# SUBUNITS OF BOVINE LUTROPIN

## HORMONAL AND IMMUNOLOGICAL ACTIVITY AFTER SEPARATION WITH REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY*

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SUMMARY

By means of reversed-phase high-performance liquid chromatography, we have fractionated bovine lutropin (LH) standard preparations. The highly purified NIAMDD-bLH-4 was fractionated into two components, while the less pure NIH-LH-B9 revealed three distinct peaks. The eluted material was further characterized by *in vitro* bioassay and by homologous radioimmunoassay for bovine LH, ovine LH-alpha and ovine LH-beta subunits. The material with the shortest retention time possessed almost no LH-activity and showed a displacement curve nearly identical with that of the ovine LH-alpha subunit. The material corresponding to the second peak exhibited 6% of the original LH-activity, and its immunoreactivity was equal to that of the ovine LH-beta subunit. Furthermore, the fractions supposed to contain the alpha and the beta subunits were rechromatographed and their aminoacid contents analyzed. The results show close similarities between the rechromatographed fractions and the pure subunits.

INTRODUCTION

Purification of lutropin (LH) (bovine/ovine), and separation of its subunits, has been achieved by using counter-current distribution[1,2,3,4], ion-exchange chromatography[5], and isoelectric focusing in a sucrose gradient[6]. Dissociation of ovine LH into subunits by urea was shown by De la Llosa *et al.*[7]. In another study, De la Llosa and Jutisz[8] reported that dissociation with use of guanidine hydrochloride was much better and quicker than use of urea. In a study with human pituitary LH,

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Loeber et al.[9] found that dissociation was spontaneous, and dependent on both time and temperature, but not due to proteolytic enzymes. Recently, gel-permeation high-performance liquid chromatography (HPLC) has been employed for studying the behaviour of follitropin (FSH), LH, LH-alpha, human chorionic gonadotropin (hCG), hCG-alpha and hCG-beta[10]. Reversed-phase HPLC has been used for fractionation of hCG and its subunits by Putterman et al.[11] as well as for human thyroid-stimulating hormone (TSH)[12]. Recently, Wilks and Butler[13] reported that hCG retained only 10–60% of its biological activity following fractionation on reversed-phase HPLC columns.

The aim of this report was to study the behaviour of two different bovine standard preparations of LH (NIH-LH-B9, NIAMDD-bLH-4) on a reversed-phase HPLC system at a low pH.

EXPERIMENTAL

*Apparatus*

A Waters HPLC system was used, which included two M 6000 solvent delivery units, a M 660 solvent programmer and a U6K injector (Waters Associates, Milford, U.S.A.). The samples were introduced into two stainless-steel columns (150 × 8 mm) connected in series. The columns were slurry-packed[14] with Spherisorb 5-$\mu$m ODS spherical silica (Phase Separations, Queensferry, U.K.) in our laboratory at 450 bar (45 MPa) pressure with a Haskel MCP 110 pneumatic amplifier (Haskel Engineering & Supply, Burbank, U.S.A.). The samples were detected at 280 nm with a LDC Spectromonitor III detector (Laboratory Data Control, Riviera Beach, U.S.A.). The fractions collected were evaporated to dryness with a Savant Speed Vac Concentrator (Savant Instruments, Hicksville, U.S.A.).

*Chemicals*

Unless otherwise stated, all reagents were of analytical grade. HPLC-grade acetonitrile and trifluoroacetic acid (TFA) of sequential grade as supplied by Fluka (Buchs, Switzerland) and ammonia solution of suprapure grade from Merck (Darmstadt, F.R.G.) were used. All the water used (HPLC grade) was prepared from distilled water deionized with a Milli-Q-Water System incorporating an Organex-Q cartridge and a sterile filter attached to its outlet (Millipore, Bedford, U.S.A.).

Double-antibody solid phase (DASP; Organon, The Netherlands) was used for the separation of free and antibody-bound hormone, and $^{125}$I-labelled sodium iodide was purchased from the Radiochemical Centre (Amersham, U.K.).

The preparations tested by HPLC were NIH-LH-B9 and NIAMDD-bLH-4. The bovine lutropin standards for radioimmunoassay (RIA) were bLH-DS, the ovine LH-beta subunit (NIAMDD-WRR-2-beta) and the ovine LH-alpha subunit (NIAMDD-WRR-1-alpha).

*HPLC*

The mobile phase flow-rate was 1.5 ml/min, which was controlled by a pressure of 1500 p.s.i. (10.2 MPa). All the tests were performed at room temperature, and the sample size was 1.0 mg of protein in 200–1500 $\mu$l of water. The solvent system consisted of two buffers (*A* and B), mixed by the solvent programmer and the pumps in appropriate proportions.

Buffer A was prepared by diluting 5 ml of TFA to 1000 ml with degassed water, and buffer B by diluting 5 ml of trifluoroacetic acid with 800 ml of degassed acetonitrile and 195 ml of degassed water. The mixtures will be referred to by their percentage concentration of buffer B. In both buffers, the pH was adjusted to 3.3 with concentrated ammonia solution. The columns were equilibrated before injection for at least 30 min with 5% of buffer B. The samples were injected and a linear gradient was run from 5% of buffer B to 63% of buffer B during 39 min (Fig. 1 and 2). Each eluted fraction was collected, quickly frozen and evaporated to dryness, and the residue was dissolved in 1000 $\mu$l of water for protein determination. These solutions were stored at $-20°C$ until the immunoassay and bioassay were performed.

*RIA*

The bovine standard bLH-DS was used for iodination by the Chloramine-T method[15] and for preparation of the calibration graph. The method for radioimmunoassay of LH was essentially that described by Stupnicki and Madej[16], except that the separation of free and antibody-bound hormone was carried out by adding 1 ml of DASP suspension, followed by an overnight incubation under rotation at room temperature. The sensitivity of the assay was 0.1 ng per tube, and the between-assay error was 6.4, 8.9 and 11% for three different plasma pools averaging 1.9, 18 and 48 $\mu$l/l, respectively. The separation of free and antibody-bound hormone was also carried out according to the method described by Eisenman and Chew[17]. In this method, 0.1 ml of the second antibody (anti-rabbit gamma-globulin) was added, followed by 0.5 ml of polyethylene glycol. Immediately afterwards, the tubes were centrifuged for 30 min at 4°C and 2000 $g$. The supernatant was decanted, and the last drops were removed by absorbent tissue. The amount of radioactivity in the dried precipitate was counted with an automatic gamma-ray counter. The sensitivity of the assay according to Eisenman and Chew[17] was also 0.1 ng per tube. All the assays were performed in 0.05 $M$ sodium phosphate buffer of pH 7.5 containing 0.85% of sodium chloride and 0.2% of bovine serum albumin.

*Calculation of RIA*

Plots of logit of the specifically bound antibody fraction *vs.* log concentration of added protein per tube were used to present our data, as suggested by Rodbard *et al.*[18]. The following identity was used: Logit $y = \ln (y/1 - y)$, where $y$ is the specifically bound fraction, *i.e.*, $y = (B - N)/(B_0 - N)$; where $B$ is the number of bound counts (cpm) in the presence of standard or unknown unlabelled hormone; $B_0$ is the number of cpm bound in the absence of unlabelled hormone; $N$ is the mean number of non-specifically cpm bound, *i.e.*, blank value.

*Protein determination*

From each standard preparation, 1.0 mg was dissolved in water to yield a concentration of 1 mg/ml. The absorbance of the solution was measured in a 1-cm quartz cell at 280 nm. The values of $A_{280\,nm}^{1\,cm}$ (1.0 mg/ml of protein) in the present study for the two standards were in the range 0.458–0.630. After the separation of each standard preparation, the total protein content of each fraction was determined. The calculation was based on the total protein absorbance.

*Purity assessment*

Further purification of the fractionated components of the bovine lutropin standard NIH-LH-B9 (Fractions abbreviated LHB9:39–41 and LHB9:54–55; Fig. 1) has been performed by HPLC rechromatography. The samples were run in the same system as described above, but detection was carried out at 230 nm and the gradient was run for 20 min.

*In vitro bioassay*

The *in vitro* bioassay was based on the production of testosterone by Leydig cells, prepared from adult mouse testes in the presence of graded doses of LH. The experiments were carried out as described by Van Damme *et al.*[19]. The substances assayed were four pooled fractions (LHB9:39–41; LHB9:42; LHB9:43–52 and LHB9:54–55) of bovine LH using NIH-LH-B9 as standard.

*Amino acid analyses*

The fractions originating from the separation of NIH-LH-B9 were hydrolyzed in sealed and evacuated tubes for 24 h with 6 $M$ hydrochloric acid at 110°C. In addition, samples of the fractions LHB9:39–41 and LHB9:54–55 were also hydrolyzed for 72 h under the same conditions. The hydrolysates were analyzed with a Durrum D-500 analyzer.

*Column efficiency*

A value for the height equivalent to a theoretical plate (HETP) was measured on the basis of the toluene peak with methanol–water (70:30) as mobile phase at varying flow-rates (1.0–3.0 ml/min). The lowest HETP values were found to be 13.2 and 15.0 $\mu$m at 1.5 ml/min for toluene with the two columns used here.
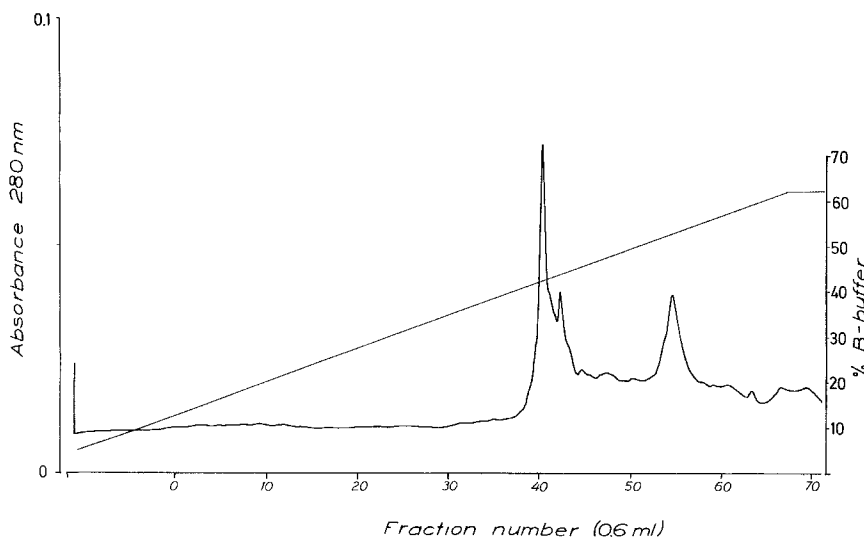


Fig. 1. Reversed-phase HPLC, profile of the bovine LH standard preparation NIH-LH-B9 (sample size 1.0 mg). Conditions: two 150 × 8 mm 5 $\mu$m ODS columns; buffer A, 0.5% TFA in water (pH 3.3), buffer B, TFA–acetonitrile–water (0.5:80:19.5), pH 3.3; gradient, 5–63% in 39 min; flow-rate, 1.5 ml/min; detection, UV 280 nm; a.u.f.s. 0.1.

RESULTS

As shown in Fig. 1 and 2, the standard preparation NIH-LH-B9 can be fractionated into three different components, whereas the elution pattern from the highly purified standard preparation NIAMDD-bLH-4 shows just two distinct peaks. The mean differences in retention time for these two peaks were 7.1 min for NIH-LH-B9 and 7.6 min for NIAMDD-bLH-4, respectively.

Based on results given in Fig. 1, the eluted fractions from the bovine standard NIH-LH-B9, which formed the first peak (LHB9:39–41), the second peak (LHB9:42), the plateau (LHB9:43–52) and finally the third peak (LHB9:54–55), were all tested in the *in vitro* bioassay (Fig. 3). The highest production of testosterone was found in the pooled fractions LHB9:43–52. However, that bioactivity was only *ca.* 13% of that found in native NIH-LH-B9. The other fractions tested showed negligible LH bioactivity (LHB9:39–41, 0.2%; LHB9:42, 2.5% and LHB9:54–55, 6.2%; respectively).

All eluted fractions up to LHB9:50, obtained from NIH-LH-B9 showed no or very little immunoreactivity in a homologous RIA of bovine LH. Fractions LHB9:54 and 55, representing the last peak at the chromatogram possessed the highest activity as shown in Fig. 4. Also, in NIAMDD-bLH-4 the highest immunoreactivity was obtained from the material eluted at the end of the gradient (not shown in Figure).

In two specific RIA (one homologous LH-beta RIA and one homologous LH-alpha RIA) the pooled fractions LHB9:39–41 and the pooled fractions LHB9:54–55 were further tested. The displacement lines for ovine LH-beta subunit
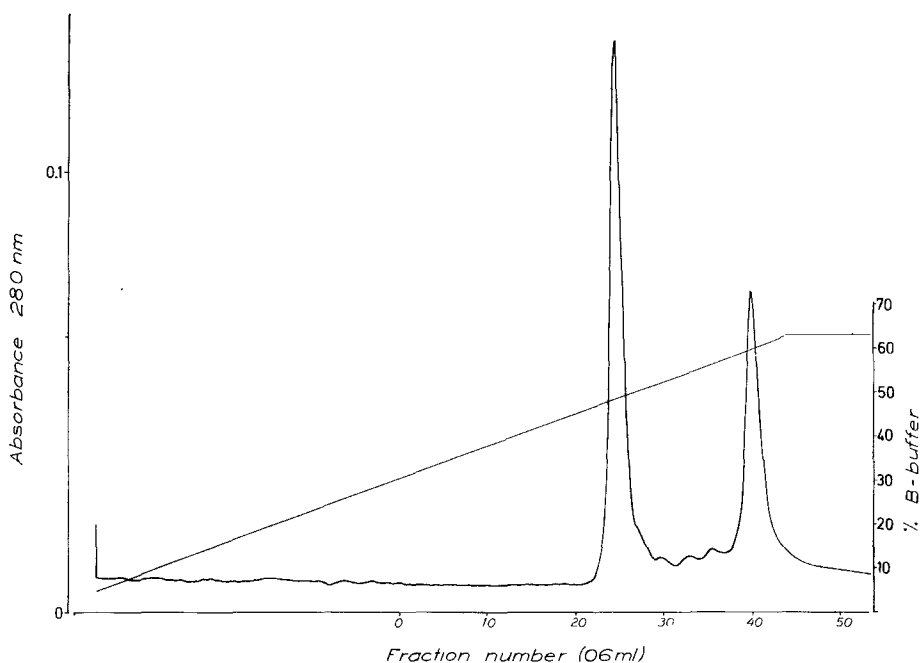


Fig. 2. Reversed-phase HPLC profile of the bovine LH standard preparation NIAMDD-bLH-4. Conditions as in Fig. 1.
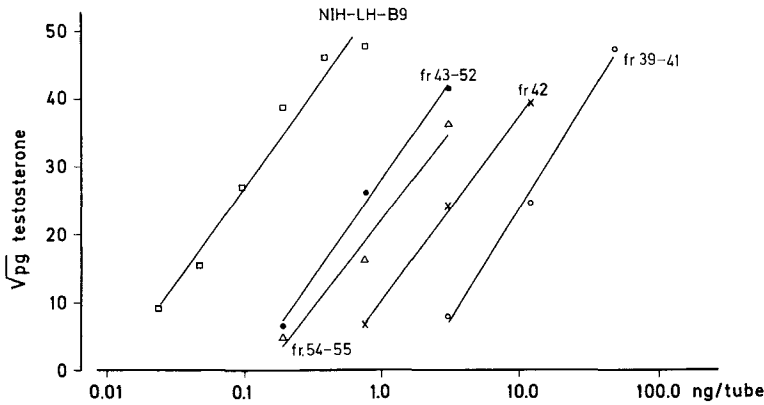
Fig. 3. Comparison in bioactivity with aid of the testosterone production assay for LH, between the collected and pooled fractions LHB9:39–41, LHB9:42, LHB9:43–52 and LHB9:54–55 isolated from the bovine LH standard preparation NIH-LH-B9. The non-fractionated NIH-LH-B9 is used as standard.

and for the pooled fractions LHB9:54–55 (Fig. 5) are almost parallel, whereas the fractions LHB9:39–41 show no beta-subunit immunoreactivity.

Comparison between the immunoreactivity of the fractions LHB9:39–41 and fractions LHB9:54–55 in the homologous assay system of ovine LH-alpha subunit is presented in Fig. 6. Here, on the other hand, fractions LHB9:39–41 show a displacement line almost identical with that of the alpha subunit. The fractions LHB9:54–55 do not contain any ovine alpha-subunit immunoreactivity.

Rechromatography of the pooled fractions (LHB9:39–41 and LHB9:54–55) with recording at 230 nm revealed only one distinct peak and no impurities could be detected. Fig. 7 shows the rechromatography of these pooled fractions on the columns containing 5-$\mu$m spherical silica, in which the same mobile phase as described
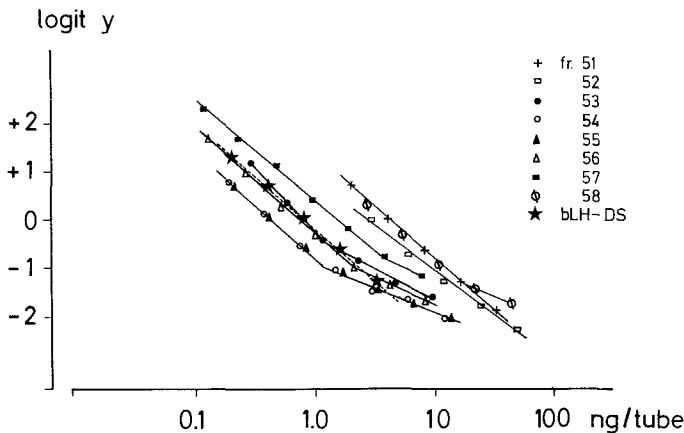


Fig. 4. LH immunoreactivity of the fractions obtained after HPLC of the bovine LH standard preparation NIH-LH-B9. No LH immunoreactivity is found in fractions LHB9:1–50. The bovine LH preparation bLH-DS is used as tracer as well as standard. The displacement curves of the bound radioactivity have been linearized by the logit transformation of $(B - N)/(B_0 - N) = y$ vs. log dose of added protein per tube.
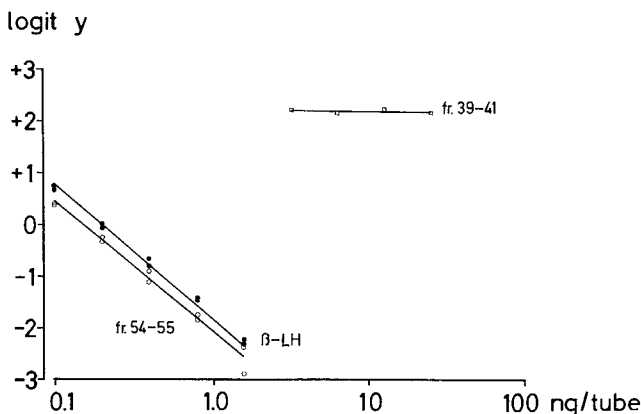
Fig. 5. Comparison in ovine LH-beta subunit immunoreactivity between the two rechromatographed fractions LHB9:39–41 and LHB9:54–55, isolated from the bovine LH (NIH-LH-B9) on HPLC and the ovine LH-beta subunit. The ovine LH-beta subunit is used as tracer as well as standard. The displacement curves of the bound radioactivity have been linearized by the logit transformation of $(B - N)/(B_0 - N)$ = $y$ vs. log dose of added protein per tube.

in the Experimental section was used. The immunoreactivity of each purified component was not affected by the rechromatography.

The pooled fractions obtained after HPLC of NIH-LH-B9 (LHB9:39–41 and LHB9:54–55) were, after rechromatography, analyzed for their amino acid contents (Table I, columns 1 and 2). The analyses are presented as residues per 100 residues analyzed, together with a compilation of reported compositions[20] of the two subunits of bovine LH (columns 3 and 4).

DISCUSSION

A report by Putterman et al.[11] has shown that it is possible to prepare subunits of hCG with HPLC, by using a $C_{18}$ column for separation of the two subunits from
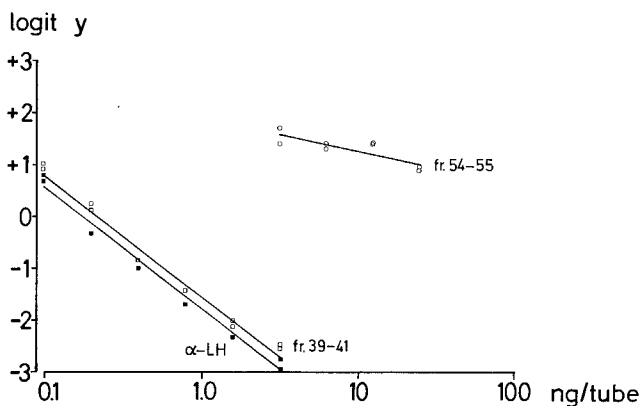


Fig. 6. Comparison in immunoreactivity, between the two rechromatographed fractions LHB9:39–41 and LHB9:54–55, isolated from the bovine LH (NIH-LH-B9) on HPLC and the ovine LH-alpha subunit. The ovine LH-alpha subunit is used as tracer as well as standard. The displacement curves of the bound radioactivity have been linearized by the logit transformation of $(B - N)/(B_0 - N)$ = $y$ vs. log dose of added protein per tube.
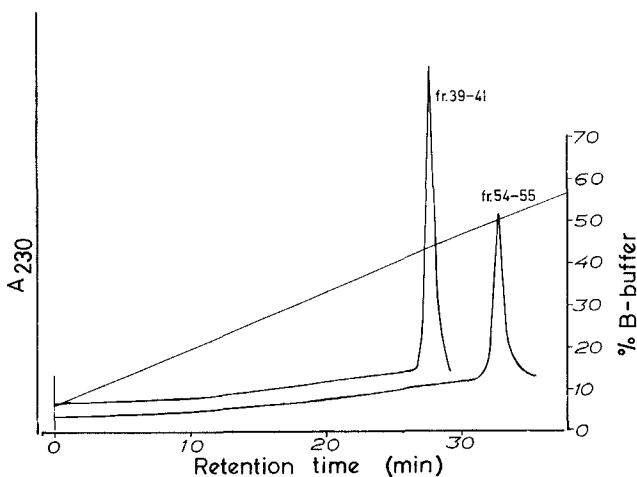
Fig. 7. Rechromatography of the following pooled fractions: LHB9:39–41 and LHB9:54–55, which were isolated from the LH standard preparation NIH-LH-B9 (Fig. 1). The samples were run under the same conditions as described in Fig. 1, except that detection was carried out at 230 nm and that the gradient was run for 20 min. The two pooled fractions were further analyzed for their amino acid composition. Retention times: fr. 39–41, 27.9 min; fr. 54–55, 32.8 min.

a crude source of hCG. The separation was carried out by incubation for 1 h at a rather low pH followed by a gradient separation with acetonitrile–TFA acid buffers for 1 h.

Bristow et al.[12] recently showed that hTSH was easily dissociated, i.e., subunits

TABLE I

AMINO ACID COMPOSITION (RESIDUES PER 100 RESIDUES ANALYZED) OF PURIFIED FRACTIONS FROM A BOVINE LH STANDARD PREPARATION (NIH-LH-B9).

Results in columns 1 and 2 are the purified components and in columns 3 and 4 are the values presented by Bahl[20].

| Amino acid | Fr. Nos. 39–41 (1) | Fr. Nos. 54–55 (2) | α-bLH (3) | β-bLH (4) |
|---|---|---|---|---|
| Lys | 11.5 | 3.9 | 9.9 | 2.1 |
| His | 3.8 | 3.6 | 3.3 | 2.3 |
| Arg | 6.4 | 9.3 | 3.3 | 5.2 |
| Asp | 5.9 | 4.7 | 6.2 | 4.2 |
| Thr | 7.8 | 5.9 | 8.4 | 6.1 |
| Ser | 5.2 | 5.7 | 4.8 | 6.1 |
| Glu | 9.8 | 7.7 | 7.9 | 6.3 |
| Pro | 7.4 | 13.8 | 8.0 | 18.0 |
| Gly | 3.0 | 3.7 | 4.7 | 7.0 |
| Ala | 5.4 | 5.2 | 9.2 | 7.6 |
| Cys | 6.9 | 8.6 | 9.4 | 8.5 |
| Val | 5.8 | 6.3 | 6.2 | 7.1 |
| Met | 3.3 | 2.6 | 4.2 | 2.2 |
| Ile | 2.5 | 3.7 | 2.3 | 3.6 |
| Leu | 3.3 | 9.8 | 3.6 | 9.8 |
| Tyr | 7.1 | 2.4 | 4.8 | 1.3 |
| Phe | 4.9 | 3.2 | 3.6 | 2.5 |

were easily fractionated at pH 2.0, but without any pre-incubation. The subunits of hTSH were separated on an alkyl-silica column by using acetonitrile–sodium chloride buffers with gradient elution. The retention time between the first and second peak in our studies was consistantly much longer than that described by Bristow et al.[12] (7 min vs. 1 min). In the present study, the content represented by the first peak corresponds to the alpha subunits, while the content by the second corresponds to the beta subunit. In contrast, Bristow et al.[12] showed that the beta subunit was less retarded than the alpha subunit.

The pooled fractions LHB9:39–41 (alpha-like) and LHB9:54–55 (beta-like) had very similar retention times to those of the pure subunits of ovine LH. When the same chromatographic system was used, as mentioned before the iodinated alpha and beta subunits had almost exactly the same retention times as the two pooled fractions LHB9:39–41 and LHB9:54–55 (ref. 21).

From the amino acid analyses, the similarities in composition between fraction LHB9:39–41 and the alpha subunit (see Table I) can be seen. It is only the Arg and Ala values that differ considerably from those of Bahl[20]. The values found by us in fractions LHB9:54–55 and the beta subunit, differ not only in Arg content, but also in, e.g., Gly. Nevertheless, the analyzed components show a great deal of similarity to the two subunits.

In conclusion, it seems that, with this applied chromatographic method, the subunits are well separated from each other, which may be useful for separation of the subunits of bovine LH. No incubation is needed and the separation takes only 40 min.

## ACKNOWLEDGEMENTS

## REFERENCES

1 H. Papkoff and T. S. A. Samy, Biochim. Biophys. Acta, 147 (1967) 175.
2 L. E. Reichert, Jr., M. A. Rasco, D. N. Ward, G. D. Niswender and A. R. Midgley, Jr., J. Biol. Chem., 244 (1969) 5110.
3 T.-H. Liao and J. G. Pierce, J. Biol. Chem., 245 (1970) 3275.
4 H. Papkoff and J. Gan, Arch. Biochem. Biophys., 136 (1970) 522.
5 M. Jutisz and C. Courte, Gen. Comp. Endocrinol., 11 (1968) 562.
6 P. Rathnam and B. B. Saxena, J. Biol. Chem., 246 (1971) 7087.
7 P. de la Llosa, C. Courte and M. Jutisz, Biochem. Biophys. Res. Commun., 26 (1967) 411.
8 P. de la Llosa and M. Jutisz, Biochim. Biophys. Acta, 181 (1969) 426.
9 J. G. Loeber, J. W. G. M. Nabben-Fleuren, L. H. Elvers, M. F. G. Segers and R. M. Lequin, Endocrinology, 103 (1978) 2240.
10 D. H. Calam and J. Davidson, J. Chromatogr., 218 (1981) 581.

11 J. G. Putterman, M. B. Spear, K. S. Meade-Cobun, M. Widra and C. V. Hixson, *J. Liq. Chromatogr.*, 5 (1982) 715.
12 A. F. Bristow, C. Wilson and N. Sutcliffe, *J. Chromatogr.*, 270 (1983) 285.
13 J. W. Wilks and S. S. Butler, *J. Chromatogr.*, 298 (1984) 123.
14 P. A. Bristow, P. N. Brittain, C. M. Riley and B. F. Williamson, *J. Chromatogr.*, 131 (1977) 57.
15 F. G. Greenwood, W. H. Hunter and J. S. Glover, *Biochem. J.*, 89 (1963) 114.
16 R. Stupnicki and A. Madej, *Endokrinologie*, 68 (1976) 6.
17 J. R. Eisenman and B. P. Chew, *J. Dairy Sci.*, 66 (1983) 1174.
18 D. Rodbard, W. Bridson and P. Rayford, *J. Lab. Clin. Med.*, 74 (1969) 770.
19 M.-P. van Damme, D. M. Robertson and E. Diczfalusy, *Acta Endocrinol. (Copenhagen)*, 77 (1974) 655.
20 O. P. Bahl, in B. B. Saxena, G. G. Beling and H. M. Gandy (Editors), *Gonadotropins*, Wiley-Interscience, New York, 1972, p. 200.
21 A. Madej and P. Hallin, unpublished results.