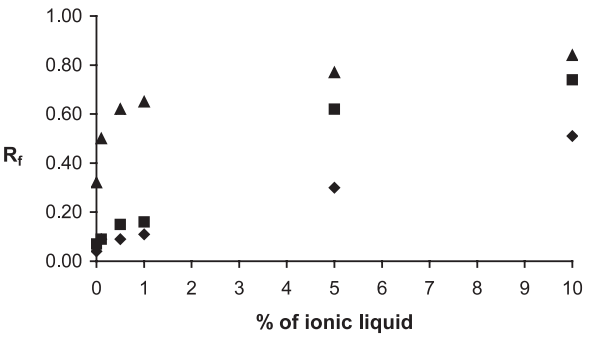

Fig. 2. The exemplary relationships between R_f values and the ionic liquid content in the mobile phase for the following peptides:
▲, LAQAVRSS-NH_2;
◆, LPPGPAVVDLTEKLEGQGG-NH_2;
■, EVHHQKLVFF.

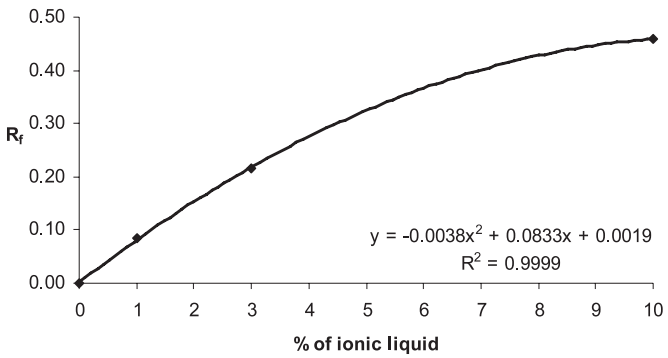

Fig. 1. The relationship between the percentage of ionic liquid and the retention factor (R_f) of angiotensin II chromatographed in 1D-TLC system.

Table I. R_f values obtained for peptides studied.

No.	Amino acid sequence	Ionic liquid content (%)					
		0	0.1	0.5	1	5	10
1	AA	0.86	0.90	0.88	0.90	0.91	0.91
2	AG	0.89	0.90	0.89	0.90	0.91	0.91
3	AF	0.85	0.82	0.84	0.85	0.87	0.87
4	YL	0.87	0.82	0.86	0.86	0.87	0.86
5	DD	–	0.90	0.92	0.92	0.68	0.81
6	ML	0.66	0.70	0.70	0.72	0.83	0.84
7	WW	0.70	0.73	0.72	0.71	0.69	0.74
8	GM	0.88	0.88	0.84	0.83	0.88	0.88
9	GH	0.58	0.60	0.67	0.65	0.82	0.87
10	GL	0.77	0.80	0.82	0.76	0.82	0.82
11	WF	0.61	0.71	0.77	0.73	0.73	0.78
12	GHG	0.46	0.53	0.64	0.64	0.81	0.86
13	VKGTEDSGTT-NH_2	0.64	0.72	0.72	0.75	0.81	0.86
14	EHADLLAVVAASQKK-NH_2	0.06	0.27	0.48	0.56	0.78	0.84
15	VVAASQKK-NH_2	0.29	0.48	0.62	0.65	0.78	0.83
16	LAQAVRSS-NH_2	0.32	0.50	0.62	0.65	0.77	0.84
17	YKIEAVKSEPVEPPLPSQ-NH_2	0.04	0.14	0.13	0.17	0.38	0.60
18	LPPGPAVVDLTEKLEGQGG-NH_2	0.04	0.09	0.09	0.11	0.30	0.51
19	VVDLTEKLEGQGG-NH_2	0.18	0.41	0.36	0.37	0.57	0.57
20	VAKETS	0.61	0.76	0.72	0.75	0.80	0.85
21	TVAKETS	0.61	0.79	0.71	0.74	0.77	0.85
22	HTVAKETS	0.12	0.19	0.26	0.29	0.48	–
23	WHTVAKETS	0.14	0.47	0.57	0.65	0.80	0.85
24	HWHTVAKETS	0.05	0.47	0.61	0.66	0.79	0.85
25	TLSYPLVSVVSESLTPER-NH_2	0.06	0.24	0.50	0.43	0.66	0.80
26	DAEFRH-NH_2	0.22	0.47	0.58	0.59	0.76	0.80
27	DAEFGH-NH_2	0.40	0.49	0.58	0.62	0.74	0.79
28	DAEFRHDSG-NH_2	–	0.45	0.51	0.54	0.69	0.76
29	DAEFGHDSG-NH_2	0.50	0.58	0.45	0.48	0.58	0.65
30	DAEFRHDSGY-NH_2	–	0.43	0.53	0.55	0.69	0.79
31	DAEFGHDSGF-NH_2	0.39	0.43	0.40	0.40	0.57	0.66
32	EVHHQKLVFF-NH_2	0.05	0.12	0.16	0.18	0.69	0.78
33	EVRHQKLVFF-NH_2	0.06	0.22	0.44	0.48	0.73	0.79
34	LVFF-NH_2	0.24	0.48	0.61	0.63	0.80	0.84
35	GSNKGAIIGLM-NH_2	0.11	0.50	0.63	0.65	0.81	0.87
36	GKTKEGVLY-NH_2	0.24	0.48	0.63	0.64	0.82	0.87
37	KTKEGVLY-NH_2	0.26	0.48	0.63	0.65	0.73	0.85
38	TKEGVLY-NH_2	0.33	0.52	0.63	0.64	0.80	0.87
39	GVLY-NH_2	0.43	0.67	0.71	0.72	0.81	0.87
40	GLSPMIETIDQVR-NH_2	0.06	0.20	0.26	0.27	0.58	0.69
41	MAGASELGTGPGA-NH_2	0.16	0.39	0.49	0.49	0.74	0.84
42	AGGYKPFNLETA-NH_2	0.20	0.44	0.58	0.60	0.78	0.84
43	GAPGGPAFPGQTQDPLYG-NH_2	0.03	0.07	0.12	0.11	0.37	0.46
44	LHWHT	0.05	0.07	0.12	0.13	–	–
45	HLHWHT	0.07	0.08	0.06	0.09	0.15	0.22
46	ETHLHWHT	0.07	0.08	0.08	0.09	0.15	0.21
47	EVRHQKLVFF	0.09	0.23	0.43	0.46	0.71	0.80
48	EVRHQK	0.16	0.42	0.51	0.54	0.80	0.83
49	EVHHQKLVFF	0.07	0.09	0.15	0.16	0.62	0.74

the addition of a small amount of acetonitrile (in this case equaled 5%) to the mobile phase was useful to solubilize the more hydrophobic peptides. Without that addition, their separation was not possible (Table I).

Further investigation was performed for the following contents of ionic liquid in the mobile phase: 0%, 0.1%, 0.5%, 1%, 5% and 10%. For all peptides tested the increase of R_f values with increasing of ionic liquid content in the mobile phase was

clearly demonstrated (Fig. 2). On the other hand, weaker influence of the ionic liquid content on the retention of dipeptides was noted (Table I).

Finally, quantitative structure-retention relationships (QSRR) (Kaliszan, 1987, 1993) were used for the studies of the predictions of peptides' retention in the TLC systems with the addition of ionic liquid in terms of the predictions performed recently in HPLC systems. Previous studies (Kaliszan *et al*., 2005; Bączek *et al*., 2005b) demonstrated a good capability of a general QSRR model to predict RP-HPLC retention of peptides on various stationary phases and at diverse HPLC conditions. The following molecular descriptors were employed in the current QSRR analysis: the logarithm of the calculated *n*-octanol/water partition coefficient, clog*P*, refraction index, R_I, the square of the angle energy, E_A^2 and the dihedral energy, E_D.

The peptides in Table II were used to derive the QSRR model.

The QSRR model has the following form:

$$R_M = -5.34\ (\pm 1.94)\ (p = 1\times10^{-2}) + 0.02\ (\pm 0.004)\ E_D\ (p = 1\times10^{-4}) + 0.07\ (\pm 0.02)\ \mathrm{clog}P\ (p = 2\times10^{-3}) + 3.20\ (\pm 1.21)\ R_I\ (p = 1\times10^{-2}) + 0.19\times10^{-3}(\pm 0.05\times10^{-3})\ E_A^2\ (p = 1\times10^{-3}) \quad (1)$$

$$n = 30,\ R = 0.908,\ F = 29,\ s = 0.23,\ p < 4\times10^{-9}.$$

The experimental R_M values, $R_{M\,exp}$, and those calculated with the use of (1), $R_{M\,calc}$, were compared and collected in Table II.

Reasonable agreement between the R_M values received experimentally and calculated with the

Table II. Experimental and calculated R_M values with the use of (1) for peptides in the case of TLC system with the addition of 1% ionic liquid.

No.	Amino acid sequence	E_D	clog*P*	R_I	E_A^2	$R_{M\,exp}$	$R_{M\,calc}$	ΔR_M
1	VKGTEDSGTT-NH_2	9.825	−6.41	1.57	96.53	−0.48	−0.57	0.09
2	EHADLLAVVAASQKK-NH_2	21.007	−3.89	1.55	757.97	−0.10	−0.07	0.03
3	VVAASQKK-NH_2	15.797	−3.24	1.54	36.34	−0.27	−0.31	0.04
4	LAQAVRSS-NH_2	14.979	−3.36	1.63	57.27	−0.27	−0.04	0,23
5	YKIEAVKSEPVEPPLPSQ-NH_2	51.500	−1.94	1.58	2307.01	0.69	1.09	0.40
6	LPPGPAVVDLTEKLEGQGG-NH_2	43.321	−3.74	1.56	1872.20	0.91	0.65	0.26
7	VVDLTEKLEGQGG-NH_2	17.977	−4.2	1.55	360.24	0.23	−0.25	0.48
8	VAKETS	0.855	−2.45	1.55	25.56	−0.48	−0.54	0.06
9	TVAKETS	1.176	−3.03	1.55	36.77	−0.45	−0.56	0.11
10	WHTVAKETS	−0.849	−2.59	1.61	1195.02	−0.27	−0.17	0.10
11	DAEFRH-NH_2	0.550	−2.57	1.68	374.90	−0.16	−0.05	0.11
12	DAEFGH-NH_2	7.569	−3.23	1.61	363.88	−0.21	−0.21	0.00
13	EVHHQKLVFF-NH_2	13.867	0.35	1.59	1667.33	0.66	0.38	0.28
14	EVRHQKLVFF-NH_2	7.738	0.56	1.64	711.09	0.03	0.26	0.23
15	LVFF-NH_2	−5.788	3.59	1.56	15.94	−0.23	−0.21	0.02
16	GSNKGAIIGLM-NH_2	22.853	−4.12	1.54	1836.97	−0.27	0.14	0.41
17	GKTKEGVLY-NH_2	8.458	−1.69	1.57	205.70	−0.25	−0.21	0.04
18	KTKEGVLY-NH_2	5.251	−0.94	1.57	158.31	−0.27	−0.23	0.04
19	TKEGVLY-NH_2	2.032	−0.5	1.58	151.07	−0.25	−0.26	0.01
20	GVLY-NH_2	3.118	1.09	1.58	31.19	−0.41	−0.13	0.28
21	MAGASELGTGPGA-NH_2	20.699	−6.46	1.56	2982.06	0.02	0.22	0.02
22	AGGYKPFNLETA-NH_2	7.732	−2.22	1.58	673.65	−0.18	−0.14	0.04
23	GAPGGPAFPGQTQDPLYG-NH_2	43.280	−4.86	1.60	2342.33	0.91	0.78	0.13
24	LHWHT	20.107	−0.3	1.64	2067.18	0.83	0.72	0.11
25	HLHWHT	18.428	−1.11	1.65	4088.30	1.00	1.07	0.07
26	ETHLHWHT	24.682	−2.27	1.64	3632.47	1.28	0.98	0.3
27	EVRHQKLVFF	5.709	1.04	1.59	1635.21	0.07	0.25	0.18
28	EVRHQK	9.567	−3.36	1.66	502.62	−0.07	0.03	0.10
29	GHG	1.244	−2.63	1.61	343.37	−0.25	−0.30	0.05
30	EVHHQKLVFF	5.171	1.25	1.64	696.70	0.72	0.24	0.48

use of the QSRR model obtained for the set of structurally diversified peptides provided its ability to be a useful tool for the preliminary explanation of the peptide retention behavior in TLC systems comprising ionic liquid.

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