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Identification of gonadorelin (LHRH) derivatives: comparison of reversed-phase high-performance liquid chromatography and micellar electrokinetic chromatography

Patrick H. Corran* and Nick Sutcliffe

Chemistry Division, National Institute for Biological Standards & Control, Blanche Lane, South Mimms, Potters Bar, Herts. EN6 3QG (UK)

ABSTRACT

A number of reversed-phase (RP) HPLC systems for the separation of gonadorelin (gonadoliberin, LHRH) and five therapeutically important analogues have been systematically examined. The selectivity of RP-HPLC has been compared with several micellar electrokinetic chromatographic (MEKC) systems and free solution capillary electrophoresis. RP-HPLC exhibits greater selectivity towards structural differences, but complete separation of the peptides in one isocratic analytical run is tedious due to the large differences in retention. Gradient elution gives satisfactory separation in an acceptable time span. Of the micellar systems examined (sodium dodecyl sulphate, cetrimide, 3-[(cholamidopropyl)dimethylamino]-1-propanesulphonate and Triton X- 100) only MEKC with cetrimide micelles gave a complete separation showing selectivity similar, but not identical, to RP-HPLC, and providing a complete separation of all six compounds as rapidly as gradient RP-HPLC.

INTRODUCTION

Over the last five years a number of gonadorelin* derivative have entered clinical use for the control of gonadotrophin-dependent conditions, in particular by inhibiting the production of gonadal steroids. Two important applications are in the treatment of cancer of the prostate, where gonadorelin analogues offer an alternative to surgical intervention, oestrogens or anti-androgens, and the treatment of endometriosis (for an overview see refs. l-3). The natural gonadotrophin-releasing hormone, gonadorelin, is a lo-residue peptide possessing a blocked N-terminal residue (pyroglutamic acid) and a C-terminal amide. It is mainly of diagnostic use clinically. The derivatives on the market at present all contain a bulky D-amino acid residue substituted for Gly at position 6 and a variety of modifications for the C-terminal glycinamide residue. They have a stronger receptor binding affinity than gonadorelin, and ultimately totally inhibit pituitary-dependent secretion of gonadal steroid hormones. The structures of these analogues and gonadorelin itself are summarised in Fig. 1. All derivatives contain one D-amino acid [D-tryptophan, D-leucine, the tertiary butyl ether of D-Ser (twice) and D-naphthylalanine] as a replacement for glycine, in three the C-terminal glycinamide is replaced with ethylamine, in one with an azaglycinamide residue and one (nafarelin) has an unmodified glycinamide. There is a considerable literature on high-performance liquid chromatography (HPLC) of gonadorelin and numerous analogues (e.g., see refs. 4-1 1), but most of these studies

* Corresponding author.

☆ Gonadorelin is the International Non-proprietary Name (INN) of the hormone also referred to as gonadoliberin (IUPAC/IUB approved name), GnRH or LHRH. For consistency, since the derivatives studied are unnatural sequences, the INN is used throughout.

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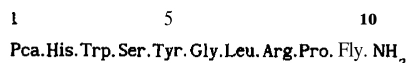
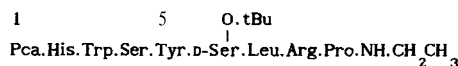
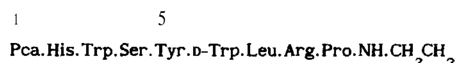
(1) Gonadorelin (gonadoliberin, LHRH)



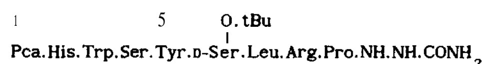
(2) Buserelin



(3) Deslorelin



(4) Goserelin



(5) Leuprorelin

(6) Nafarelin

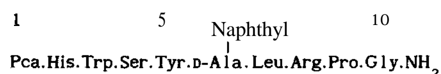


Fig. 1. Structure of gonadorelin and five analogues. tBu = *tert*.-butyl, Pca = pyrrolidone carboxylyl (pyroglutamyl).

have been concerned with the assay, identification or purity of a single peptide, and there is little information on comparative chromatographic behaviour. For the purposes of identification it is necessary to be able to distinguish between the six closely related peptides, and the first part of the study reported below was designed to systematically examine several candidate reversed-phase (RP) HPLC systems likely to be useful as the basis of an identity test in an official specification such as a pharmacopoeia. However, the set of peptides also provides a series of closely related structures with which to explore the differences in selectivity between the different forms of capillary electrophoresis (CE) and RP-HPLC. There have been a number of reports of free CE of peptides (e.g., see refs. 12-15) including gonadorelin [16,171. Since all the analogues possess the same number and type of ionisable residues as gonadorelin itself, limited success was expected from free CE. However a number of additives have been shown to affect the electrophoretic mobility of peptides when included in the electrolyte. These include alkyl sulphonates [18] and alkyl quaternary ammonium salts [19,20], cyclodextrins [20] and the replacement of H_2O by ^2H_2O [21]. The addition of charged detergents at concentrations above the critical micelle limit in the hybrid technique micellar electrokinetic chromatography (MEKC) [22] may also powerfully affect electrophoretic separations, since there may be wide differences in the way that different solutes partition to the micelles, and since uncharged solutes can be separated through differences in their partition coefficients, but there have been few reports of the use of MEKC to separate peptides (one exception is ref. 20). The study was therefore extended to compare the selectivity observed in RP-HPLC with free CE and electrophoresis in a number of micellar systems.

MATERIALS AND METHODS

Apparatus

RP-HPLC was carried out on a system consisting of a Waters WISP Model 712 automatic injector (Millipore, Watford, UK) and a Spectra-Physics SP8800 or SP8700 gradient pump, SP100 UV detector and SP4400 integrator with Lab BASIC (Spectra-Physics, Hemel Hempstead, UK). Raw data was captured via Labnet to an IBM Model 55SX computer running WINner software and reprocessed with the supplied utility DISPLAY, if necessary (Spectra-Physics). The columns compared were a single or double cartridge (100 or 200 x 3 mm I.D.) of Spherisorb S5ODS-2 (Chrompack, Milharbour, London, UK), a column (250 x 5 mm I.D.) packed in-house with Nucleosil 5 C_{18} (Camlab, Cambridge, UK) and a column (150 x 4.6 mm I.D.) of the polymeric reversed-phase packing PLRP-S 300 (d_p 8 μm) (Polymer Labs., Church Stretton, UK).

CE was carried out on a Model 270HT system (Applied Biosystems, Warrington, UK). Data was collected and integrated using a PC with data acquisition card and Summit software modified for

use with CE (Comus, Humberside, UK). Polymicro Technologies fused-silica capillary tubing (50 μm I.D. x 375 μm O.D.) was purchased from Composite Metal Services (Hallow, UK) and prepared before use by flushing with 1 A4 NaOH for 30 min followed by water for 10 min.

Materials

Ammonia (specific gravity 0.880), triethylamine (TEA) and tetramethylammonium hydroxide (TMA) (25%, w/w, aqueous solution) were purchased from Aldrich (Gillingham, UK), the sodium salts of butane and pentane sulphonic acid from Phase Separations (Deeside, UK), of hexanesulphonic acid from Fisons (Loughborough, UK), of heptane sulphonic acid from BDH (Poole, UK) and of octanesulphonic acid from Aldrich. Sodium dodecyl sulphate (SDS) for electrophoresis and cetyltrimethylammonium bromide (CTAB) were from BDH and 3-[(cholamidopropyl)dimethylamino]-l-propanesulphonate (CHAPS) and Triton X-100R-S from Sigma (Poole, UK). Other reagent were of analytical-reagent or HPLC-grade as appropriate.

Gonadorelin and buserelin were supplied by Hoechst (Milton Keynes, UK), goserelin by ICI Pharmaceuticals (Maccleslield, UK), nafarelin by Syntex Pharmaceuticals (Ivor, UK), leuprorelin by Cyanamid (Gosport, UK) and deslorelin by Bachem (Torrance, CA, USA), all as peptide acetates. They were dissolved in 20% acetonitrile at a concentration of 2 mg/ml and further diluted as necessary. For CE the samples were further diluted to 200 μg/ml in 10 m*M* sodium citrate pH 2.5.

Methods

HPLC solvents were made up by titrating a 0.1 *M* solution of phosphoric acid (final concentration) to pH 2.5 with the appropriate base: for system a 10 *M* NaOH, for system b 0.880 NH_3, for system c 25% TMA, and for system d TEA. System e was 0.05% (v/v) phosphoric acid and 0.5% (w/v) $(NH_4)_2SO_4$. These aqueous solutions were used in the gradient pumps as solvent A. A portion was then mixed with an appropriate volume of acetonitrile to give a 60:40 or 50:50 mixture acetonitrile-buffer for use as solvent B. All solvents were filtered through a 0.2-μm filter before use, and solvent reservoirs were sparged with helium and maintained with a low flow of helium throughout chromatography.

For studies of the effect of acetonitrile concentration on retention a short Lab BASIC routine was written for the SP4400 integrator which reprogrammed the solvent composition supplied by the pump. The BASIC pseudo-random number generator was used to select the next composition from one of a series of steps (usually 10) covering a given range of compositions until a specified number of replicates (usually two) at each composition had been completed. This approach was chosen to avoid possible problems with systematic alterations in solvent composition over protracted analytical runs due to helium degassing of solvents. There was a good distribution of compositions throughout any given series of runs, with no systematic bias readily disernable. Duplicate retentions agreed well.

CE was carried out in capillaries of from 45 cm (23 cm to detector) to 100 cm (78 cm to detector). Capillaries were flushed before each run for 2 min with 0.1 *M* NaOH followed by the appropriate running buffer (for 4 or 5 min). Sampling was by vacuum for l-5 s depending on capillary length, and for identification of individual components a marker injection cycle was included to inject a small amount of a mixture of pure components. Benzyl alcohol was used to estimate endosmosis in free electrophoresis and methanol in charged micellar systems. Micellar mobilities of charged micelles were estimated with Sudan III [22]. Electropherograms were monitored at 200 nm except for the CTAB system (280 nm, see text). All buffers were passed through a 0.2-μm filter before use. Electrophoresis was carried out towards the anode for the CTAB systems and towards the cathode for all others. Free solution electrophoresis was normally carried out in 0.2 *M* sodium phosphate pH 2.5, but for the determination of the dependence of mobility on pH 0.1 *M* sodium dihydrogenphosphate adjusted to pH values between 2 and 7.5 with H_3PO_4 or NaOH as appropriate, or 0.05 *M* Tris-0.02 *M* sodium borate pH 8.5 and pH 9.5 was used. To examine the effect of hydrophobic pairing agents 50 m*M* sodium salt of the appropriate ion pair was added to the pH 2.5 phosphate electrolyte. MEKC was carried out in 0.05 *M* Tris-borate adjusted to pH values from 7 to 9.5 with 5-25 m*M* CTAB or 40 m*M* SDS, or in 0.2 *M* phosphate pH 2.5 with 5-50 m*M* CHAPS or 0.1–0.5% (v/v) Triton X-100. All pH values were determined at ambient temperature (20–25°C during the period of the study).

RESULTS

Reversed-phase chromatography

All the analogues were eluted in the same order and well separated in all systems with the general exception of the pair goserelin-leuprorelin, which was often poorly resolved. For the silica-based columns the systems a, b and e showed the best separation of this pair, but systems a and b showed significant curvature when $\ln k'$ was plotted against the acetonitrile concentration (see the sample plots in Fig. 2 for deslorelin), and systems c and d consistently gave smaller k' values for equivalent acetonitrile concentrations suggesting that a degree of mixed-mode interaction between peptide and packing was occurring. This is supported by the fact that the curvature and difference in retention was not apparent for the polymeric column, which would not be expected to show this type of behaviour. Nor did the polymeric column show the improved resolution of goserelin and leuprorelin in the systems where mixed-mode retention was suspected. System e was intermediate, giving a smaller curvature than a and b, but preserving the separation of leuprorelin and goserelin. Overall there was no significant difference in the slopes of the retention plots with the exception of gonadorelin, which consistently showed a steeper dependence on acetonitrile concentration than did the five analogues. The effects of varying acetonitrile concentration are summarised in Fig. 4 for the systems illustrated in Fig. 3a–b. The main problem in securing an isocratic

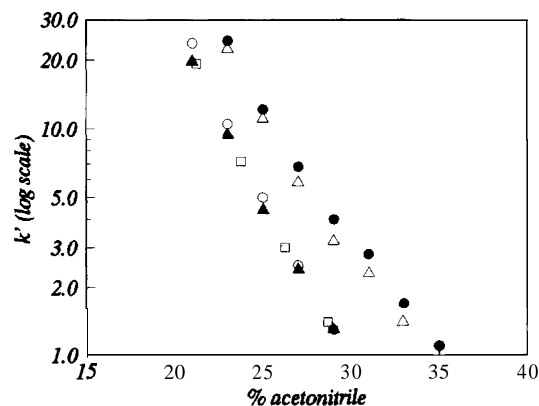


Fig. 2. Dependence of ln k' on proportion of acetonitrile for deslorelin: 100 x 3 mm I.D. Spherisorb S5ODS-2, 0.5 ml/min. Mobile phase systems: ● (a), △ (b), 0 (c), A (d), □ (e) (see Methods).

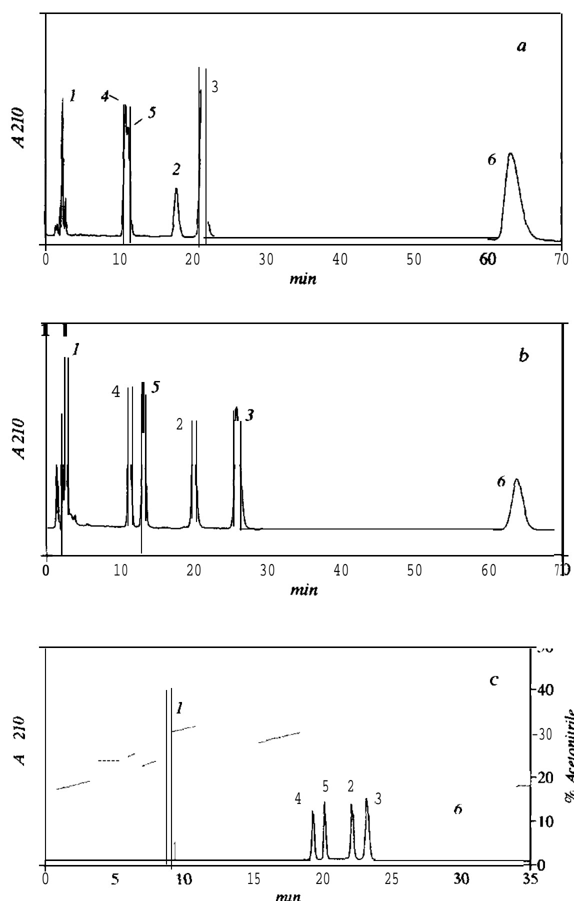


Fig. 3. RP-HPLC separation of gonadorelin and analogues. (a) Isocratic, mobile phase system d, 24% CH_3CN; (b) isocratic, system e, 24% CH_3CN; (c) gradient RP-HPLC separation of analogues, system e. Numbering as in Fig. 1. Column in all cases: 200 × 3 mm I.D. Spherisorb S5ODS-2 cartridge; detection: UV 210 nm; flow-rate: 0.5 ml/min. For mobile phase systems see Methods section.

separation of all peptides in one run was the large difference in retention between gonadorelin (much the least retained) and nafarelin (the most strongly retained) which made it difficult to carry out a meaningful separation in a reasonable time (say 1 h). The best compromises are shown in Fig. 3a–b, but the retention of gonadorelin is low ($k' \ll 1$) and the use of a shallow gradient clearly preferable (Fig. 3c).

Capillary electrophoresis

Free solution electrophoresis. Free solution electrophoresis over the pH range 2-9.5 showed the

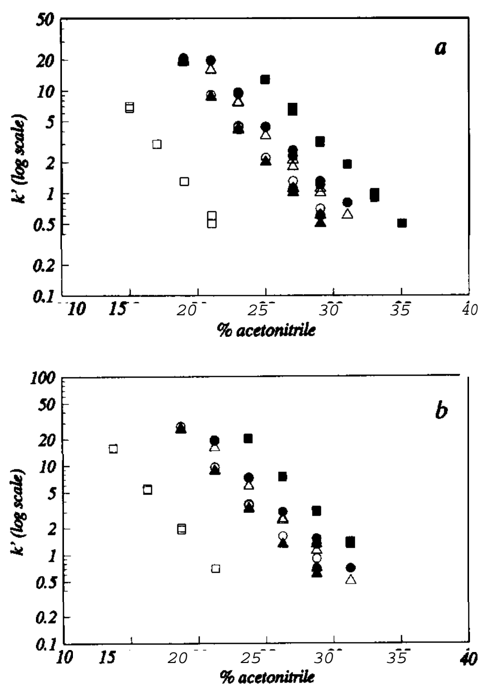


Fig. 4. Dependence of $\ln k'$ on proportion of acetonitrile for the two isocratic systems illustrated in Fig. 3. (a) System d (Fig. 3a); (b) system e (Fig. 3b). □ = Gonadorelin, A = buserelin, ● = deslorelin, A = goserelin, 0 = leuprorelin, ■ = nafarelin.

expected reduction in mobility corresponding to the titration of the imidazole side-chain of histidine with a pK_a of approximately 6 (Fig. 5). Unexpectedly there was a clear difference in mobility at pH 2.5 between gonadorelin and nafarelin on the one hand

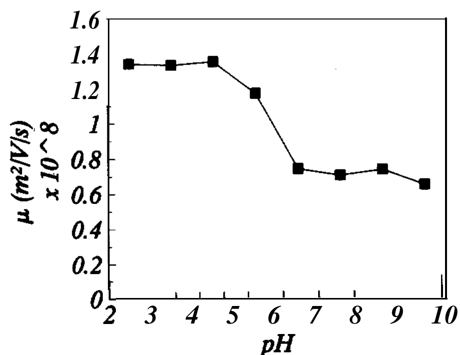


Fig. 5. Dependence of free-solution mobility of gonadorelin on pH. Conditions: 52 cm x 50 μm I.D. capillary (30 cm to detector); 15 kV; 30°C; buffers: pH 2.5-7.5, 0.1 M sodium phosphate, pH 8.5 and 9.5, 0.1 M Tris Cl-40 mM borate.

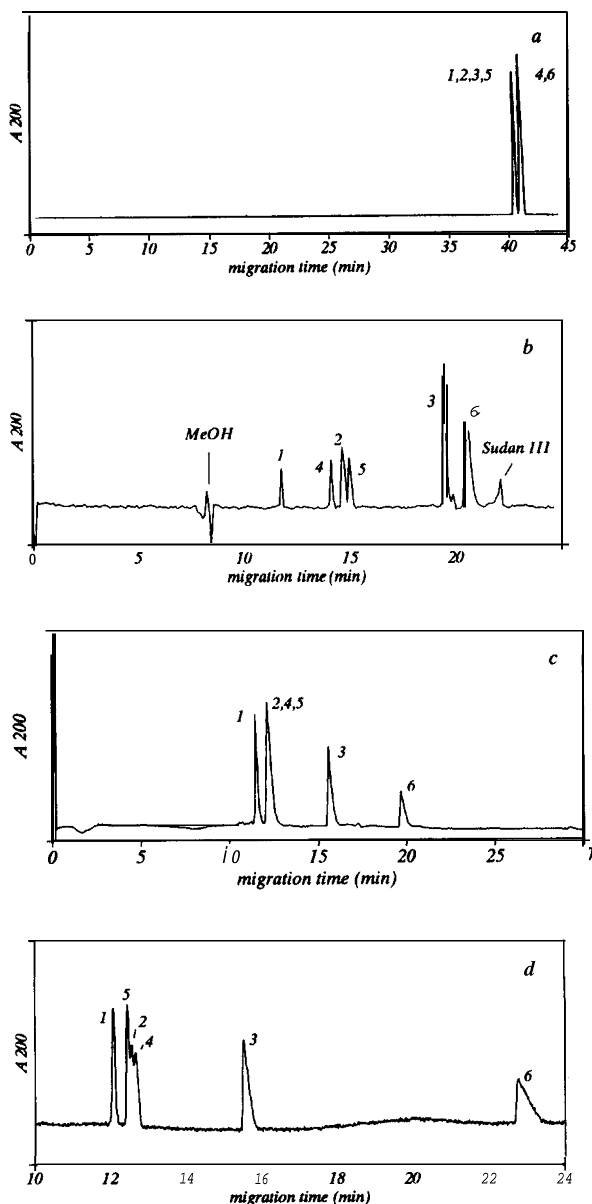


Fig. 6. Free and micellar electrophoresis of gonadorelin and derivatives, Numbering as in Fig. 1. (a) Free solution electrophoresis in 0.2 M sodium phosphate, pH 2.5; 1 m x 50 μm I.D. capillary (78 cm to detector); 18 kV; 30°C; 200 nm (mixture spiked with additional goserelin). (b) MEKC in 20 mM CTAB-0.05 M Tris Cl-20 mM borate, pH 7.5; 1 m x 50 μm I.D. capillary (78 cm to detector); -20 kV; 35°C; 280 mn. (c) Electrophoresis in 20 mM CHAPS-O.2 M sodium phosphates, pH 2.5; 45 cm x 50 μm I.D. capillary (32 cm to detector); 7 kV; 30°C; 200 nm. (d) Electrophoresis in 0.5% (v/v) Triton X-100 with 0.2 M sodium phosphate, pH 2.5; 52 cm x 50 μm I.D. capillary (30 cm to detector); 15 kV; 30°C; 200 nm.

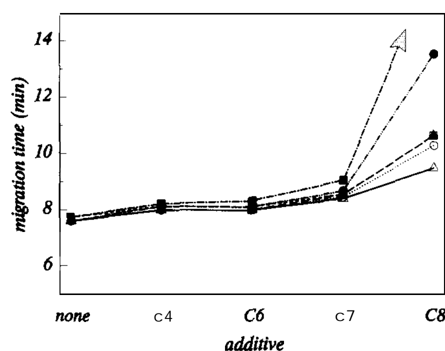


Fig. 7. Effect of addition of sodium salts of n-alkyl sulphonates (50 mM) on migration time in 0.2 M phosphate, pH 2.5. C_4 = Butane sulphonate, C_6 = hexane sulphonate, C_7 = heptane sulphonate, C_8 = octane sulphonate. △ = Gonadorelin, □ = goserelin, 0 = leuprorelin, A = buserelin, ● = deslorelin, ■ = nafarelin.

and the remaining peptides on the other, which had a signlicantly higher mobility (Fig. 6a).

Effect of hydrophobic pairing agents

The addition of 50 mM of n-alkyl sulphonate showed a modest selective effect on mobility which increased with increasing n-alkyl chain length up to C_7, but which dramatically increased from *n*-heptyl- to n-octylsulphonate (Fig. 7) -in fact nafarelin did not emerge at all with n-octylsulphonate. The selectivity of the modifiers was similar to that of the micellar systems, and it was clear that at pH 2.5 any increase of modifier to obtain improved resolution of the earlier peaks would result in unacceptably long analysis times for the later peaks.

Micellar electrophoresis in charged micellar systems

In the SDS system it was clear that the behaviour of the peptides was dominated by a strong ionic interaction between the positively charged peptides and the negatively charged micelles, and separation was poor. With the positively charged micelles of CTAB a much greater range of mobility was observed (Fig. 6b), with the order very much following that of RP-HPLC except inversion of buserelin and leuprorelin. The baseline disturbance associated with injection made detection difficult at low wavelength. However at 280 nm the baseline was almost free of disturbance and sensitivity quite adequate for identification. The effect of temperature over the range 30–60°C, the effect of pH over the range 7-9.5 and the addition of 5 or 10% (v/v) methanol were examined in order to optimise the peak shape of nafarelin (which became excessively asymmetrical when migrating too close to the micelle marker) without adversely affecting the resolution of the poorest resolved pair, buserelin and leuprorelin. The best compromise was at pH 7.5 at 35–40°C. Methanol improved the shape of the nafarelin peak, but seriously affected the resolution of buserelin and leuprorelin. All the peptides were much more strongly

TABLE I

COMPARISON OF DISTRIBUTION COEFFICIENTS (k') FOR MEKC [22] WITH RP-HPLC

	k' Value			
	RP-HPLC without detergent[a]	Background electrolyte for CE		
		20 mM CTAB[b]	20 mM CHAPS	0.2% (w/v) (3.2 mM) in Triton X-100[d]
Gonadorelin	0.21	0.682	-0.03	0.08
Goserelin	3.39	1.598	0.05	0.14
Leuprorelin	3.67	2.128	0.05	0.12
Buserelin	5.15	1.931	0.05	0.13
Deslorelin	6.96	10.096	0.62	0.44
Nafarelin	17.29	19.724	1.66	1.32

[a] 24% Acetonitrile in 0.05% H_3PO_4–0.5% $(NH_4)_2SO_4$, Spherisorb S5ODS-2.
[b] Critical micelle concentration (CMC) = 26 μM; aggregation number = 170 (data from supplier's literature).
[c] CMC = 8 mM; aggregation number = 10.
[d] CMC = 0.5 mM; aggregation number = 140.

distributed to the CTAB micelles than for the neutral detergents (Table I).

Micellar electrophoresis in neutral and zwitterionic systems

In the case of CHAPS the migration order was similar to that of CTAB except that there was no resolution between goserelin, buserelin and leuprorelin, and the separation on the 45 cm capillary took as long as with CTAB on the 1 m capillary (Fig. 6c). With Triton X-100 resolution between goserelin, buserelin and leuprorelin was better, but complete separation of this group would take an unacceptably long time (Fig. 6d).

DISCUSSION

For RP-HPLC a popular and UV-transparent anion (phosphate) was chosen, and the effect of modifying the counter ion studied. Since there are no groups in the compounds expected to ionise over the pH range 2-5, the effect of pH on selectivity was not expected to be very significant, and for simplicity a standard pH of 2.5 was maintained. In addition we included a mobile phase system we have found to give good results and which does not require titration. The evidence for mixed-mode retention suggests that systems c and d may be intrinsically more robust than systems a and b which are based on cations which compete less effectively for unreacted silanol groups. This is also borne out by other observations (not described) that system d is more satisfactory for the analysis of related impurities in peptide samples such as these (see ref. 23). Nevertheless, for the purposes of identification the improved resolution between goserelin and leuprorelin seemed worthwhile, and we prefer system e (Fig. 3c). An attempt was made to examine the retention of the peptides at pH 9.5 on the polymeric RP column PLRP-S 300, but peak shapes were so poor that it proved impossible to define retentions with any accuracy.

Free solution CE has been used extensively for the separation of peptides and proteins, and it is clear that quite subtle effects may lead to the resolution of peptides of comparable size and carrying the same nominal charge. There is no obvious common explanation for the slightly lower mobility at pH 2.5 of nafarelin and goserelin with respect to the other analogues since no ionisable groups should be titrating at this pH. Nafarelin has the bulky and very hydrophobic unnatural amino acid naphthylalanine, but it is not clear how this might confer electrophoretic properties significantly different from the equally bulky D-tryptophan in deslorelin: one possibility might be an increased tendency to self-association which might lower mobility. In the case of goserelin the difference is even less easy to rationalise, but it is clear that in many cases differences in uncharged residues may affect electrophoretic mobility through perturbation of the pK_a of a neighbouring residue (though this is not likely to be the explanation here), or through differences in charge masking and solvation [12]. It is unlikely that peptides of this size would exhibit any preferred secondary structure in aqueous solution. The addition of aliphatic sulphonic acids as pairing agent [18] leads to selective retardation similar to that seen in the case of the micellar systems and RP-HPLC, which suggests that the interaction is truly hydrophobic rather than ionic. The poor separations achieved with micelles of opposite charge to the peptides of interest have previously been noted [20].

Micellar systems employing either an uncharged detergent, Triton X-100, or a neutral (at pH 2.5) zwitterionic detergent, CHAPS, (not strictly MEKC since the micelles do not migrate and transport is by normal electrophoretic migration) gave separations which were slower than MEKC with charged micelles. Nevertheless clear differences in selectivity were apparent -for instance leuprorelin migrated slightly before buserelin and goserelin in 0.2% Triton X-100, although all resolution between goserelin and buserelin was lost at 0.5%. It is possible that higher concentrations of detergent and a move to a pH where endosmotic flow is greater would bring about a full separation, particularly in the case of Triton X-100. However electrolytes containing high concentrations of Triton X-100 are very viscous.

MEKC with CTAB was the only electrophoretic system which gave full resolution of all six peptides under the conditions examined: even so it was necessary to extend the capillary to obtain complete resolution. Fortunately all analogues possessed a tryptophan, and the baseline disturbance at low wavelength could be circumvented by detection at 280 nm. It is possible that other quaternary alkylammonium detergent may give a quieter baseline,

and that these may be preferable to CTAB (which is anyway a mixture of homologues). One noticable feature of all the micellar separations was significant peak asymmetry, worsening with the components distributed more to the micelle (Fig. 6b-d). This was particularly noticable with nafarelin in the CTAB system, where distorted peak shapes were a problem when the nafarelin peak migrated too close to the micelle marker. The distribution coefficient of the peptides to the micelles was much greater for CTAB than for the neutral detergents (Table I). This partly reflects the concentration of detergent (we found Triton X-100 difficult to work with at concentrations above 0.5% w/v) and micelles, but must also be a function of the very different aggregation properties and structures of the detergents. Clearly the choice of detergent for any given pH depends on the charges of the peptides of interest -little is to be gained by combining neutral peptides and neutral detergents- but in all cases detergents are likely to be of most general use at the ends of the pH spectrum, where all peptides are likely to carry an overall charge of the same sign.

CONCLUSIONS

Three RP-HPLC systems suitable for the separation of LHRH and its derivatives have been identified: our favoured system (Fig. 3c) has the merit of not requiring a pH meter. Comparison with micellar CE shows similar selectivity amongst the derivatives, but a selectivity which can be modulated by suitable choice of detergent and which is distinct from the RP-HPLC columns examined. This feature may be useful under certain circumstances, and the rapid method-development possible with automated commercially available CE equipment might make this an efficient way of developing particular separations. None of the CE systems was superior to the RP-HPLC systems favoured, either in speed or in selectivity.

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