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High-performance separation methods in the analysis of a new peptide family: the galanins

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Abstract

An analytical investigation of a new peptide family, the human galanins and their fragments, was carried out by reversed-phase HPLC, capillary zone electrophoresis (CZE) at different pH values and micellar electrokinetic capillary chromatography (MECC) in phosphate-borate-sodium dodecyl sulphate buffer. None of the methods seems to be superior to the others. The complementary nature of the electrophoretic methods is obvious when the profiles of peptides are compared; impurities not separated by HPLC are separated by CZE or MECC and vice versa. With these three different separation methods, a more complex analytical control of the synthetic work can be achieved.

Keywords: Galanins; Peptides

1. Introduction

Galanins (GALs) are relatively new 29/30 amino acid-containing multifunctional peptides in the neuroendocrine system. The first biologically active GAL was isolated in 1983 from porcine intestine (pGAL). Since that time, rat, bovine, chicken and recently sheep and alligator GALs have also been discovered, identified and chemically characterized [1]. They are widely distributed in the central and peripheral nervous systems, urogenital and gastrointestinal tracts and adrenals. All known endogenous animal GALs are linear 29-mer peptides amidated at the C-terminal. The amino acid sequence of their N-terminal 1–15 part is identical in all species, however, the C-terminal part is more species-specific.

The primary structure and the identification of two molecular forms of human galanin (hGAL) were described in 1991 [1]. Human galanins contain 30 or 19 amino acids that share the sequence of the amino-terminal fifteen residues with other mammalian GALs, all amino acid substitutions are restricted to the C-terminal part of the molecule (16–30). The carboxy terminus of hGALs is not amidated. The endogenous hGAL1–19 (the N-terminal part of hGAL1–30) is generated by enzymatic cleavage of the longer form [2].

Biological studies carried out mainly with porcine and rat GAL have shown that they have various physiological/pharmacological effects in endocrine and nervous systems, such as controlling hormone release (insulin, gastrin, somatostatin, growth hormone, prolactin), neurotransmitter release (acetylcholine, serotonin, dopamine) and species-specific effects on the neurons, smooth muscle, gut

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and feeding [3]. They exhibit contractile action on the rat jejunum, ileum, fundus and urinary bladder [4]. Receptor-binding and structure–activity studies have led to many important results and a number of effective receptor antagonists have been developed and are successfully applied in physiological studies [5].

Biologists and especially neuroendocrinologists expect hGALs to have different and new physiological, pharmacological, endocrinological and psychiatric effects. For this reason, we have synthesized and purified hGALs1–30 and 1–19 and some of their fragments and investigated their purity by means of different chromatographic techniques.

2. Experimental

2.1. Materials

All the hGAL peptides were synthesized manually in our laboratory by the solid-phase peptide synthesis method on 1% cross-linked polystyrene-divinylbenzene resin (1 mmol/g) by using the Boc technology [6]. Dicyclohexylcarbodiimide–hydroxy-benztriazole (1:1) and a three-fold excess of Boc-amino acid were applied for coupling. All couplings were controlled by the test of Kaiser et al. [7]. Deprotection of the peptides and cleavage of the peptides from the resin were performed with the hydrogen fluoride (HF) procedure (0°C, 45–60 min) in the presence of anisole–dimethyl-sulphide–*p*-thiocresol (1:3:1, v/v/v). The crude peptides were purified by preparative reversed-phase high-performance liquid chromatography (RP-HPLC) and structural confirmation achieved by fast atom bombardment (FAB)-MS, amino acid analysis, circular dichroism (CD) spectra and specific rotations ($[\alpha]_D^{20}$).

Trifluoroacetic acid (TFA), acetonitrile, disodium hydrogenphosphate, potassium dihydrogenphosphate, sodium borate and sodium dodecyl sulphate (SDS) were supplied by Merck (Darmstadt, Germany). Purified water obtained from a Milli-Q system from Millipore (Bedford, MA, USA) was used to prepare all solutions. All other reagents were of analytical grade and were used without further purification.

2.2. Instruments

The purification and analysis of synthetic peptides were carried out on a Pharmacia-LKB HPLC system using a semipreparative LiChrosorb RP-18 column (250×16 mm I.D., particle size 10 μm) and an analytical Nucleosil $5C_{18}$ column (250×4 mm I.D., particle size 5 μm, pore size 300 Å) equipped with an appropriate precolumn (30×16 mm I.D. or 30×4 mm I.D., respectively) obtained from Knauer (Berlin, Germany).

For electrophoretic analysis, a BioFocus 3000 capillary electrophoresis system from BioRad (Hercules, CA, USA) equipped with a fused-silica capillary-containing cartridge (50 cm×50 μm I.D., BioRad Laboratories), was used. Data acquisition was performed on a DTK-486 PC with the BioFocus Integrator software.

2.3. Methods

Analytical HPLC of the peptide samples was performed by using a linear gradient of 0.1% aqueous TFA (eluent A) and 80% aqueous acetonitrile solution containing 0.1% TFA (eluent B). A gradient profile of 20% B to 80% B in 30 min) with a flow-rate of 1.0 ml/min was used at ambient temperature. Peptides dissolved in eluent A (1 mg/ml) were injected (20 μl) and column eluates monitored at 215 nm.

Fused-silica capillaries with an effective length of 45.5 cm were used for capillary zone electrophoresis (CZE) and micellar electrokinetic capillary chromatography (MECC). The capillary was conditioned by rinsing with 0.1 *M* sodium hydroxide for 20 min, then filled with the running buffer and equilibrated for another 20 min. Before each run, the capillary was rinsed with the appropriate running buffer (6 min for CZE and 8 min for MECC). Run buffers for CZE were prepared by titration of 0.1 *M* phosphoric acid with 1 *M* sodium hydroxide solution to the desired pH (2.5–5.0). MECC was carried out in 0.01 *M* sodium borate buffer (pH 8.5) containing 0.005 *M* sodium phosphate and 0.05 *M* SDS. Samples were made up as 1 mg/ml solutions in water. All buffers and samples were filtered

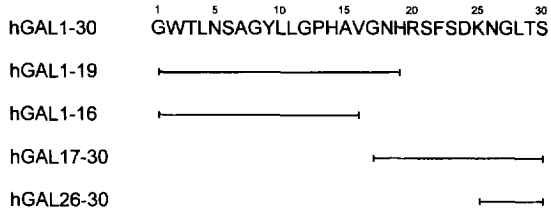

Fig. 1. Amino acid sequences of hGALs and their fragments. The C-termini are free acids.

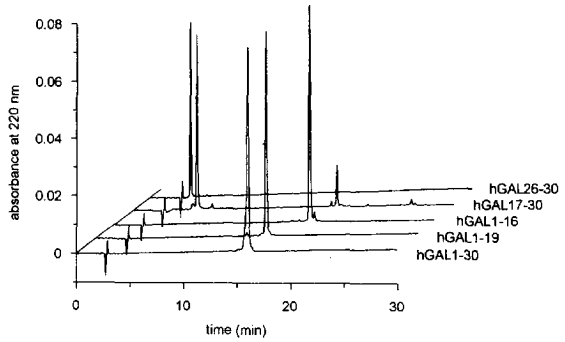

Fig. 2. RP-HPLC analysis of purified synthetic human galanins and their segments on a 250×4 mm I.D. analytical column (Nucleosil $5C_{18}$) connected to 30×4 mm I.D. pre-column. Chromatographic conditions: solvents, A=0.1% TFA in water, B=0.1% TFA in 80% aqueous acetonitrile; gradient, 20 to 80% B in 30 min; flow-rate, 1.0 ml/min; detection, 215 nm.

through a 0.45 μm pore filter (Millipore) and degassed prior to use. Injection of the samples was accomplished by pressure injection for 2 s (about 14 nl). The running voltage was 15 kV for both methods with a maximal current of 80 μA; and the polarity was towards the cathode. Sample monitoring was performed at 200 nm (on column). During runs, the capillary temperature was set to 20°C (water cooling).

3. Results

The amino acid sequences of hGAL1–30 and its C- and N-terminal fragments are shown in Fig. 1. The middle region (14–25) of hGAL1–30 contains the polar amino acids (H, H, R, D, K), while the C-terminal part is less polar.

Preparative HPLC using an acetonitrile–water–TFA gradient on a reversed-phase column proved useful for purifying synthesized GALs. The purity of the collected fractions was more than 97% (except for hGAL17–30), as determined by analytical HPLC, CZE and MECC. Fig. 2, Fig. 3 and Fig. 5 show the chromatographic and electrophoretic profiles of hGAL1–30 and its fragments.

The hydrophobicity of the peptides increases with increase in size, as depicted in Table 1, and, as a consequence, retention time generally correlates with the hydrophobicity. The C-terminal pentapeptide reveals either lowest hydrophobicity or retention time, whilst the obviously most hydrophobic hGAL1–30 (assuming an additive effect of contributions of hydrophobic increments from the individual amino acids) nevertheless was not the last-eluting component on a C_{18} RP-HPLC column. The high resolution of HPLC is demonstrated in Fig. 2, which shows a minute amount of impurities before and after the main peak of each peptide.

CZE was performed at six different pH values.

Table 1
Physicochemical properties, migration and retention time for galanin 1–30 and its fragments

Peptide	Hydrophobicity[a]	Retention time (min)	Charge[b]	CZE migration time (min)	MECC migration time (min)
hGAL1–30	37.61	15.94	+4.74	14.19	19.85
hGAL1–19	31.79	15.67	+0.83	15.80	19.35
hGAL1–16	29.28	18.29	+1.83	18.59	21.51
hGAL17–30	8.33	5.96	+3.74	11.27	18.37
hGAL26-30	4.84	3.67	+0.83	20.02	6.50

[a]Sum of hydrophobic increments of amino acids [8,9].
[b]Calculated according to Henderson–Hasselbalch equation; pK data obtained from Rickard et al. [10].

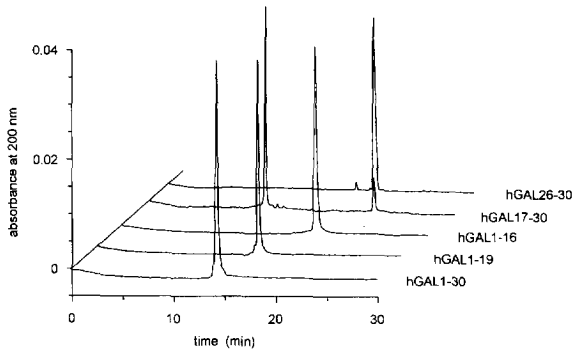

Fig. 3. CZE analysis of synthetic hGALs and their fragments. Electrophoretic conditions: capillary, 50 cm×50 μm I.D. fused silica, separation length 45.5 cm; buffer, 0.1 *M* sodium phosphate, pH 2.5; injection, pressure mode for 5 p.s.i.×s (pressure×time); voltage, 15 kV; temperature, 20°C.

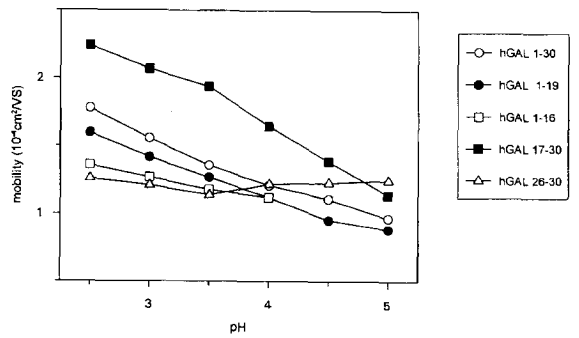

Fig. 4. Dependence of electrophoretic mobility for human galanin peptides on pH of running buffer. Experimental conditions: as in Fig. 3. Buffers: 0.1 *M* sodium phosphate, pH range 2.5–5.0.

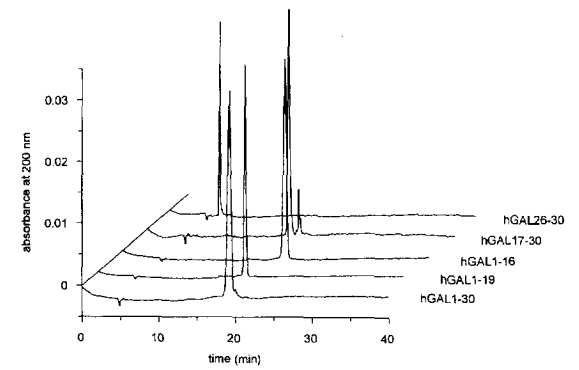

Fig. 5. Analysis of synthetic human galanins and their fragments by MECC. Buffer, 0.01 *M* sodium borate +0.005 *M* sodium phosphate +0.05 *M* SDS. All other conditions were the same as for Fig. 3.

hGAL17–30 had the highest mobility, whereas hGAL26–30 migrated most slowly at pH 2.5 (Fig. 3). Again, some impurities were found in all peptide samples except hGAL1–19. There was a better separation for hGAL26–30, but worse for hGAL1–16 as compared to results from HPLC studies.

Plotting of the observed electrophoretic mobilities determined at the different pH values resulted in parallel declining curves, with a slight disturbance in the case of peptides carrying more than one positive charge (Fig. 4). The migration of the shortest GAL fragment is less dependent on the pH value and a slight increase of electrophoretic mobility was observed at higher pH. The migration time for hGAL1–16 was out of the time window at higher pH.

To determine the influence of the physicochemical properties of the peptides on their mobility, the electrophoretic mobility was plotted versus the calculated charge (q) divided by the molecular mass (M) or amino acid number (n) to various exponential functions [10,11]. A good linear correlation was obtained for every calculated charge-to-size function ($\ln[q+1]/n^{0.43}$, $q/M^{1/3}$, $q/M^{2/3}$) at pH 2.5 (r=0.9958, r=0.8973, r=0.9745, respectively), but it decreased on increase of the pH and it completely vanished above pH 4.0.

The migration times for the larger peptides were close together in MECC despite the differences in hydrophobicity. hGAL1-16, with the highest retention time in RP-HPLC showed the lowest mobility in MECC and there was a good correlation between the retention times from RP-HPLC and MECC for three other peptides, but nevertheless hGAL17–30 did not fit into this series. Some impurities occurred on micellar electrokinetic chromatograms, but the separation was less efficient compared with the previous methods (Fig. 5).

4. Discussion

The parallel use of three different separation techniques demonstrated the feasibility of all the systems and the information acquired substan-

tially enhanced when compared with the results obtained by the use of one method alone. RP-HPLC as a well-known and highly reproducible technique will be the basis of quality control in peptide synthesis, but the additional data from the different capillary electrophoretic methods should not be overlooked [12,13].

The major factor governing the retention behaviour of peptides during reversed-phase chromatography is the relative hydrophilic/hydrophobic character of the side chain of amino acid residues contributing to the overall hydrophobicity of the molecule. Despite of the fact that the sum of individual contributions of hydrophobic increments from the individual amino acids would implicate the highest retention for hGAL1–30, the peptide did not elute last in our experiment. However, the amino acid composition itself does not determine the retention time of a peptide, ionic and other intramolecular interactions between neighbouring amino acids, and conformational effects also have to be considered. Though secondary structure (e.g. α-helix or β-sheet) is generally absent even in favourable aqueous conditions for small peptides (up to approximately fifteen residues), the potential for a defined secondary and tertiary structure increases with increasing polypeptide chain length. For peptides greater than 20–35 residues, folding to internalize hydrophobic residues to stabilize the secondary and tertiary structure in aqueous solution is likely become a significant conformational feature. NMR experiments [14] performed on hGAL1–30 dissolved in aqueous solution (150 m*M* KCl, pH 4.0) indicate that region 3–11, which incorporates a hydrophobic core from residues 6–11, form a nascent helix, and residues 14–18 and 22–30 adopt nonrandom conformation. CD studies showed that ca. six residues of peptide transformed to a stable helix in the presence of 30–60% TFE. These conformational data would explain altered retention behaviour of hGAL1–30, i.e. the hiding of hydrophobic amino acids resulted in a shorter retention time.

Separation in CZE is based on the differential migration of charged peptides in an electric field due to differences in the charge-to-size ratios of the peptides. McCormick [15] described the advantages in the lower pH range for the separation of peptides in CZE. At this pH range all of the amino groups are protonated and the ionization of carboxyl groups is nearly completely suppressed, which results in the migration of all molecules towards the cathode. The mobility plot (Fig. 4) indicates that the peptides have different charges at different pH values. The net charge of a peptide can be modified by a small change in buffer pH, particularly near the pK_a of the amino and carboxyl groups from either N- or C-terminal parts as well as side chains. Under these low pH conditions, the aspartic acid side group and the carboxyl terminal group are completely protonated.

In addition to affecting the solute charge, a change of pH will also cause a concomitant change in the electroosmotic flow, which in turn affects the separation efficiency and the resolution. A buffer of low pH protonates the capillary wall, decreasing the electroosmotic flow almost to zero. An increasing pH alters the surface of the silica capillary: it becomes more negative which elevates the electroosmotic flow. Although the p*I* of fused silica differs from vendor to vendor, the electroosmotic flow generally becomes only significant above pH 4.0. Thus a change of pH affects the interactions of the peptide with the silanol groups on the capillary wall but conformative changes of the peptide as a consequence of a change in pH have to be additionally considered.

The correlation of electrophoretic mobility with physicochemical parameters is difficult [10,16]. We must account for the movement of a charged ion of finite size through an ionic medium in the presence of an electric field. The mobility of an analyte depends on its size, shape and charge. The charge and size of the analyte must be determined, but both of these factors are affected by the pH and the composition of the buffer. In calculations of electrophoretic mobilities, the shape of peptides has traditionally been assumed to be spherical, whereas the net charge has been calculated from the amino acid composition by using the Henderson–Hasselbalch equation [10]. The good correlation in either type of calculation ($\ln[q+1]/n^{0.43}$ and $q/M^{2/3}$) at

low pH indicates that both models are valid and that there is a strong connection between the electrophoretic mobility and the charge/molecular mass or peptide chain length. The lack of a correlation at pH>4.0 can be explained by the different secondary and tertiary structures of the peptide molecules at different pH values.

The MECC method, which is based on the partitioning between the micelles (acting as the stationary phase in HPLC) and the free buffer solution, is very similar to RP-HPLC, the proposed purity check method for peptide analysis [17,18]. Because of the analogous separation techniques, the two methods can readily be compared. With this experimental system, the acceptable HPLC purification of peptides could be proved by CE run in the MECC mode, where the resolution was almost the same as in RP-HPLC.

Our results indicate that sufficiently pure hGALs and their fragments can be obtained in one or two steps by preparative RP-HPLC. At the same time, besides the analytical RP-HPLC, amino acid analysis, FAB-MS and other characterization methods, the two different HPCE methods we have used in this case proved to be an additional reliable means of quality control. Since the electrophoretic separation mechanism of capillary electrophoresis is different from the analyte–stationary phase interaction employed by RP-HPLC, the two methods are complementary and can be used in parallel in the analytical characterization of the hGALs and other similar peptides.

Acknowledgments

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