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Analytical and semi-preparative separation of diastereomeric lipidic amino acid conjugates

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Abstract: A series of biologically active peptides and their conjugates with lipidic amino acids were investigated by systematic change of the mobile phase composition using traditional octadecylsilica stationary phase and the newly developed Supelcosil™ LC–ABZ column. The mobile phases contained various concentrations of methanol and acetonitrile combined with 0.1% trifluoroacetic acid (TFA). Better peak shapes and higher resolution of the isomers could be observed when the mobile phase contained 0.1% TFA. More symmetrical peaks and much higher *S* values (slope of the log *k'* vs organic phase concentration plots) were obtained on the special reversed-phase column developed for anionic, basic or zwitterionic compounds. The optimum separation conditions were scaled up to a semi-preparative reversed-phase column (15 mm i.d.) to collect mg quantities of isomers for further studies.

Keywords: *HPLC; lipidic amino acids; diastereomer separation.*

Introduction

The chemical modification of existing biologically active molecules can lead to a new drug with improved absorption, less toxic or more specific molecules. Lipidic amino acids and peptides were suggested as possible conjugates to pharmacologically active compounds by Gibbons *et al.* [1] and Sakarellos *et al.* [2]. They are expected to possess a degree of membrane-like character which is sufficient to facilitate the passage of poorly absorbed drugs across biological membranes to reach their active sites. Quantitative structure activity relationships were revealed for a series of lipidic amino acid conjugates of β-lactam antibiotics [3] which showed the importance of the lipidic side chain in the *in vivo* action.

The lipidic amino acid conjugates of the peptides investigated were synthesized in order to reveal the essential amino acid sequence for its biological activity. Autoantibodies against the acetylcholine receptor (AchR) cause Myasthenia Gravis (MG) and autoimmune neuromuscular disease characterized by the weakness of the skeletal muscles. The majority of the anti-AchR antibodies from MG patients is directed against an extracellular area of the AChr, a subunit called "Main Immunogenic Region" (MIR) with sequence W^{67}-N-P-A-D-Y-G-G-I-K^{76} for Torpedo electric organ [4]. Substitution of each residue in the MIR, by alanine revealed the critical role of N^{68}, P^{69}, D^{71} for binding to the anti-MIR mAbs [5]. Additionally, ^{2}H-NMR studies on a series of MIR-analogues showed that enhanced conformational mobility of the C-terminals with simultaneous preservation of the β-folding at the N-terminal of the MIR-peptides are highly favourable to the mAbs recognition [6]. On the other hand the [A^{76}]-MIR analogue was twice as easily recognized by different anti-MIR mAbs than the natural sequence containing lysine in position 76. This observation addressed the question of the role of lysine in the Abs recognition and we have stated the hypothesis that an ionic interaction between K^{76} and D^{71} side chains could be involved in an

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intra-MIR interaction, opposed to an optimal mAb recognition. On the other hand, the K^{76} and D^{71} charged side chains of the interact AChR could participate in longer-range interactions, more propitious for binding to the mAbs. It was also found that the [Gly^{70}, Nle^{76}]-MIR analogue was selectively recognized by the mAb198 with 180% binding capacity compared to the natural Torpedo A67-76 sequence. With the aim to gain further insight into the contribution of a relatively long and hydrophobic side-chain in position 76 we have synthesized and studied the [amino-DL-decanoic $acid^{76}$]-MIR analogue. Moreover, the [Gly^{70}, amino-DL-$decanoic^{76}$]-MIR derivative was designed to further facilitate the formation of the β-folding at the N-terminal sequence N^{68}-P-A-D^{71} with the substitution of the Ala at the third position of the β-turn by Gly.

By using the racemic lipidic amino acids for conjugation the synthesis always results in diastereomeric mixtures. For the final separation and purification and also for quality control, HPLC can be one of the most important tools.

The lipidic amino acids and peptides have amphophilic character (cationic and anionic plus long hydrophobic side chain) so they can be regarded as "difficult" compounds in the chromatography. Various analytical and preparative columns were tested and the mobile phase composition was also systematically varied to reveal the retention behaviour of the compounds and optimize the separation of diastereomeric isomers.

Experimental

The compounds were synthesized as described by Gibbons *et al.* [1] and Sakarellos *et al.* [2]. They were chromatographically pure. The chemical structure of the compounds can be seen in Table 1.

The HPLC measurements were carried out on a Gilson HPLC system consisting of Model 303 pumps, Model 115 variable wavelength UV detector and Rheodyne injector (Anachem, Luton, UK). Samples were dissolved at 1 mg ml^{-1} concentration in water or aqueous methanol and 20 μl was injected onto the column. Detection was carried out at 210 nm (0.1 AUFS). Spherisorb ODS 5 μm (4.6 × 50 mm) column (PhaseSep, Deeside, UK), Supelcosil™ LC-ABZ (4.6 × 150 mm) (Supelco Inc., Bellefonte, PA, USA) and Partisil 10 ODS-2 (15 mm i.d. × 250 mm) (Whatman Scientific Ltd, Maidstone, UK) columns were used. The mobile phases were various concentrations of methanol–water (mobile phase A: MeOH ranging from 95 to 35%), acetonitrile–water (Mobile phase B: AcN ranging from 95 to 15%), and acetonitrile, 0.1% TFA–water mixtures (mobile phase C: AcN ranging from 95 to 15%). The flow rate was 1 ml min^{-1} in the analytical experiments and 2.00 ml min^{-1} with the preparative column.

The retention time measurements were repeated three times consecutively and the average was taken into account in the calculations of the capacity ratio (k'). The first solvent peak was regarded to be the dead time.

In order to prove that the separated peaks are true isomers the collected fractions from the semi-preparative reversed-phase column were subjected to further FAB-MS analysis. The spectra were obtained by using thioglycerol, TFA matrix to enhance the ionization and solubility.

Results and Discussion

The chemical structures of the compounds investigated are listed in Table 1. Compounds 1 and 2 differ from each other only with the optical isomers of two amino acids from the 10. The retention behaviour of these peptides shows how this part of compound 3 contributes to the retention. Compound 4 had a similar lipidic side chain to compound 3, but it contains only nine amino acids in the peptide chain. The separated diastereomers of compounds 3 and 4 resulted in two peaks. As the exact configuration of the separated isomers

Table 1

No.	Structures of the compounds
1	Trp-dAsn-Pro-Ala-Asp-Tyr-Gly-Gly-Ile-Lys
2	Trp-Asn-Pro-Ala-dAsp-Tyr-Gly-Gly-Ile-Lys
3	Trp-Asn-Pro-Gly-Asp-Tyr-Gly-Gly-Ile CO-NH-(D,L)$CH(CH_2)_{10}CH_3$-COOH
4	Trp-Asn-Pro-Ala-Asp-Tyr-Gly-Gly-Ile CO-NH-(D,L)$CH(CH_2)_{10}CH_3$-COOH

Table 2
The measured log k' values for the compounds investigated by using mobile phase C (acetonitrile–0.1% TFA–water mixtures) and Spherisorb ODS column

	Acetonitrile concentration										
No.	45	50	55	60	65	70	75	80	85	90	95
1	0.16	0.05	−0.04	−0.21	−0.27	−0.34	−0.46	—	–	—	—
2	0.15	0.02	−0.08	−0.24	−0.30	−0.37	−0.46	−0.55	−0.70	—	—
3	0.17	0.03	−0.06	−0.27	−0.34	−0.46	−0.57	−0.80	—	—	—
4	0.18	0.03	−0.04	−0.27	−0.30	−0.37	−0.51	−0.62	—	—	—
5	−0.19	−0.30	−0.24	−0.42	−0.46	−0.51	−0.57	−0.62	—	—	—
6	−0.22	−0.30	−0.28	−0.37	−0.46	−0.51	−0.64	−0.70	—	—	—
7a	0.43	0.06	−0.12	−0.46	−0.57	−0.64	−0.72	−0.80	—	—	—
7b	0.62	0.27	0.05	−0.27	−0.37	−0.46	−0.57	−0.62	—	—	—

Table 3
The measured log k' values for the compounds investigated by using mobile phase C (acetonitrile–0.1% TFA–water mixtures) and Supelcosil LC–ABZ™ column

	Acetonitrile concentration													
No.	10	15	20	25	30	35	40	45	50	55	60	65	70	75
1	—	—	—	—	—	—	0.21	0.09	−0.07	−0.22	−0.30	−0.43	−0.65	—
2	—	—	—	—	—	—	0.06	−0.08	−0.19	−0.28	−0.40	−0.49	−0.60	−0.76
3	—	—	—	—	—	—	0.30	0.18	0.03	−0.19	−0.26	−0.43	−0.52	−0.70
4	—	—	—	—	—	—	0.20	0.01	−0.10	−0.17	−0.28	−0.37	−0.49	−0.65
5	0.97	0.52	0.03	−0.56	−1.13	—	—	—	—	—	—	—	—	—
6	1.02	0.61	0.00	−0.46	−0.90	—	—	—	—	—	—	—	—	—
7a	—	—	—	—	1.01	0.51	0.00	−0.56	—	—	—	—	—	—
7b	—	—	—	—	1.30	0.76	0.24	−0.24	—	—	—	—	—	—

are not known, the letters *a* and *b* denote the first and later eluting peaks, respectively.

The retention of the compounds (log k') was measured by using methanol–water mixtures (mobile phase A), acetonitrile–water mixtures (mobile phase B) and acetonitrile–water–0.1% TFA mixtures (mobile phase C) on two types of reversed phase column. The retention parameters obtained with mobile phase C (acetonitrile, water and 0.1% TFA mixtures) are listed in Tables 2 and 3, on the Spherisorb ODS and on the Supelcosil™ LC–ABZ columns, respectively. In general, wider peaks were observed on both columns when using only methanol and acetonitrile without buffer or TFA. This is probably due to the charges on the compounds and secondary equilibria. Figure 1 shows the chromatogram of compound 3 on Spherisorb ODS using acetonitrile–water (60:40 v/v). The two isomers were not separated. Figure 2 shows the chromatogram of the same compound (3) obtained with the same mobile phase on the Supelcosil LC–ABZ™ column which was specially designed for the separation of anionic, basic or zwitterionic compounds without silanophilic effect. Addition of 0.1% TFA to the mobile phase improved the peak shape on both columns. Figure 3 shows the two chromatograms obtained on Spherisorb ODS (a) and Supelcosil™ LC–ABZ (b) columns for compound 3. Separation of the diastereomeric isomers was observed on both columns, but more symmetrical peaks were obtained on the special reversed-phase stationary phase. When calculating the linear relationship between the organic phase composition of the mobile phase and the log k' values from the data presented in Tables 2 and 3, the calculated S values (slope values) are listed in Table 4. It can be seen that the S values are much higher on the Supelcosil column than on the Spherisorb ODS column. It means that by changing the acetonitrile concentration in the mobile phase the retention time changed more significantly on the Supelcosil LC–ABZ stationary phase, therefore narrower range of mobile phase could be used for the separations. In general, for the same separation lower organic phase concentration was needed and the retention changed with higher degree by changing the organic phase concentration on the Supelcosil™ LC–ABZ column.

As the two diastereomeric isomers might have different biological activity the prepara-

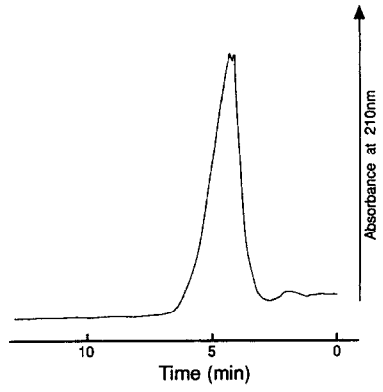

Figure 1
The chromatogram obtained by injecting compound 3 (a and b) on Spherisorb ODS 5 μm (4.6 × 50 mm) column, with 60% (v/v) acetonitrile and 40% (v/v) water mobile phase at flow rate 1.00 ml min^{-1}. Detection was carried out at 210 nm.

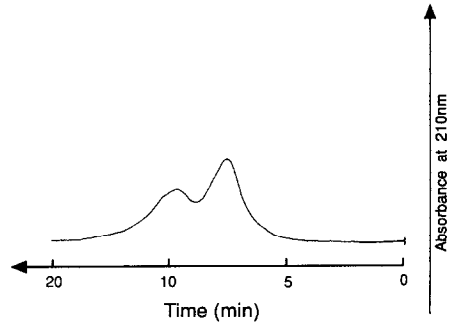

Figure 2
The chromatogram obtained by injecting compound 3 (a and b) on Supelcosil™ LC–ABZ (4.6 × 150 mm) column, with 60% (v/v) acetonitrile and 40% (v/v) water mobile phase at flow rate 1.00 ml min^{-1}. Detection was carried out at 210 nm.

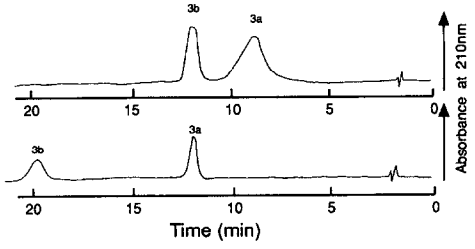

Figure 3
The comparison of the chromatograms obtained on Spherisorb ODS 5 μm (4.6 × 50 mm) (a) and on Supelcosil™ LC–ABZ (4.6 × 150 mm) columns for compound 3 (a and b) by using 35% (v/v) acetonitrile, 0.1% TFA and 65% water as mobile phase with a flow rate 1.00 ml min^{-1}. Detection was carried out at 210 nm.

Table 4
The slope values of the log k' vs acetonitrile concentration plots obtained on the two analytical reversed phase column

Compound no.	Spherisorb ODS	Supelcosil LC–ABZ
1	−0.0124	−0.1056
2	−0.0139	−0.0982
3	−0.0338	−0.1044
4	−0.0346	−0.1028

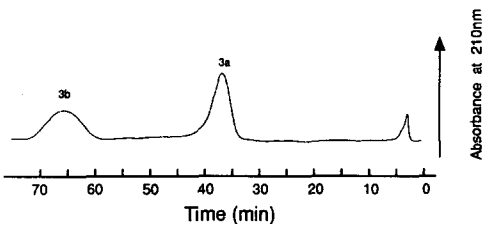

Figure 4
The preparative chromatogram of compound 3 (a and b) obtained by injecting 400 μl from 10 mg ml^{-1} solution on Partisil 10 μl ODS-2 column. The mobile phase was 35% acetonitrile, 0.1% TFA, and 65% water with the flow rate 2.00 ml min^{-1}.

tive separation is of great importance. Figure 4 shows the preparative chromatogram about the separation of the diastereomers of compound 3 on Partisil 10 ODS-2 stationary phase by using acetonitrile–aqueous 0.1% TFA (35:65 v/v). The semi-preparative separation of the diastereomer pairs of compound 4 was carried out using the same mobile phase at a flow rate of 2.00 ml min^{-1}. The retention times were 24 min and 38 min for the first and later eluting diastereomers, respectively.

The separated diastereomer fractions were investigated by mass spectrometry, and they showed similar spectra proving that the diastereomers were isolated. Intensive molecular ion peaks were detected at *m/z* 1185 for both peaks presented in Fig. 4 for compound 3. Also intensive molecular ion peaks were obtained from the two fractions of the separated isomers of compound 4 at *m/z* 1162. By repeating the semi-preparative RP–HPLC separations several times, mg quantities of diastereomers could be collected. The purity of the fractions was tested on the analytical column again, and the pure components are subjected to further biological evaluation.

In conclusion, the slight differences of the retention behaviour of lipidic peptides were observed by using various reversed-phase columns and mobile phase compositions. Better peak shapes and higher resolutions were obtained on all columns investigated by using acetonitrile and TFA in the mobile phase. The retention change due to the organic modifier concentration was more significant on the Supelcosil LC–ABZ column than on the other regular reversed-phase columns. The separation conditions could be easily scaled up for semi-preparative isolation of the diastereomeric isomers.

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