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Capillary zone electrophoresis studies of motilin peptides

Effects of charge, hydrophobicity, secondary structure and length

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ABSTRACT

Motilin is a gut hormone, which is involved in gastrointestinal motility. Capillary electrophoresis studies were made on 24 peptides that are N-terminal, C-terminal or internal fragments of motilin. The isoelectric point, total charge and hydrophobicity were calculated for all of the peptides. The effects of buffers and pH on migration time and resolution were studied. These included citrate buffer, pH 2.5; phosphate buffer, pH 7.0 and borate buffer, pH 10.0. A capillary zone electrophoresis method was developed to resolve 14 of the motilin peptides. Secondary structure predictions were made using the Chou–Fasman method. Circular dichroism spectra were collected to confirm presence of α-helix in several fragments. Effects of charge, hydrophobicity, secondary structure and length of the motilin fragments on migration time were studied.

INTRODUCTION

Motilin is a 22-residue peptide hormone (FVPIFTYGELQRLQEKERNKGQ) that is synthesized in the antrum of the stomach. Its biological effect is to stimulate phase III migrating motor complex (MMC) which increases gut motility [1]. Recently a two-dimensional NMR study was published which had suggested a three-dimensional structure for native motilin [2]. A circular dichroism study was also done indicating that an α-helix was present in native motilin in hexafluoropropanol–water (30:70).

Motilin contains ten charged residues distributed throughout the peptide. Capillary zone electrophoresis (CZE), with separation determined predominantly by charge and mass, seemed to be an ideal technique for analysis of motilin peptides as an alternative to reversed-phase high-performance liquid chromatography (RP-HPLC). No HPLC or CZE studies have been reported on motilin peptides. Previous CZE studies have been done on factors affecting resolution in a series of heptapeptides. These factors included charge, hydrophobicity and amino acid sequence of the peptides [3–5].

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In the present study a series of 24 motilin fragments were synthesized where Leu replaces Met-13 using the standard 9-fluorenylmethyoxycarbonyl (FMOC) [6] solid-phase peptide method. A number of factors including charge, hydrophobicity and secondary structure were correlated with CZE migration times.

METHODS

Capillary electrophoresis was carried out under the following conditions. Instrument: Applied Biosystems (ABI, Foster City, CA, USA) 270 CZE; injection: 2 s vacuum injection, samples *ca.* 10 μg/ml; buffers: pH 2.5 20 mM sodium citrate, pH 7.0 50 mM sodium phosphate, pH 9.4 50 mM sodium borate; voltage: 30 kV, *ca.* 20–30 mA with anode at the autosampler end; capillary: 72 cm (48 cm to detector) × 50 μm, 30°C; detection: UV at 200 nm; running procedure: 2 min wash with 0.1 M NaOH, followed by a 5-min buffer wash, then sample injection.

The purity of the motilin peptides was confirmed by gradient RP-HPLC. The primary structure of all peptides was confirmed by quantitive amino acid analysis and fast atom bombardment mass spectrometry (FAB-MS). In addition the primary sequence of motilin 1-14 was confirmed by peptide sequencing.

The secondary structure was predicted using PROSEC (CHEMLAB, Molecular Design Limited, Hayward, CA, USA) a program which is based on Chou–Fasman methods [7]. Helical wheels were also generated for the regions of the peptides that were predicted to contain α-helix. The isoelectric point (pI) was calculated using MacVector (International Biotechnology, New Haven, CT, USA). The pK_a and pK_b values used for the calculation of pI are based on published data with several modifications [8][a]. The total charges on the peptides were calculated at pH increments of 0.1 units using a program called PIAA, supplied by Applied Biosystems.

The total hydrophobicity values were calculated by summing up the Kyte and Doolittle (K&D) parameters for each residue in the peptide [10]. The charge/mass ratio was calculated using the total calculated charge divided by the molecular mass of the peptide.

All figures were plotted with R/S1 using the MAKEGRAPH function (Bolt, Beranek and Newman, Cambridge, MA, USA) and lines were drawn using the least-squares linear fitting program from R/S1 called FITLINE.

The circular dichroism (CD) spectra were recorded on a Jasco 720 instrument (Japan Spectroscopic Co., Tokyo, Japan) using conditions as listed in the legend to Fig. 2.

RESULTS AND DISCUSSION

The isoelectric points and total charges for the 24 N-terminal, C-terminal and internal peptide fragments of motilin are shown in Table I. The charges range from +0.36 to +4.36 at pH 2.5, −2 to +2 at pH 7.0 and −2.5 to +1.4 at pH 10.0. The

[a] The following values were used for pK_a and pK_b values: Asp 3.86, Glu 4.25, Tyr 10.10, Lys 9.80, Arg 12.48, NH_3 end 8.00 and COOH end 3.00. They are taken with some modifications from ref. 9.

TABLE I

ISOELECTRIC POINT AND CALCULATED TOTAL CHARGE AT pH 2.5, 7.0 AND 10.0

Motilin fragment	p*I*	Calculated charge		
		pH 2.5	pH 7.0	pH 10.0
1-5	5.496	0.398	−0.002	−0.376
1-7	5.496	0.398	−0.003	−0.542
1-9	3.625	0.382	−1.001	−1.542
1-10	3.625	0.382	−1.001	−1.542
1-11	3.625	0.367	−1.999	−2.542
1-12	6.135	1.382	−0.001	−0.543
1-13	6.135	1.367	−0.999	−1.543
1-14	6.135	1.367	−0.999	−1.543
1-15	4.292	1.351	−1.997	−2.543
1-16	6.278	2.351	−0.998	−1.617
1-19	6.365	3.351	0.002	−0.617
1-22	8.673	4.351	1.002	0.309
15-22	8.730	3.367	1.001	0.476
12-22	9.81	4.367	2.001	1.475
10-22	9.81	4.367	2.001	1.475
8-22	8.73	4.351	1.003	0.475
6-22	8.673	4.351	1.002	0.309
3-22	8.673	4.351	1.002	0.309
7-19	6.365	3.351	0.002	−0.617
5-17	4.568	3.351	0.002	−0.617
1-5/17-22	8.899	2.382	0.999	0.550
1-7/19-22	8.797	1.398	0.997	0.384
2-14	6.135	1.367	−0.999	−1.543
3-14	6.135	1.367	−0.999	−1.543

isoelectric points range from 3.6 to 9.8. The p*I* values for the 24 motilin peptides provided a large and varied data base for this CZE study.

Table II lists the migration times at pH 2.5, pH 7.0 and pH 10.0. A marker such as mesityl oxide was not used, so electrophoretic mobilities were not calculated. The migration times range from 5.2 to 13.7 min at pH 2.5; 5.2 to 8.9 min at pH 7.0; 4.7 to 7.3 min at pH 10.0. The greatest resolution for motilin peptides was found in citrate buffer at pH 2.5.

Table III lists the residues that were involved in the predicted secondary structure (α-helix, β-turn and β-sheet). The rationale for including the secondary structure predictions was to study if the secondary structure in these small model peptides would affect CZE migration time. Most of the motilin fragments larger than Mot_{1-14} are predicted to have a helical region, except Mot_{15-22} and $Mot_{1-5/17-22}$. Many of the peptides are predicted to contain a β-turn.

Fig. 1 shows the log of $q/mw^{2/3}$ plotted against the migration time for the 24 peptides. This plot yields a linear relationship ($R = 0.89$), with several outlying points including Mot_{1-14}. Interestingly Mot_{1-14} is predicted to contain regions of α-helix from residues 8-14. Mot_{1-15}, Mot_{1-16} and Mot_{1-19} are also predicted to contain α-helix but are not outlying points in Fig. 1.

TABLE II

CZE MIGRATION TIMES OF MOTILIN PEPTIDES

CZE performed with an ABI 270A at 30 kV, 30°C, with a 72 cm × 50 μm capillary. Buffers were pH 2.5 citrate, pH 7.0 phosphate, and pH 10.0 borate. UV detection at 200 nm.

Motilin fragment	Migration times (min)		
	pH 2.5	pH 7.0	pH 10.0
1-5	10.9	8.99	6.48
1-7	12.3	7.80	6.33
1-9	13.7	8.63	7.15
1-10	12.6	7.31	7.37
1-11	13.7	7.06	4.76
1-12	8.67	7.11	6.11
1-13	9.16	7.22	6.08
1-14	5.96	5.3	6.2
1-15	10.45	5.3	6.77
1-16	7.92	5.29	5.89
1-19	7.13	5.13	5.86
1-22	6.89	5.61	5.47
15-22	5-21	5.52	5.34
12-22	5.29	5.48	4.76
10-22	5.47	5.78	
8-22	5.84	6.81	5.35
6-22	5.96	5.26	5.62
3-22	6.32	5.24	5.67
7-19	6.75	5.61	6.28
5-17	7.78	5.52	7.15
1-5/17-22	7.13	5.82	
1-7/19-22	9.6	5.85	5.80
2-14	9.78	6.41	6.09
3-14	9.39	6.79	6.08

Fig. 2 shows a circular dichroism spectra of $Mot_{1\text{-}22}$ and $Mot_{1\text{-}14}$ in 20 mM acetic acid pH 3.8 with 20% hexafluoropropanol (HFP). HFP tends to induce or stabilize helical regions of peptides. A secondary structure prediction package for CD data from Jasco called SSE-338 was used to calculate amounts of secondary structure including α-helix in $Mot_{1\text{-}14}$ and $Mot_{1\text{-}22}$. This prediction package is based on published methods [11]. The CD results with 20% HFP indicate that $Mot_{1\text{-}14}$ contains 47% α-helix, while $Mot_{1\text{-}22}$ contains 42% helix. These values are similar to the Chou–Fasman predictions of *ca.* 50% α-helix for both peptides. CD studies done in the absence of HFP indicate that both 1-14 and 1-22 still maintain some small amount of helical content.

All CZE studies were performed in aqueous buffers with no addition of HFP.

Table IV lists the Kyte and Doolittle [10] values for the total hydrophobicity, log $(q/\text{mw}^{2/3})$ and the number of residues in the 24 motilin peptides. The calculated charge at pH 2.5 and the K&D hydrophobicity values are 1.3, 2.9 for $Mot_{1\text{-}14}$ and 2.3, -4.5 for $Mot_{1\text{-}16}$, respectively. Motilin 1-19 and 1-22 and many of the other motilin peptides have more negative calculated hydrophobicity and greater positive charges but are well correlated with the linear relationship in Fig. 1. The deviation of $Mot_{1\text{-}14}$ may be due to differences in charge and hydrophobicity.

TABLE III

PREDICTED SECONDARY STRUCTURE OF MOTILIN PEPTIDES USING CHOU–FASMAN METHOD

	1 10 20
Motilin (Leu-13)	FVPIFTYGELQRLQEKERNKGQ

Motilin fragment	Residues contained in secondary structure		
	α-Helix	β-Turn	β-Sheet
1-5			
1-7			
1-9			
1-10		5-9	3-9
1-11		5-9	3-9
1-12		5-9	3-9
1-13		5-9	3-9
1-14	8-14	5-9	3-9
1-15	8-15	5-9	3-9
1-16	8-16	5-9	3-9
1-19	8-19	5-9	3-9
1-22	8-20	5-9, 17-22	3-9
15-22			
12-22	12-20	17-22	
10-22	10-20	17-22	
8-22	8-20	17-22	
6-2	8-20	17-22	
3-22	8-20	3-7, 17-22	3-9
7-19	8-19		
5-17	10-17	7-11	
1-5/17-22		17-22	
1-7/19-22	3-7/19-20		
2-14	8-14	5-9	3-9
3-14	8-14	5-9	3-9

Fig. 3 depicts the migration time *versus* hydrophobicity. The data points are not well correlated with a linear function (R = 0.67). The plot indicates that the more hydrophobic (less hydrophilic) the peptide, the longer the migration time. Presumably the hydrophilicity is related to the relative charge of the peptide and thus CZE migration time.

Fig. 4 is a plot of the log ($q/\mathrm{mw}^{2/3}$) *versus* hydrophobicity. The relationship can be fitted to a linear function with R = 0.81. Many of the deviating points, such as motilin 1-9, 1-10 and 1-11, have small charge/mass ratios (due to high percentage of charged residues) as well as large hydrophobicities.

Fig. 5 is an electropherogram of 14 of the motilin fragments. This figure indicates the resolving power of CZE. Many of the peptide fragments differ by only one residue, while others have only a small difference in total charge.

In this study several different factors affecting CZE of motilin peptides were investigated. These included the affects of charge/mass; hydrophobicity and secondary structure on migration time.

The CZE migration times for this series of 24 motilin peptides is linearly corre-

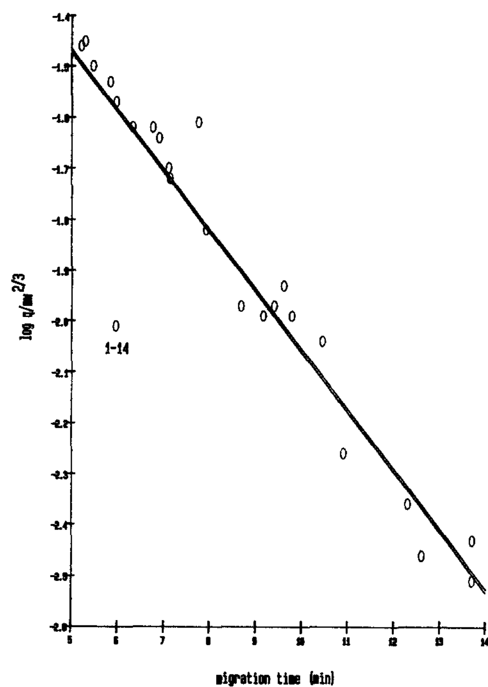

Fig. 1. Log (q/mw$^{2/3}$) *versus* CZE migration time (min) for 24 motilin peptide fragments run at 30 kV in pH 2.5 citrate buffer.

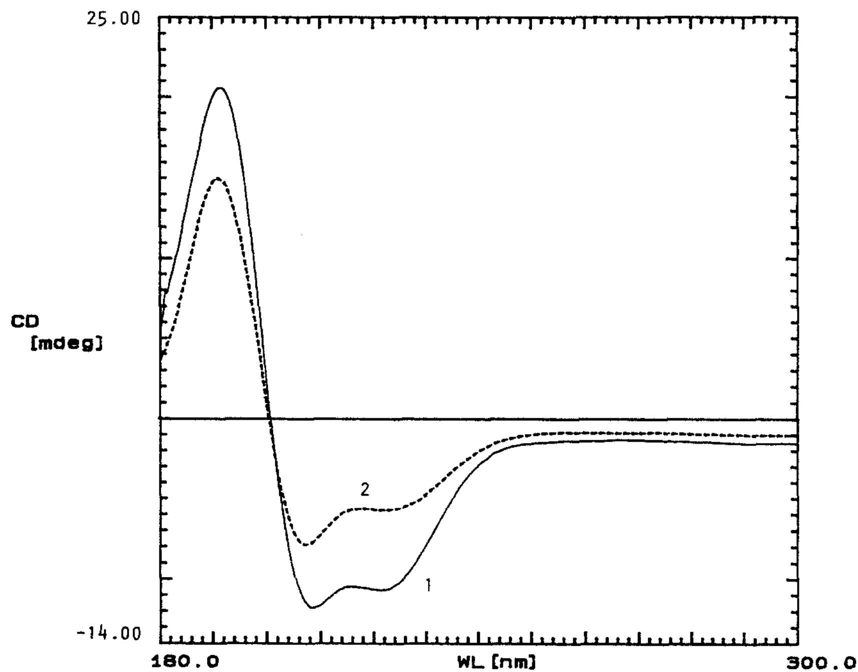

Fig. 2. Circular dichroism spectra, ellipticity (millidegree) *versus* wavelength (WL) (nm) of $Mot_{1\text{-}22}$ and $Mot_{1\text{-}14}$. Sample is 0.7 mg/ml in 20 mM acetic acid pH 3.8 with 20% hexafluoropropanol. CD method: Scan 300 to 180 nm at 50 nm/min using a step of 0.1 nm/data. Bandwidth 1.0 nm, sensitivity 20 millidegree with response of 8 s. Path length of cell was 0.1 mm, temperature was 25°C. Curves: 1 = $Mot_{1\text{-}22}$; 2 = $Mot_{1\text{-}14}$.

TABLE IV

MASS/CHARGE RATIO, HYDROPHOBICITY AND NUMBER OF RESIDUES IN MOTILIN PEPTIDES

Motilin fragment	$\mathrm{Log}(q/\mathrm{mw}^{2/3})$	Hydrophobicity (Kyte and Doolittle)	Number of residues
1-5	−2.26	12.7	5
1-7	−2.36	10.7	7
1-9	−2.43	6.8	9
1-10	−2.46	10.6	10
1-11	−2.51	7.10	11
1-12	−1.97	2.60	12
1-13	−1.99	6.40	13
1-14	−2.01	2.90	14
1-15	−2.04	− 0.60	15
1-16	−1.82	− 4.50	16
1-19	−1.72	−16.00	19
1-22	−1.64	−23.80	22
15-22	−1.46	−26.70	8
12-22	−1.45	−30.90	11
10-22	−1.50	−30.60	13
8.22	−1.53	−34.50	15
6.22	−1.57	−36.50	17
3-22	−1.62	−30.80	20
7-19	−1.62	−31.15	13
5-17	−1.61	−21.05	12
1-5/17-22	−1.70	−6.60	11
1-7/19-22	−1.93	−4.10	11
2-14	−1.99	0.10	13
3-14	−1.97	1.70	12

lated with the log of the charge/mass ratio when $q/\mathrm{mw}^{2/3}$ is plotted, with several exceptions. When q/mw is plotted the linear correlation is less ($R = 0.86$). Similar results have been shown in CZE studies of other peptides [12]. The correlation between migration time and hydrophobicity is poor. In this series, the hydrophobicity is not a major factor in determining migration in CZE, although generally more hydrophobic motilin peptides have longer migration times. The hydrophobicity of these fragments has a higher correlation with the charge/mass ratio than with the migration time. The N-terminal region of $\mathrm{Mot}_{1\text{-}22}$ and similar fragments is hydrophobic, while the carboxy terminal region is hydrophilic. In general the 24 fragments have charged groups that are distributed throughout the length of the peptides, but there is no correlation between the number of residues and CZE migration time.

In the motilin family of peptides, the presence of secondary structure doesn't appear to be a major factor in deviations from linearity in Fig. 1. Smaller peptides such as motilin 1-5, 1-7, 1-9, 1-11, 1-12 and 1-13 are not predicted to contain α-helix and are not outlying points. An exception is $\mathrm{Mot}_{1\text{-}14}$ which contains helix and also has a much faster migration time than would be expected from the charge/mass ratio. Presumably a small peptide containing a significant amount of helical structure could possess an altered hydrodynamic profile compared to a peptide having no secondary structure. This could possibly affect migration time and offer an explanation for the

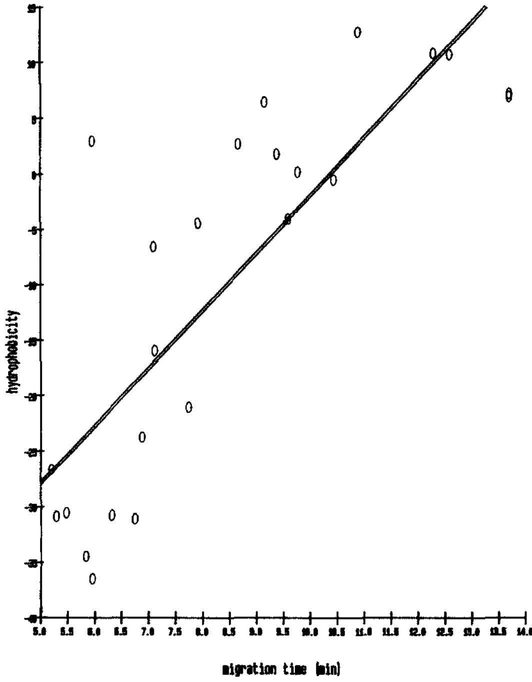

Fig. 3. Hydrophobicity (Kyte and Doolittle parameters) *versus* CZE migration time (min) for 24 motilin fragments at 30 kV in pH 2.5 citrate buffer.

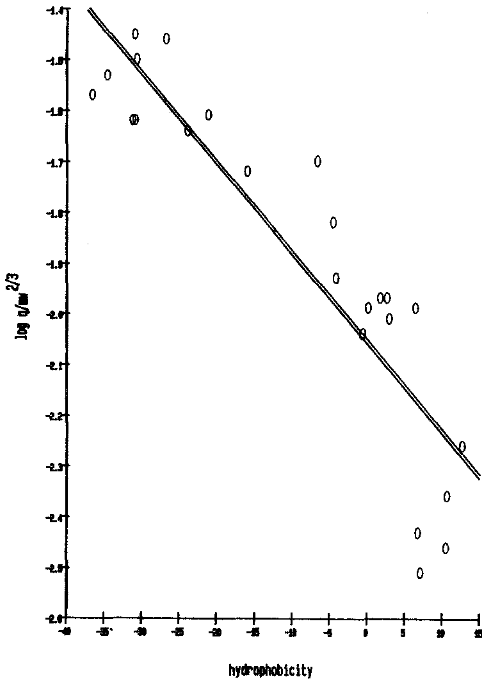

Fig. 4. Log $(q/mw^{2/3})$ *versus* hydrophobicity (K&D parameters) for 24 motilin fragments.

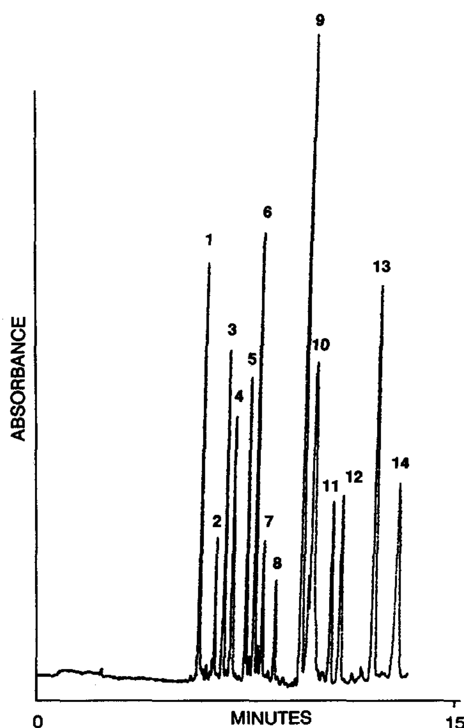

Fig. 5. Electropherogram of fourteen motilin peptides. 1 = Mot_{15-22}; 2 = Mot_{10-22}; 3 = Mot_{1-14}; 4 = Mot_{3-22}; 5 = Mot_{1-22}; 6 = Mot_{1-19}; 7 = Mot_{1-16};8 = Mot_{1-12}; 9 = Mot_{3-14}; 10 = Mot_{2-14}; 11 = Mot_{1-15}; 12 = Mot_{1-5}; 13 = Mot_{1-7} 14 = Mot_{1-9}. Conditions as described in Methods using pH 2.5 citrate buffer at 20 kV. Absorbance range 0–0.01 a.u.f.s. Each motilin peptide was run individually to correlate migration times in the mixture.

deviation of Mot_{1-14} seen in Fig. 1. It is not clear why a peptide with a similarly predicted helical content such as Mot_{1-16} is linearly correlated in Fig. 1. Further CD studies will be needed to confirm helical content and to evaluate the contributions of secondary structure to CZE migration time in the motilin peptides.

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