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Note

Simultaneous separation of β-lipotrophin, adrenocorticotropic hormone, endorphins and enkephalins by high-performance liquid chromatography

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The object of this study has been to develop and evaluate methods of high-performance liquid chromatography (HPLC) for the separation of polypeptide hormones. Tissues active in the synthesis of such hormones often produce and/or contain a spectrum of related materials which may be precursors and fragments. This is especially apparent in their ectopic production by tumours when the normal balance of synthesis, intracellular processing, secretion and degradation may be altered. Our aim has been, therefore, to ascertain whether HPLC can achieve their rapid separation, identification and measurement in a manner analogous to that which we have already described for steroid hormones [1].

Several recent papers have shown that peptides can be separated using reversed-phase partition HPLC systems [2—6]. A number of hormones, including somatostatin [2], oxytocin [3], melanotrophin (α-MSH) and angiotensin [4], releasing factors [5], insulin [6] and some endorphins [7], have been individually chromatographed under a variety of different HPLC conditions. Systematic examinations of the capability of single optimised systems for separating simultaneously a wide range of related hormones are, however, few at present. As part of such a study we present here an HPLC system capable of rapidly separating at least 13 members of the adrenocorticotropic hormone (ACTH)—lipotrophin series, ranging in size from β-lipotrophin (91 residues) to enkephalin (5 residues). This chromatographic system does not destroy the bio- and immunoactivity of ACTH.

MATERIALS

Chemicals

Synthetic human adrenocorticotrophin ($ACTH_{1-39}$) and [D-Ser^1, $Lys^{17,18}$]-ACTH-(1—18)-amide (Ciba 41'795-Ba) were a gift from Dr. W. Rittel (Ciba-Geigy, Basle, Switzerland) and corticotrophin-(1—24)-tetracosapeptide (Synacthen, $ACTH_{1-24}$) was obtained from Ciba, Horsham, Great Britain. Corticotrophin-like intermediate lobe peptide (CLIP, synthetic human sequence $ACTH_{18-39}$), and β-lipotrophin (LPH) extracted from human pituitaries, were gifts from Dr. P.J. Lowry (St. Bartholomew's Hospital, London, Great Britain). Chromatographically purified porcine pituitary $ACTH_{1-39}$ (150 U/mg) was obtained from Sigma (Poole, Great Britain) and all other peptides from Bachem (Torrance, Calif., U.S.A.). A full list of polypeptides tested is given in Table I.

Acetonitrile (HPLC S grade) was obtained from Rathburn Chemicals (Walkerburn, Great Britain) and other reagents for chromatography were AnalaR (BDH, Poole, Great Britain) dissolved in double glass-distilled water.

Apparatus

Chromatography was carried out using a DuPont Model 830 chromatograph with 838 programmable gradient module, coupled in series to a DuPont Model 837 variable wavelength spectrophotometer and a Schoeffel Model 970 fluorescence detector. Samples were injected via a Rheodyne Model 7120 septumless valve fitted with a 175-μl injector loop on to stainless-steel columns (100 × 5 mm I.D.) slurry-packed with Hypersil-ODS (5 μm) according to the manufacturer's instructions (Shandon Southern, Runcorn, Great Britain). Eluted polypeptides were collected for subsequent bio- and radioimmunoassay into polyethylene minitubes, using an LKB 2112 Redirac fraction collector.

TABLE I

LIST OF POLYPEPTIDES TESTED

+ = Strong fluorescence. — = Weak or no fluorescence.

Peptide	Trivial name	Amino-acid residues	Fluorescence (275 nm)
$ACTH_{1-39}$	Adrenocorticotrophin	39	+
$ACTH_{1-24}$	Synacthen	24	+
*$ACTH_{1-18}$	41'795-Ba	18	+
$ACTH_{1-13}$	α-MSH	13	+
$ACTH_{4-10}$	Memory peptide	7	—
$ACTH_{18-39}$	CLIP	22	—
$ACTH_{34-39}$	—	6	—
β-LPH	β-Lipotrophin	91	+
$\beta\text{-LPH}_{39-45}$	—	7	—
$\beta\text{-LPH}_{61-65}$	Met-enkephalin	5	—
*$\beta\text{-LPH}_{61-65}$	Leu-enkephalin	5	—
$\beta\text{-LPH}_{61-76}$	α-Endorphin	16	—
$\beta\text{-LPH}_{61-91}$	β-Endorphin	31	—

*Analogues of naturally occurring peptide hormone sequences.

METHODS

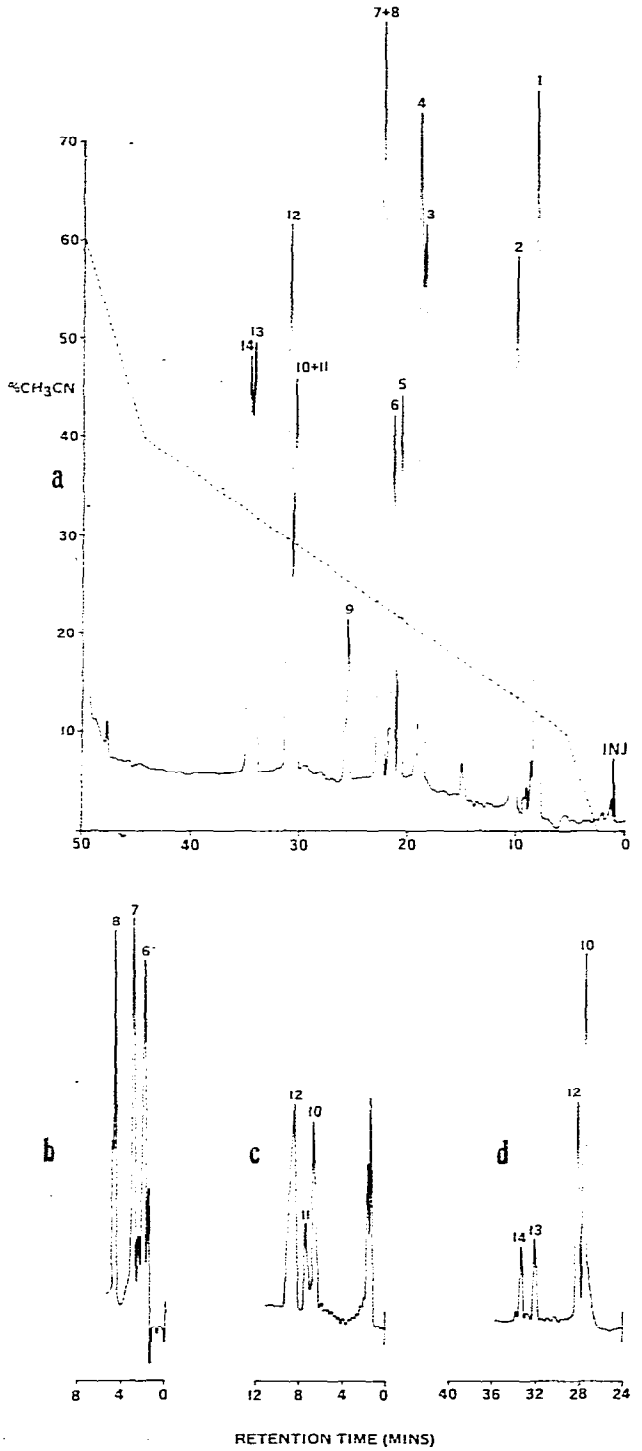
For chromatography, polypeptide standards were dissolved in the primary solvent (0.1 *M* sodium dihydrogen phosphate adjusted to pH 2.1 with orthophosphoric acid). Between 250 ng and 10 μg of each peptide was injected onto the columns, in 10—20 μl primary solvent, together with 250 ng of L-tryptophan as an internal standard. A loading period of 2.5 min pumping primary solvent only was employed to concentrate the polypeptides on the head of the column. They were then sequentially eluted over 50 min using a binary gradient with acetonitrile as the secondary solvent (Fig. 1a). The final concentration of organic modifier reached was 60% (v/v). Chromatography was carried out at ambient temperatures with an initial flow-rate of 1.0 ml/min (625 p.s.i.). These conditions, which are based on one of the systems used by Molnár and Horváth [4] were empirically optimised for the separation of compounds in Table I. The eluted polypeptides were detected by UV absorbance at 225 nm, and by fluorescence (see Table I) at an activation wavelength of 275 nm (emission filter 370 nm). Column efficiencies determined by isocratic peptide elution were 10,000—20,000 theoretical plates per metre.

RESULTS AND DISCUSSION

Complete or partial separation of 11 of the 13 polypeptides tested was achieved in a single run of 50 min at the ambient temperature using a linear gradient of acetonitrile in acid phosphate buffer with Hypersil-ODS (Fig. 1a). The system will effectively separate the endorphins, enkephalins, $ACTH_{1-39}$ and a number of related peptide fragments, and partially separate β-endorphin from β-lipotrophin. Compounds which failed to separate completely on this system could be resolved by a simple modification of the chromatographic conditions. α-Endorphin and leu-enkephalin could be separated by isocratic chromatography with 20% acetonitrile, CLIP and $ACTH_{1-39}$ at 24%, and separation of β-endorphin from β-lipotrophin was achieved by raising the temperature to 45° (see Fig. 1, b—d). Thus a potential for the complete separation of all polypeptides tested was apparent.

No deterioration of peak shape or peak broadening occurred with late-eluting polypeptides such as $ACTH_{1-39}$ and β-endorphin. Injection of the individual polypeptides under isocratic conditions showed that the chromatography of each occurred only over a very narrow range (3—4%) of organic modifier concentration with no retention or irreversible binding above or below these limits. This feature of the chromatography of the polypeptide hormones is probably responsible for the lack of peak broadening and also for the very high precision with which individual retention times could be reproduced. Thus, repeated injections of $ACTH_{1-24}$ with a mean retention time of 21.5 min gave a coefficient of variation of 0.57% (n = 7), i.e. ± 8 sec. This feature is of considerable importance for predictable chromatography of complex biological polypeptide-containing samples.

Retention times were minimally influenced (< 10%) by different flow-rates (0.5—1.5 ml/min) or different temperatures (15—70°) although the latter could be used to enhance certain separations, as shown above. Nevertheless, to avoid


7+8
4
1
3
12
2
13
14
10+11
5
6
%CH3CN
a
9
INJ
70
60
50
40
30
20
10
50
40
30
20
10
0
8
7
6
b
12
10
c
11
d
10
12
14
13
8
4
0
12
8
4
0
40
36
32
28
24
RETENTION TIME (MINS)

possible decomposition of unstable polypeptides we opted for routine chromatography at ambient temperature. The presence of both acid (pH 2.1) and salt (0.1 *M* NaH_2PO_4) in the primary solvent was essential for efficient reproducible chromatography. Omission of either resulted in marked deterioration of peak shape and loss of resolution of late-eluting compounds, and omission of both in the irreversible binding of many polypeptides to the ODS column packing.

It is evident from the behaviour of ACTH and lipotrophin-related hormones that separation and retention orders are not dictated by molecular weight with compounds of similar size eluted both very early (β-LPH_{39-45} at 8.5 min) and very late ($ACTH_{34-39}$ at 31 min). Furthermore, a very small change in the sequence of a large polypeptide could alter its retention time significantly, particularly if a hydrophobic amino-acid residue was involved. Thus porcine $ACTH_{1-39}$ was completely separated from human $ACTH_{1-39}$ (Fig. 2) although it differs from the latter only in the substitution of leucine for serine at position 31 [8].

Detection limits for individual peptides varied according to their size and amino-acid composition. A tryptophan-containing hormone such as $ACTH_{1-24}$ could be detected at levels down to 15 ng per peak by fluorescence while the corresponding minimum value for UV detection (see Methods) was 120 ng, with a linear response up to at least 100 μg. The conditions of fluorescence measurement used in this study were optimised for the detection of endogenous tryptophan present in some polypeptide hormones. The molar fluorescence ratio of tryptophan—tyrosine was 30:1, with the latter, and other amino-acid residues therefore not contributing significantly to polypeptide detection by this method. Table I indicates those fluorescent hormones which contain tryptophan (one residue per molecule in each case), and for which detection limits were 5—15-fold lower than by using the UV absorption contributed by the peptide bonds. These limits, while low in comparison with conventional methods of chromatography, are still high with respect to hormone levels in vivo.

A capability for retaining bio- and immunoactivity of eluted polypeptides, as well as the capacity to resolve a series of closely related materials in biological extracts, is a prerequisite for a biologically useful HPLC system. To test whether HPLC meets these criteria, we have used commercially available "chromatographically purified" porcine pituitary ACTH known to contain several components on polyacrylamide gel electrophoresis. Fig. 2 illustrates results

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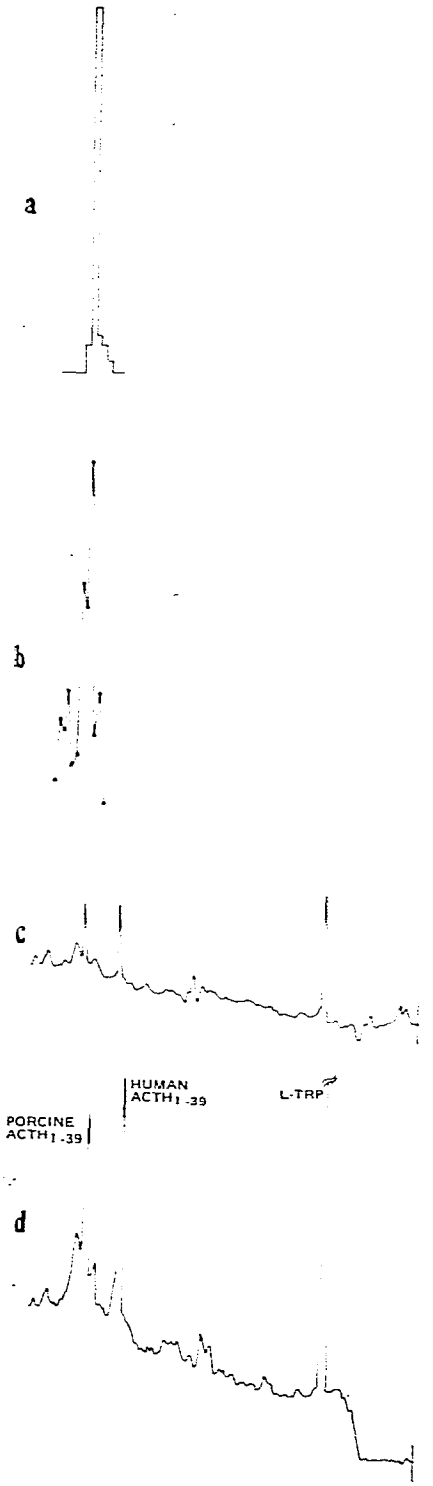
Fig. 1. Separation of polypeptide hormones by HPLC on Hypersil-ODS. (a) Chromatogram of 250 ng—10 μg of polypeptide standards in 0.1 *M* NaH_2PO_4—H_3PO_4 buffer (pH 2.1) eluted with an acetonitrile gradient (dotted line) at ambient temperature. Detection by UV absorbance (225 nm, 0.16 a.u.f.s.). INJ = injection artifact; 1 = β-LPH_{39-45}; 2 = L-tryptophan; 3 = $ACTH_{1-18}$; 4 = met-enkephalin; 5 = $ACTH_{4-10}$; 6 = $ACTH_{1-24}$; 7 = α-endorphin; 8 = leu-enkephalin; 9 = α-MSH; 10 = h$ACTH_{1-39}$; 11 = CLIP; 12 = $ACTH_{34-39}$; 13 = β-endorphin; 14 = β-lipotrophin. See Table I for abbreviations. (b) Chromatogram of $ACTH_{1-24}$ (6), α-endorphin (7) and leu-enkephalin (8) eluted isocratically with 20% acetonitrile in acid-phosphate buffer at ambient temperature. (c) Chromatogram of h$ACTH_{1-39}$ (10), CLIP (11) and $ACTH_{34-39}$ (12) eluted isocratically with 24% acetonitrile in acid-phosphate buffer at ambient temperature. (d) Chromatogram of h$ACTH_{1-39}$ (10), $ACTH_{34-39}$ (12) β-endorphin (13) and β-lipotrophin (14) with gradient elution at 45°, other conditions as in (a).


a
b
c
HUMAN
ACTH1-39
L-TRP
PORCINE
ACTH1-39
d

obtained with this preparation. First, several UV-absorbing and fluorescent peaks are resolved. These compounds can be individually re-chromatographed with identical retention times indicating that they are not degraded by chromatography and also suggesting that they are individual compounds, not conformational isomers. Second, the eluted peaks have been successfully radioimmunoassayed for ACTH after evaporation of the organic solvent, neutralisation of the acid and dilution of buffer 1:4 to reduce its ionic strength, giving a detection limit of 200 pg per peak. Last, the eluted peaks have been successfully bioassayed for ACTH, using an isolated adrenal cell suspension [9] and a fluorescence assay for corticosteroid [10]. Only one peak showed significant bioactivity with over 90% of the activity recovered therein, in contrast to the multiple peaks revealed by radioimmunoassay with an N-terminal-directed antibody (Fig. 2). Thus the HPLC system described here conforms to these criteria for biological use.

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REFERENCES

1 M.J. O'Hare, E.C. Nice, R. Magee-Brown and H. Bullman, J. Chromatogr., 125 (1976) 357.
2 C. Martin and G.E. Purdon, J. Endocrinol. 77 (1978) 67.
3 K. Krummen and R.W. Frei, J. Chromatogr., 132 (1977) 27.
4 I. Molnár and C. Horváth, J. Chromatogr., 142 (1977) 623.
5 R. Burgus and J. Rivier, in A. Loffett (Editor), Proc. 14th European Peptide Symposium, University of Brussels, Brussels, 1976, p. 85.
6 W.S. Hancock, C.A. Bishop, R.L. Prestidge, D.R.K. Harding and M.T.W. Hearn, Science, 200 (1978) 1168.
7 N. Ling, Biochem. Biophys. Res. Commun., 74 (1977) 248.
8 B. Riniker, P. Sieber, W. Rittel and H. Zuber, Nature, New Biol., 235 (1972) 114.
9 M.C. Richardson and D. Schulster, J. Endocrinol., 55 (1970) 127.
10 G. Sayers, R.L. Swallow and N.D. Giordano, Endocrinology, 88 (1971) 1063.

Fig. 2. HPLC of porcine pituitary ACTH preparation (25 μg) with L-tryptophan and synthetic human $ACTH_{1-39}$ as internal standards. Conditions of chromatography as Fig. 1a. Chromatograms show (a) bioactive and (b) immunoreactive materials eluted in 30 sec fractions. compared with (c) UV absorbance (225 nm, 0.16 a.u.f.s.) and (d) fluorescence profiles (275 nm activation, 0.2 μA) obtained from this preparation. Retention times of standards were identical to those given in Fig. 1a and that of the bioactive porcine pituitary ACTH peak was 33.25 min.