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EVALUATION OF SEVERAL STRATEGIES FOR PREPARING A BOVINE GONADOTROPIN-LIKE PEPTIDE USING ISOTACHOPHORESIS, ISOELECTROFOCUSING AND HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

Several strategies for preparing a gonadotropin-like, thermostable peptide from the bovine adenohypophysis were compared. Initially, we extracted bovine pituitaries, in the form of an acetone powder, with acetic acid and water.

Using one strategy, we isolated the peptide from the crude extract by (1) gel filtration, (2) isotachophoresis, and (3) isoelectrofocusing. The yield was *ca.* 50 μg of peptide from 100 pituitaries.

Using another strategy, we isolated the peptide from the crude extract by precipitating the active material with ethanol followed by high-performance liquid chromatography (HPLC). The yield was *ca.* 500 μg of peptide from 100 pituitaries.

Using analytical HPLC, we found that material obtained using the different strategies gave similar results. The HPLC route was, however, more efficient and faster than the other routes, and we recommend it for preparing this gonadotropin-like peptide.

INTRODUCTION

A bovine adenohypophysial extract, prepared as described by Kihlström and Danninge[1], Kihlström *et al.*[2], Lakoma[3], and Renlund and Hallin[4], contains two biologically active fractions: one consists of substances of fairly low relative molecular mass (M_r *ca.* 1000) and the other of substances of higher relative molecular mass (M_r *ca.* 10 000). The former fraction proved to contain the neurohypophysial hormones oxytocin and vasopressin[5]. The active principle of the latter fraction was purified by Lakoma[3] by means of gel filtration, isotachophoresis (ITP), and isoelectrofocusing (IEF). She named the principle "sperm-releasing substance" (SPRS), since it induced spermiation when injected intralymphatically into male frogs. A number of biochemical and biophysical tests showed SPRS to be a glycopeptide non-identical with the gonadotropins and their subunits. In addition, we recently reported that SPRS binds to a rat ovarian follitropin receptor and also stimulates aromatase activity in intact Sertoli cells from immature male rats *in vitro*[4]. We have

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also described a method of preparing the peptide by means of ethanol precipitation and reversed-phase high-performance liquid chromatography (HPLC).

The aim of the present paper is to compare the method of preparing SPRS developed by Lakoma[3] (here called the liquid chromatography–electrophoretic route) with our method, *i.e.* the HPLC route, and with another method incorporating ion-exchange chromatography, in addition to HPLC.

EXPERIMENTAL

Materials

An acetone powder prepared from the *pars distalis* of bovine adenohypophyses was obtained from Biofac (Copenhagen, Denmark). The material was purchased in batches containing lyophilized acetone powder corresponding to 500 hypophyses. After arrival at our laboratory, each batch was stored in an exsiccator (+4°C) prior to use. The acetone powder was further treated as described by Renlund and Hallin[4]. Thus the material was subjected to heated extraction with acetic acid (HAc, 44 m*M*, 100°C, 20 min) and water (100°C, 5 min) to yield the so-called "Hac–H_2O extract". The material with sperm-releasing activity in this extract was precipitated with 72% (final concentration) ethanol to yield a material called the "ethanol precipitate". These two types of extract were the starting materials used to prepare SPRS via the different routes described below.

Methods

The liquid chromatography–electrophoretic route. The HAc–H_2O extract obtained as described above was applied to a Pharmacia K5/100 column containing Sephadex G-50F gel (Pharmacia, Uppsala, Sweden) with water as eluent at a flow-rate of 1.5 ml/min. The chromatographed material was detected at 280 nm with the aid of a Uvicord absorptiometer (units 8301A and 8303A, LKB, Bromma, Sweden). The biologically active material eluted during the gel filtration was subjected to ITP using a LKB Uniphor 7900 apparatus according to the procedure described by the manufacturer[6] and Lakomaa[3].

The separations were carried out in a LKB Ampholine column (110 ml), in which the gel (7.5% polyacrylamide, *ca.* 13 cm high) was prepared by the polymerization for 1 h of a solution containing 3.64 g of acrylamide, 0.11 g of N,N-methylenebisacrylamide, 0.025 g of ammonium persulphate and 25 μl of tetramethylethylenediamine, 6 ml of leading electrolyte (see below), and water to a total volume of 50 ml.

The buffers used were the leading electrolyte [0.30 *M* phosphoric acid in 0.50 *M* tris(hydroxy)methylaminomethane pH 6.7], the terminating electrolyte [0.23 *M* 6-aminohexanoic acid in 12 m*M* tris(hydroxy)methylaminomethane, pH 9.0], and the elution buffer [66 m*M* tris(hydroxy)methylaminomethane in 30 m*M* sulphuric acid, pH 7.1].

The sample obtained by gel filtration was diluted in 5 ml of terminating buffer and 2.4 ml of Ampholine (pH 7–9) and was layered on top of the polyacrylamide gel. ITP was achieved by applying a current that was stabilized at 15 mA with the aid of a Pharmacia Power Supply 2000/3000 Unit, giving an initial power of 12 W over the column. The duration of each ITP run was 24 h, during which the column

was cooled with running tap water (+ 10°C). The flow-rate of the elution buffer was 0.3 ml/min throughout the experiments, and eluted fractions were detected at 280 nm with the aid of a Uvicord absorptiometer (LKB). The recovered material was kept frozen until desalted prior to bioassay using Sephadex G-15 in a Pharmacia SR 25/45 column.

The biologically active material, recovered from ITP, was purified further by IEF using the LKB 7900 apparatus, as described by the manufacturer[6] and Lakomaa[3]. The separations were achieved in a sucrose density gradient containing carrier ampholytes (Ampholine, LKB) in the pH range 5–7.

The solutions used were applied to the column in the following order, starting at the anode: (1) dense electrode solution (100 ml of 2.0 *M* sucrose in 22 m*M* phosphoric acid); (2) dense gradient solution [50 ml of 1.8 *M* sucrose containing 0.8 ml of Ampholine (pH 5–8) and 0.8 ml of Ampholine pH (7–9)] mixed with light gradient solution [65 ml water containing 0.8 ml Ampholine (pH 5–8) and 0.8 ml Ampholine (pH 7–9)] to give the density gradient; (3) the sample (20 mg, obtained by ITP and diluted in 2 ml of light gradient solution); and (4) light electrode solution (150 ml of 6 m*M* sodium hydroxide) at the cathode.

The electric power over the column was stabilized at 10 W using a Pharmacia power supply 2000/3000 unit, and the column was cooled with running tap water (+ 10°C). Electrofocusing was completed after 20 h.

The sucrose was pumped (0.5 ml/min) through an Uvicord (LKB) detector cell, and the focused samples were detected at 280 nm. The pH of each fraction collected was measured and plotted against the UV absorption of the fraction.

The sucrose was removed from each fraction with the aid of a Pharmacia SR 25/45 column containing Sephadex G-50F using water as eluent, and the material was lyophilized and then assayed based on its biological activity.

The HPLC route. The separations were performed according to the method described by Renlund and Hallin[4]. A Waters chromatographic system was used, including two M-6000 pumps, a U6K injector, and an M-660 solvent programmer (Waters Assoc., Milford, MA, U.S.A.). Chromatographed material was detected at 280 or 220 nm with the aid of an LDC Spectromonitor III (Laboratory Data Control, Riviera beach, FL, U.S.A.) and a dual-channel recorder (W & W, Model 302). The samples were run either in a single stainless-steel column (150–300 × 8 mm I.D.) or in two columns connected in series. The columns were slurry-packed in our laboratory according to Bristow *et al.*[7] with Spherisorb 5 μm ODS spherical silica (Phase Separations, Queensferry, U.K.). The efficiencies of the columns were based on the toluene peak obtained with methanol–water (70:30) as eluent at a flow-rate of 1.5 ml/min. The heights equivalent to a theoretical plate (HETP) ranged between 10 and 20 μm.

The solvent system consisted of two buffers, A and B, mixed by the solvent programmer and the pumps to appropriate proportions. The mixtures will be referred to by their percentage concentration of buffer B. Buffer A was prepared by diluting 5 ml of trifluoroacetic acid to 1000 ml with degassed water. Buffer A was prepared by diluting 5 ml of trifluoroacetic acid with 800 ml of degassed acetonitrile and 195 ml of degassed water. The pH of both buffers was adjusted to 3.3 with concentrated ammonia.

The separations were performed using a step gradient composed of 5, 19, 38,

and 63% of buffer B. Material eluted at the different steps of the gradient was collected separately and will be referred to as the 5% fraction, etc. The fractions were evaporated to dryness and stored at −20°C until further purified or assayed.

Material corresponding to the 38% fraction was redissolved in water, injected into two columns (each 250 × 8 mm I.D.) connected in series, and eluted isocratically with 38% of buffer B at a flow-rate of 1.5 ml/min.

Bioassay

An *in vivo* method based on the induction of spermiation in amphibians, described by Galli-Mainini[8] and Licht[9], was used as modified by Renlund and Hallin[4]. The lyophilized material from each fraction to be assayed was dissolved in 0.65% sodium chloride and injected into the dorsal lymph sacs of male *Rana esculenta* (L). The number of sperm in the cloaca of the frogs was estimated for 3 h after the injection. The sperm density was indexed from 0 to 5, and the sum of indices taken as a measure of the potency of the fraction assayed. Gonadex (Leo, Helsingborg, Sweden) was used as standard in the *in vivo* bioassay.

Protein determination

The amount of material obtained in each purification step was determined by weight. The protein contents of the fractions eluted after HPLC were determined according to Lowry *et al.*[10].

Evaluation of the different strategies of purification

To evaluate the different routes of preparing SPRS, samples of biologically active material obtained by means of ITP, IEF, and HPLC (the fraction eluted with 38% of buffer B) were subjected to analytical HPLC. The chromatographic system was the same as described above, except that one column (10 μm ODS silica, HETP value 20 μm, measured as described above) was used instead of two connected in series. Small amounts of material (30–40 μg) were injected into the column and were eluted isocratically with 38% of buffer B.

Also, the yields of the different procedures and the time schedules for the preparation of SPRS from acetone powder representing 100 pituitaries using the different routes were compared.

RESULTS

The liquid chromatography–electrophoretic route

In a typical experiment, 2.4 g of HAc–H_2O extract, the equivalent of 150 pituitaries, were subjected to gel filtration using Sephadex G-50F. The material eluted was collected in five fractions as shown in Fig. 1. The frog spermiation assay indicated that the main activity was in fraction II; fraction I had only half the activity of II and the other fractions were inactive.

Lyophilized material (15 mg representing 150 pituitaries) of fraction II, obtained by gel filtration, was subjected to ITP (Fig. 2). Two distinct peaks representing tubes 150–159 and 160–169 were obtained at the end of the chromatogram. These two fractions were both associated with sperm-releasing activity; however, the latter (450 μg of protein) was three times as active as the former.

Desalted and lyophilized material, representing the last peak obtained in the

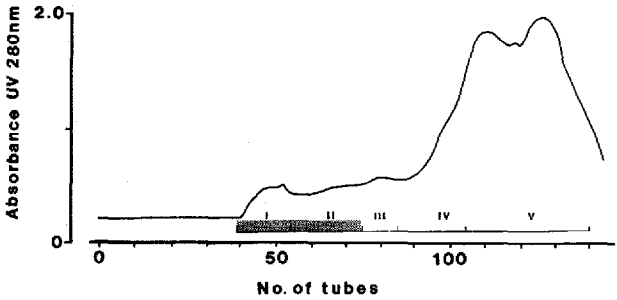

Fig. 1. Purification of the sperm-releasing substance (SPRS) by means of gel filtration (Sephadex G-50F, column 100 × 5 cm I.D., flow-rate 1.5 ml/min, 15 ml/tube) of a bovine adenohypophysial extract with sperm-releasing activity. The sample was obtained from an acetone powder of bovine adenohypophyses by heated extraction with acetic acid and water. Fractions were collected as indicated on the horizontal line above the *x*-axis. The material in fractions I and II contained sperm-releasing activity. Fraction II had the higher activity and was used for further purification of SPRS.

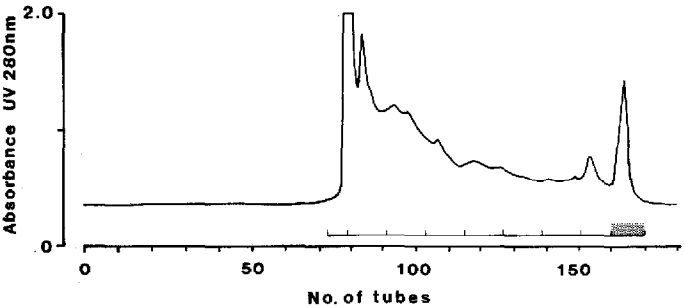

Fig. 2. A chromatogram from a preparative isotachophoretic purification of SPRS (130 mg) obtained as described in Fig. 1. The separation was performed for 24 h on a polyacrylamide gel column (13 cm, 50 ml). The current was stabilized at 15 mA (initial power 12 W). The samples were eluted at a flow-rate of 0.3 ml/min (3 ml/tube). Fractions were collected as indicated on the horizontal line above the *x*-axis. The two last fractions (tubes 150–159 and 160–169) contained material with sperm-releasing activity. The final fraction was the more active and was used for further purification.

ITP separation, was purified further by means of IEF (Fig. 3). The biological activity, however weak, was, after removal of sugar, recovered from tubes representing pH values in the range 8.4–9.4 (the peak fraction having a pH of 8.8). The recovery from 100 pituitaries was *ca.* 50 μg of protein.

The HPLC route

When the HPLC system and the step gradient were used, essentially all sperm-releasing activity was eluted with 38% of buffer B in a single, but heterogenous peak. After isocratic rechromatography of this material with 38% of buffer B as eluent, the sperm-releasing activity was associated with a peak that had a retention volume of 22 ml. A narrow fraction, represented by the front part of this peak, was collected and rerun in the same system without intermediate evaporation of the buffer. The procedure was normally repeated three or four times until a symmetrical peak, indicating pure material, was obtained (Fig. 4).

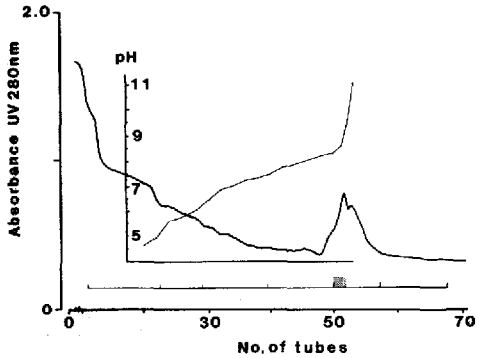

Fig. 3. A chromatogram from a 20-h long isoelectrofocusing of SPRS (20 mg) obtained as described in Fig. 2, in a sucrose density gradient using a stabilizing effect of 10 W. When the focusing had been completed the sucrose was eluted at a flow-rate of 0.5 ml/min (3 ml/tube). Fractions were combined as indicated on the horizontal line above the x-axis. The hatched area represents the sperm-releasing activity.

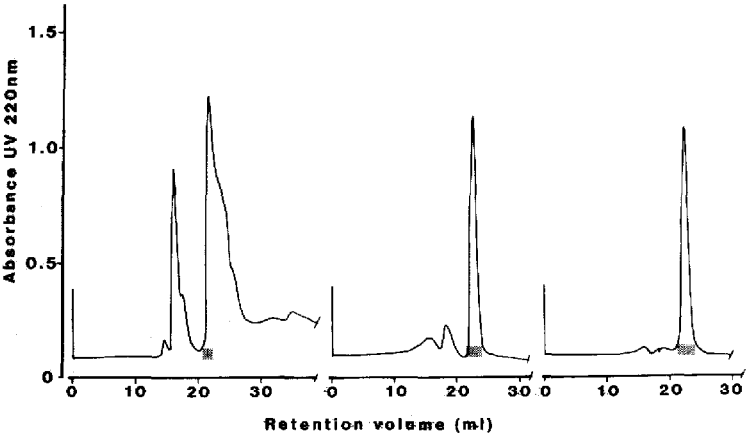

Fig. 4. Purification of SPRS by means of HPLC. The sample (130 μg) obtained as described by Renlund and Hallin[4] was injected into two stainless-steel columns (each 250 × 8 mm I.D., 5 μm Spherisorb ODS), connected in series, and was eluted isocratically with buffers B–A (38:62) at a flow-rate of 1.5 ml/min. Material known to contain SPRS activity (the hatched area) was collected separately and was repeatedly rerun in the same system until a symmetrical peak was obtained (data from Renlund and Hallin[4]).

Evaluation of the different strategies of preparing SPRS

When one HPLC column was used, the sperm-releasing material was eluted with a retention volume of 12.4 ml. As seen from Fig. 5, SPRS purified by means of ITP or IEF was eluted in a main peak that had the same retention volume. Also, the reverse has been shown to be true, *i.e.* SPRS purified by means of HPLC was, when subjected to ITP, eluted with the typical SPRS peak.

The different preparation steps in the two strategies are presented in Tables I and II. The yields were *ca.* 50 μg and 500 μg per pituitary equivalents, respectively.

TABLE I

PREPARATION OF SPRS BY THE LIQUID CHROMATOGRAPHY–ELECTROPHORETIC ROUTE

Procedure	*Time schedule for the preparation of 100 pituitaries (days)*	*Product*	*Yield per 100 pituitaries*
1 Extraction with acetic acid*	2	Acetone powder	35 g
2 Lyophilization (supernatant)	7	HAc extract	–
3 Extraction with water**	1	HAc–H_2O	1600 mg
4 Gel filtration, Sephadex G-50F	3		
5 Lyophilization	7	SPRS–G50F	10 mg
6 Isotachophoresis	1		
7 Lyophilization	2		
8 Desalting, Sephadex G-15	1		
9 Lyophilization	2	SPRS–ITP	0.3 mg
10 Isoelectrofocusing	2		
11 Lyophilization	2		
12 Removal of sugar	1		
13 Lyophilization	2	SPRS–IEF	0.05 mg

* 44 m*M*, 100°C, 20 min.
** 100°C, 5 min.

TABLE II

PREPARATION OF SPRS BY THE HPLC ROUTE

Procedure	*Time schedule for the preparation of 100 pituitaries (days)*	*Product*	*Yield per 100 pituitaries*
1 Extraction with acetic acid*	2	Acetone powder	35 g
2 Lyophilization (supernatant)	7	HAc extract	–
3 Extraction with water**	1	HAc–H_2O	1600 mg
4 Precipitation in 72% ethanol	1	Ethanol pellet	20 mg
5 HPLC (step gradient)	2		
6 Evaporation	2	SPRS–38% fraction	4 mg
7 HPLC (isocratic)	5		
8 Evaporation	1	SPRS	0.5 mg

* 44 m*M*, 100°C, 20 min.
** 100°C, 5 min.

DISCUSSION

The results in Fig. 5 indicate that the two methods of purifying the crude SPRS extract, *i.e.* (1) gel filtration and ITP followed by IEF and (2) ethanol precipitation followed by HPLC, give similar results; however, the latter route is faster and gives higher yields (Tables I and II). The latter route may also be more efficient at recovering biological activity, partly because it requires fewer procedures and particularly

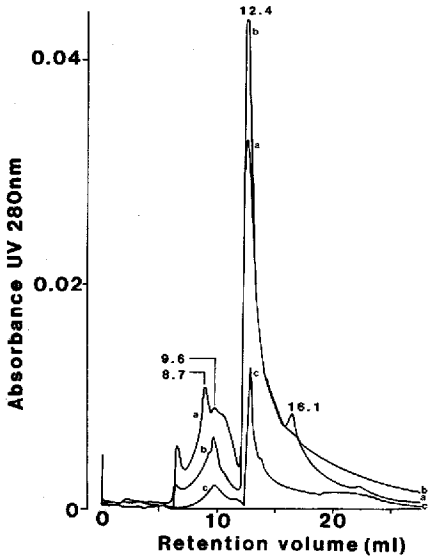

Fig. 5. Analytical chromatograms from HPLC of different preparations of SPRS. The samples were obtained by means of (a) HPLC (30 μg), (b) ITP as described in Fig. 2 (40 μg), and (c) IEF as described in Fig. 3 (30 μg). The column (300 × 8 mm I.D., 10 μm Spherisorb ODS) was eluted isocratically with a combination of 38% of buffer B (the organic phase) and 62% of buffer A (the inorganic phase) at a flow-rate of 1.0 ml/min.

because the number of lyophilizations and for evaporations is smaller (three compared with six).

Fig. 5 also shows that none of the samples chromatographed was homogenous. The least pure sample was in fact that of the 38% fraction obtained from the HPLC route, as judged by the ratio of 3.1 between the height of the SPRS peak (retention volume 12.4 ml) and the highest peak eluted in front of it (Fig. 5, chromatogram a). SPRS from the 38% fraction was, however, easily obtained in a homogenous state after successive isocratic chromatographies (Fig. 4).

The corresponding ratio of the fraction obtained by ITP was 7.0 (Fig. 5, chromatogram b), indicating relatively high purity. However, the subsequent IEF step did not improve the purity of SPRS, as judged by the ratio of 6.5 between the SPRS peak and the front peak of the IEF material, *i.e.* even less than that of the ITP material (Fig. 5, chromatogram c).

This implies that when the liquid chromatography–electropohoretic route is used to prepare SPRS, the IEF step should be excluded. This is also supported by a poor recovery of biological activity in SPRS purified by IEF. However, ITP followed by HPLC might prove advantageous when purifying SPRS, as indicated by the relatively high purity of the ITP fraction.

To develop a third route for preparing SPRS, ion-exchange chromatography was combined with the HPLC route in a preliminary experiment. The adenohypophysial extract, precipitated with ethanol, was applied to a Pharmacia SR 25/45 column containing SP-Sephadex C-25. The material was eluted for 30 h with a linear

gradient of buffer A (8.7 *M* acetic acid and 66 m*M* formic acid) to buffer B (0.5 *M* sodium chloride dissolved in buffer A). The eluent was pumped through the column at a flow-rate of 2.0 ml/min and the eluted material was detected at 280 nm. The fractions collected were desalted using Sephadex G-15, and the sperm-releasing activity was assayed after lyophilization. The elution profile is shown in Fig. 6. The sperm-releasing activity was eluted with the last peak (tubes 168–180), and the other fractions were inactive.

Samples of lyophilized material from the fraction containing sperm-releasing activity (tubes 168–180) and from a fraction containing biologically inactive material

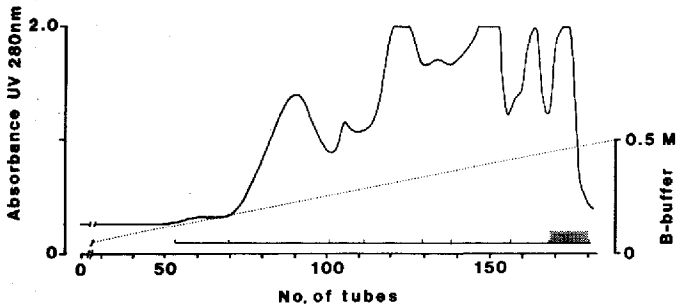

Fig. 6. Ion-exchange chromatography using an ethanol precipitate containing SPRS (SP-Sephadex C-25, column 45 × 2.5 cm I.D.; flow-rate, 2.0 ml/min; 20 ml/tube). Fractions were collected as indicated on the horizontal line above the *x*-axis. The fraction representing tubes 168–180 (the hatched area) contained material with sperm-releasing activity.

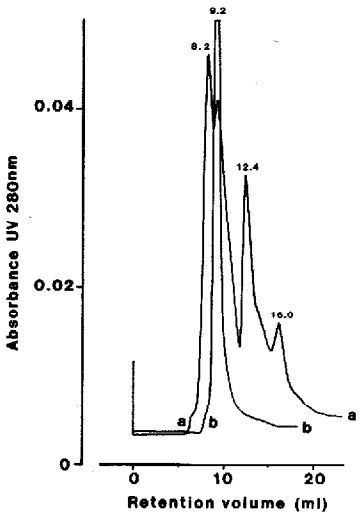

Fig. 7. Analytical chromatograms from HPLC of two fractions, one with sperm-releasing activity (a) and one without (b). The fractions were obtained as indicated in Fig. 6 and were represented by tubes 138–156 and 168–180, respectively. The column (300 × 8 mm I.D., 10 μm Spherisorb ODS) was eluted isocratically with a combination of 38% of buffer B (the organic phase) and 62% of buffer A (the inorganic phase) at a flow-rate of 1.0 ml/min.

(tubes 138–156) were subjected to isocratic HPLC as described above, on one HPLC column (10 μm ODS silica-packed, 300 × 8 mm I.D., HETP value 20 μm).

The elution profiles are shown in Fig. 7. The biologically active material (chromatogram a) has one significant peak with a retention volume of 12.4 ml, which probably represents SPRS, but the inactive fraction has no such peak (chromatogram b). The fraction containing SPRS obtained by ion-exchange chromatography was, however, much more impure than those obtained by ITP or HPLC, as judged by the ratio between the SPRS peak and the front peak, which was less than 1.

A second peak, with a retention volume of 16.0 ml, was present on the chromatogram of the biologically active fraction (Fig. 7, chromatogram a). A similar peak is also associated with the chromatogram of the 38% fraction of the HPLC route (Fig. 5, chromatogram a) and probably represents the SPRS-code 2 material discussed by us elsewhere[4]. The relative difference between the heights of the peaks with retention volumes of 12.4 ml and *ca.* 16 ml is less in the material obtained by ion-exchange chromatography (Fig. 7) than in that obtained by HPLC (Fig. 5). Thus a route combining ion-exchange chromatography and HPLC might constitute an efficient method of purifying SPRS-code 2 material.

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REFERENCES

1 J. E. Kihlström and I. Danninge, *Gen. Comp. Endocrinol.*, 14 (1970) 592.
2 J. E. Kihlström, E. Lakomaa and H. Hall, *Gen. Comp. Endocrinol.*, 17 (1971) 573.
3 E. Lakomaa, *Acta Univ. Ups. Abstr., Uppsala Diss. Fac. Sci.*, 316 (1974) 1.
4 S. Renlund and P. Hallin, *Gen. Comp. Endocrinol.*, 60 (1985) 333.
5 S. Renlund, *Acta Univ. Ups., Uppsala Diss. Fac. Sci.*, 17 (1978) 1.
6 P. J. Svendsen, *Sci. Tools*, 20 (1973) 1.
7 P. A. Bristow, P. N. Brittain, C. M. Riley and B. F. Williamson, *J. Chromatogr.*, 131 (1977) 57.
8 C. Galli-Mainini, *J. Clin. Endocrinol.*, 7 (1947) 653.
9 P. Licht, *Gen. Comp. Endocrinol.*, 20 (1973) 522.
10 O. H. Lowry, N. J. Rosebrough and A. L. Farr and R. J. Randall, *J. Biol. Chem.*, 193 (1951) 265.