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RAPID EXTRACTION AND SEPARATION OF PLASMA β-ENDORPHIN BY CATION-EXCHANGE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

Cation-exchange high-performance liquid chromatography (HPLC) was used to increase the sensitivity and specificity of the radioimmunoassay of plasma β-endorphin. Proteins were precipitated from a 0.5 to 2.5 ml sample of plasma with 60% acetonitrile at pH 4.7. The supernatant was subjected to cation-exchange HPLC. Gradient elution with volatile buffers was used to separate β-endorphin from β-lipotropin. The β-endorphin fraction (1.8 ml) was concentrated by lyophilization and subjected to radioimmunoassay. In healthy pregnant women at labour plasma concentration of β-endorphin varied from 105 to 403 pg/ml. In healthy non-pregnant women plasma concentration of β-endorphin was low, exceeding the detection limit (4 pg/ml) of the assay in only one of the 7 subjects studied.

INTRODUCTION

The pituitary secretes opioid peptides, β-endorphin and β-lipotropin, into the peripheral blood. β-Endorphin has a potent morphine-like activity, whereas β-lipotropin does not seem to have any defined biological role. Most antisera available cross-react with β-endorphin and β-lipotropin on a equimolar basis. Therefore, the separation of β-endorphin and β-lipotropin is necessary for specific radioimmunoassay of β-endorphin in plasma. Gel filtration is commonly used for this purpose[1,2]. We have applied cation-exchange high-performance liquid chromatography (HPLC) to separate fractions of β-endorphin and β-lipotropin from plasma and found it to be a convenient method to increase the specificity of the radioimmunoassay of β-endorphin.

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EXPERIMENTAL

Reference peptides

Human β-endorphin was a gift from Dr. N. Ling, the Salk Institute, La Jolla, CA, and it was also purchased from Sigma (St. Louis, MO, U.S.A.). Purified human β-lipotropin was supplied by P. J. Lowry, St. Bartholomew's Hospital, London. [^{125}I] β-Endorphin was prepared by the chloramine-T method, as described previously[3].

Collection of blood samples

Blood samples (10 ml) were collected from non-pregnant women and from pregnant women in labour, into polyethylene tubes containing 50 μl of heparin and 250 μl of a protease inhibitor, aprotinin (Apronin®; Medica, Helsinki, Finland). After centrifugation at 1000 *g* for 15 min, the plasma was separated and stored at −18°C.

Extraction of plasma samples for HPLC

Plasma (2.5 ml) from non-pregnant women and plasma (0.5 ml) from pregnant women were used for the assay. The recovery was monitored by adding 2000 cpm of [^{125}I] β-endorphin to an 0.5-ml sample of plasma. Proteins were precipitated by acidifying the sample to pH 4.7 with 0.6 ml of 0.1 *M* acetic acid and 1.65 ml of acetonitrile, the final proportion of acetonitrile being 60% of the total volume. The sample was centrifuged at 4500 *g* for 10 min at 4°C and the supernatant decanted and diluted in 5 ml of 0.05 *M* ammonium acetate. This brought the pH of the sample to 5.23, the conductivity to 3.7 mS and the proportion of acetonitrile to 20% of the total volume. The loss of labelled β-endorphin in the precipitate was counted.

Cation-exchange HPLC

A Varian Model 5020 liquid chromatograph and a cation-exchange column (Mono S HR 5/5; Pharmacia, Uppsala, Sweden) were used. Two elution buffers were used; A, 0.05 *M* ammonium acetate pH 5.5 containing 20% (v/v) of acetonitrile; B, 0.50 *M* ammonium acetate pH 5.5 containing 20% of acetonitrile. During the preparation steps the volume of the 0.5-ml plasma sample was increased to 7.5 ml, which was transferred to the column using four injections with a 2-ml syringe and a 2-ml loop. After each injection the content of the loop was flushed into the column with 2.4 ml of buffer A. The final volume of the 2.5-ml plasma sample increased to 38 ml, which was applied to the column using a 50-ml loop (Superloop, Pharmacia) the flow being 1.2 ml/min. The large loop was disconnected from the system before the start of the gradient.

Before initiation of the gradient, the column was eluted with buffer A for 2 min, then the proportion of buffer B was increased to 100% in 17 min. The flow-rate was 1.2 ml/min and fractions of 0.6 ml were collected. Before the next run the column was eluted for 10 min with 100% buffer A. Fractions 22–35 were counted to locate the labelled β-endorphin (fractions 29–31, Fig. 1). Fractions containing unlabelled β-endorphin (26–28) were combined, lyophilized and measured by radioimmunoassay.

Radioimmunoassay (RIA) of β-endorphin

The RIA buffer contained 0.2 *M* Na_2HPO_4–NaH_2PO_4, pH 6.0, gelatin 1.5 g/l

and 7.7 m*M* NaN_3. The specificity of the antiserum "K_2" has been studied previously[3]. It showed 100% cross-reaction with β-endorphin and β-lipotropin, 15% with γ-endorphin, 0.05% with α-endorphin and less than 0.01% with enkephalins and adrenocorticotropin (ACTH). The lyophilized sample was dissolved in 200 μl of RIA buffer containing 1% (w/v) of bovine serum albumin (Sigma). The antiserum was diluted 1:5000 in RIA buffer containing 1% (v/v) normal rabbit serum. About 10^4 cpm of [^{125}I] β-endorphin in 100 μl of RIA buffer containing 1% of bovine serum albumin (w%v) and 100 μl of the antiserum were added to the sample, and the mixture was incubated at 4°C for 48 h. The antigen–antibody complexes were precipitated by addition of 200 μl of sheep anti-rabbit-IgG serum (diluted 1:20). After incubation for 2 h at room temperature, 750 μl of 15% polyethylene glycol in 0.05 *M* phosphate buffer, pH 7.5–8.0, containing 0.15 *M* NaCl and 15 m*M* NaN_3 were added. After centrifugation, the supernatant was removed by aspiration and the contents of the tubes were counted.

RESULTS

Fig. 1 shows the β-endorphin immunoreactivity in the fractions obtained by chromatography of plasma from a pregnant woman at labour. Two major immunoreactive peaks were found. These showed elution patterns identical to those of the purified β-endorphin and β-lipotropin, respectively. The standard solution of [^{125}I] β-endorphin was eluted after unlabelled reference β-endorphin. The eluate of the plasma sample was monitored for absorbance at 280 nm (Fig. 1). A main absorbance peak was eluted with β-lipotropin. Because [^{125}I] β-endorphin was eluted immediately after the unlabelled β-endorphin, it was used as a marker substance to

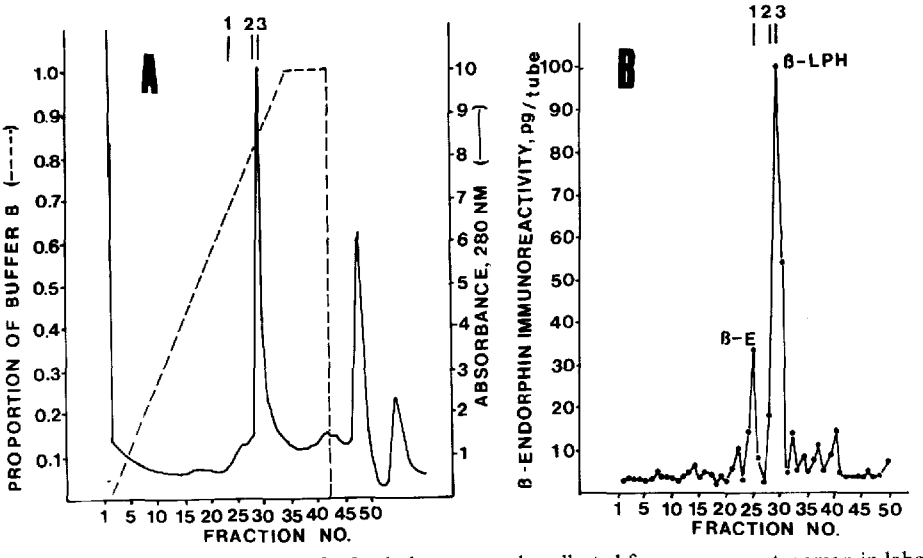

Fig. 1. Cation-exchange HPLC of a 2-ml plasma sample collected from a pregnant woman in labour. A, The elution pattern (———) of UV-absorbing material, and the concentration of buffer B (---------). B, Elution of β-endorphin immunoreactivity; the positions of elution of reference β-endorphin (1), [^{125}I] β-endorphin (2) and β-lipotropin (3) are shown by the vertical lines.

confirm the location of the three 0.6-ml fractions of the eluate containing plasma β-endorphin.

The mean loss of [^{125}I] β-endorphin into the precipitate obtained after acidification and acetonitrile precipitation of the plasma sample was 27.4%, and the mean recovery of [^{125}I] β-endorphin carried throughout the whole procedure was 62.9%. The mean recovery (± S.E.) of unlabelled β-endorphin (100–2000 pg) added to the plasma sample and carried throughout the whole procedure was 64.0 ± 2.1%. The coefficient of variation of replicate determinations of plasma β-endorphin within the concentration range of 5–235 pg/ml was 13.7% (n = 16).

The plasma β-endorphin concentration was analyzed in seven non-pregnant healthy women, 28–36 years of age, who had not used oral contraceptives. Plasma samples were obtained at times between 0800 and 0900. In one of these subjects a detectable amount of β-endorphin, 9 pg/ml, was found in the 2.5-ml sample of plasma, whereas in the other subjects the amount of β-endorphin did not exceed the sensitivity limit (4 pg/ml) of the assay. Blood samples were also collected from six healthy pregnant women in labour. The plasma β-endorphin concentration varied from 105 to 403 pg/ml, the mean value (± S.E.) being 198 ± 52 pg/ml. The values were corrected for methodological losses according to the recovery of labelled β-endorphin carried throughout the whole procedure.

DISCUSSION

Opioid peptides have earlier been extracted from plasma samples with silicic acid[4]. In our hands the recovery of labelled β-endorphin by the silicic acid procedure was 62%. With a mean recovery of 73% of labelled β-endorphin, the present acetonitrile precipitation proved to be a relatively effective extraction step, allowing chromatography of the supernatant without preceding concentration. The pH used (4.7) was chosen to give maximum precipitation of albumin. Although the sample was diluted during precipitation of the proteins, the opioid peptides were concentrated on the column and were eluted in a small volume, *e.g.*, β-endorphin in 1.8 ml. The capacity of the column was high enough to allow separation of the peptides in 2.5 ml of plasma. The use of acetonitrile in the chromatography buffer was necessary for good separation of opioid peptides. Without acetonitrile, β-endorphin and β-lipotropin were not completely separated. Using the present HPLC method β-endorphin was completely separated from β-lipotropin.

Earlier studies of the plasma concentration of β-endorphin in healthy subjects show large discrepancies between results. Using an antiserum having a very small cross-reaction with β-lipotropin and a direct radioimmunoassay of β-endorphin without preceding chromatography, Wilkes *et al.*[5] found a mean plasma level of 115 pg/ml of β-endorphin in healthy subjects, but with a similar antiserum and a sensitivity limit of 10 pg/ml, Bramnert *et al.*[6] found a detectable level (17 pg/ml) of β-endorphin in only one of 27 healthy subjects studied. After purification of serum β-endorphin followed by radioimmunoassay, Nakao *et al.*[7] found only $\leqslant$3.8 pg/ml of β-endorphin in the plasma from healthy subjects, whereas about 30 pg/ml were reported by Genazzani *et al.*[8]. Our findings are in agreement with those of Bramnert *et al.*[6] and Nakao *et al.*[7]. It seems that in healthy non-pregnant women the plasma β-endorphin concentration is very low or undetectable, but significant amounts of

β-endorphin can be detected in maternal plasma during pregnancy and even higher levels during labour.

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REFERENCES

1 V. Höllt, O. A. Müller and R. Fahlbusch, *Life Sci.*, 25 (1979) 37.
2 V. E. Ghazarossian, R. R. Dent, K. Otsu, M. Ross, B. Cox and A. Goldstein, *Anal. Biochem.*, 102 (1980) 80.
3 O. Vuolteenaho, J. Leppäluoto, O. Vakkuri, J. Karppinen, M. Höyhtyä and N. Ling, *Acta Physiol. Scand.*, 112 (1981) 313.
4 D. T. Krieger, A. Liotta and C. H. Ling, *Life Sci.*, 21 (1977) 1771.
5 M. M. Wilkes, R. D. Stewart, J. F. Bruni, M. E. Quigley, S. S. C. Yen, N. Ling and M. Chretien, *J. Clin. Endocrinol. Metab.*, 50 (1980) 309.
6 M. Bramnert, R. Ekman, I. Larsson and J. I. Thorell, *Regulatory Peptides*, 5 (1982) 65.
7 K. Nakao, Y. Nakai, S. Oki, S. Matsubara, T. Konishi, N. Nishitani and H. Imura, *J. Clin. Endocrinol. Metab.*, 50 (1980) 230.
8 A. R. Genazzani, F. Facchinetti and D. Parrini, *Clin. Endocrinol. (Oxford)*, 14 (1981) 409.