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Note

Direct isolation of β-endorphin from plasma by column chromatography

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The neuropeptides β-endorphin and β-lipotropin (β-LPH) are secreted in parallel with adrenocorticotrophin (ACTH) into peripheral blood by the pituitary in response to stress and other ACTH-releasing stimuli in the normal human adult [1]. Although β-LPH has no defined biological role, β-endorphin has potent morphine-like activity [2].

The currently available radioimmunoassay (RIA) procedures for measurement of β-endorphin cross-react with β-LPH [1]. To measure the endogenous opiate activity attributable to β-endorphin it is therefore necessary to separate β-LPH from β-endorphin. This paper describes a simplified column chromatography procedure for the isolation of β-endorphin directly from plasma, eliminating the need for an initial extraction procedure [1, 3, 4]. The column chromatography procedure is compared with octadecasilyl-silica (ODS-silica) cartridge extraction [5].

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MATERIALS AND METHODS

[^{125}I]β-LPH, [^{125}I]β-endorphin and ODS-silica cartridges were obtained commercially from Immuno Nuclear Corp., Stillwater, MN, U.S.A.

Collection of samples

Samples of placental blood (10 ml) were collected in disposable plastic syringes, immediately transferred to tubes containing EDTA as anti-coagulant (Sequestrene) and centrifuged (1000 *g*) at 4°C. Plasma was carefully separated and frozen at −20°C until used for assay.

Chromatography

Plasma (1 ml) was chromatographed directly (without prior extraction) on a 40 × 1 cm Sephadex G-50 (superfine) column (Pharmacia Fine Chemicals, Uppsala, Sweden) with 0.5% bovine serum albumin, fraction V (BSA) in 0.05 *M* borate buffer (pH 8.4). An LKB 2111 Multirac fraction collector (Bromma, Sweden) was utilized and with a slight modification eluates from three columns were collected. A waste volume of 35 min was allowed before collecting fractions at 2.5-min intervals. The column flow-rate (gravity) was 15 ml/h. Each column was primed before β-endorphin isolation by chromatographing a blank plasma sample. Prior to extraction of placental plasma samples, the columns were calibrated by chromatographing a plasma sample containing [^{125}I]β-endorphin. Each fraction (tube) collected was counted for radioactivity (Packard, PGD-Auto-Gamma, Downers Grove, IL, U.S.A.) to identify the endorphin peak. Placental samples were then chromatographed and buffer from the collection tubes corresponding to the β-endorphin fraction was pooled and lyophilized.

Buffer (1 ml 1% BSA in 0.1 *M* borate) spiked with [^{125}I]β-endorphin and [^{125}I]β-LPH was chromatographed on a 40 × 1 cm Sephadex G-75 (superfine) column similar to that described above. Due to the slower flow-rate (4.5 ml/h) a waste volume time of 90 min was allowed before collecting fractions at 7.5-min intervals.

ODS-silica cartridge extraction

Plasma samples (1 ml) were extracted and assayed for β-endorphin according to the procedure of Immuno Nuclear Corp. [5]. Briefly, the plasma was firstly agitated (4°C) with Sepharose anti-β-LPH particles (3 or 24 h) in an attempt to remove β-LPH. Plasma was then acidified and applied to the cartridges. The cartridges were washed with acetic acid (4%) and then β-endorphin was eluted with methanol. The methanol eluate was evaporated to dryness using a gentle flow of compressed air. The resulting residue was reconstituted in 1% BSA—borate buffer prior to RIA.

Radioimmunoassay

Plasma β-endorphin levels were measured using the RIA kit purchased from Immuno Nuclear Corp. The lyophilized samples were reconstituted in distilled water (1 ml) prior to RIA.

RESULTS AND DISCUSSION

[^{125}I]β-Endorphin added to buffer (1 ml 1% BSA in 0.1 *M* borate) was adequately separated from [^{125}I] LPH using Sephadex G-50 (Fig. 1A) but not Sephadex G-75 (Fig. 1B). A higher concentration of [^{125}I]β-LPH than [^{125}I]β-endorphin was used and is depicted in Fig. 1A to confirm there was little carry over of the β-LPH into the β-endorphin peak. This was necessary as β-LPH concentrations in plasma are considerably higher than β-endorphin [1, 6]. Spiked plasma chromatographed slightly slower than the spiked buffer solution (Fig. 1A) probably due to plasma protein constituents. Previous methods for the separation of β-LPH and β-endorphin have involved extraction of plasma (5 ml or greater) with talc [1, 4] and silicic acid [3]. The extracted plasma samples were then chromatographed in Bio-Gel P60 [3] or Sephadex G-50 [1, 4]. Sephadex G-75 has also been used for cerebrospinal fluid [2] and plasma separation [5].

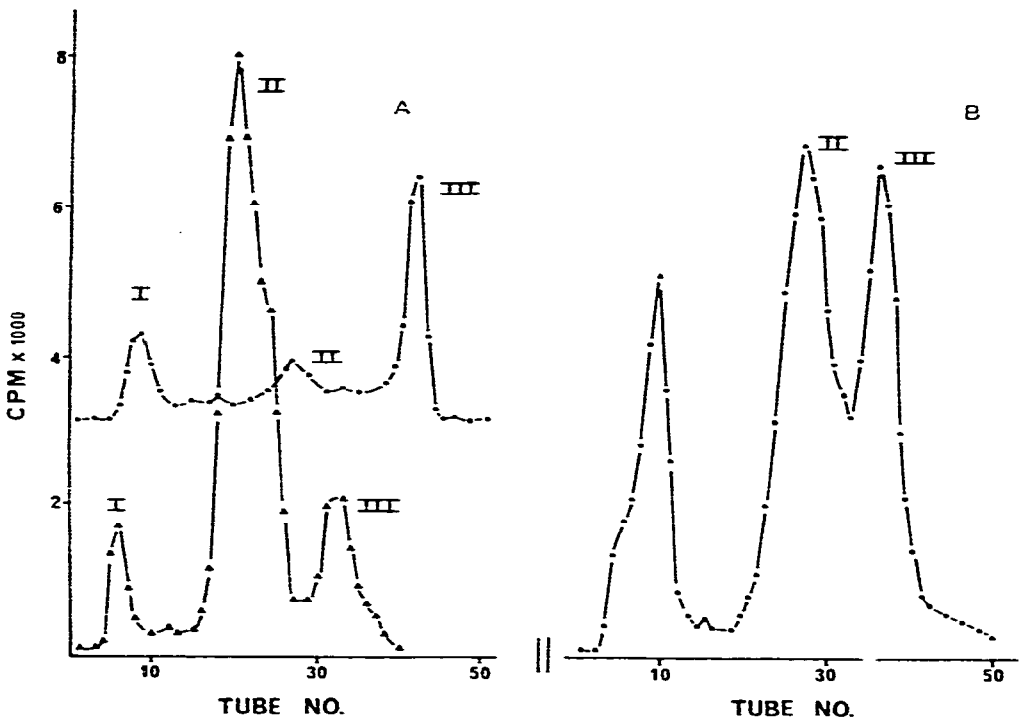

Fig. 1. (A) Elution pattern of a high-molecular-weight immunoreactive substance (I), β-LPH (II) and β-endorphin (III) standards in buffer (▲—▲) and plasma (●—●) from a Sephadex G-50 column. (B) Elution pattern of β-LPH (II) and β-endorphin standards (III) in buffer from a Sephadex G-75 column.

Unlike uniodinated endorphin, [^{125}I]endorphin appears to bind to plasma protein [5]. Peak I (Fig. 1A) may be due to contamination of the ^{125}I-standards with high-molecular-weight immunoreactive material and/or some β-endorphin covalently bound to plasma proteins [3]. We found that if [^{125}I]β-endorphin was added to plasma and left to stand (4°C) for 7 days and then

chromatographed (Sephadex G-50), the separation was lost. The radioactivity was fairly evenly spread over all collected fractions. Wilkes et al. [3] observed that 15% of the radioactivity migrated at the void volume after chromatography of plasma incubated overnight with [^{125}I]β-endorphin. In the present study a freshly prepared [^{125}I]β-endorphin-spiked plasma sample was chromatographed. The buffer eluents corresponding to peak III (Fig. 1A) were pooled, lyophilized and subsequently reconstituted in plasma. The position of the immunoreactive β-endorphin did not alter nor did the radioactivity redistribute on rechromatography of the reconstituted sample. The loss of total radioactivity during lyophilization is negligible. Furthermore, recovery of radioactivity from the column chromatograph was always greater than 94%.

Buffer eluents from tubes 35—44 after placental plasma chromatography were pooled, lyophilized and reconstituted in distilled water for RIA. The mean β-endorphin level of these placental plasma samples chromatographed on Sephadex G-50 was 133 ± 37.7 pg/ml (S.D.) (Table I), a level close to previously reported [6] umbilical arterial concentrations (114 ± 44 pg/ml).

TABLE I

β-ENDORPHIN LEVELS DETECTED BY RIA AFTER (A) COLUMN CHROMATOGRAPHY (SEPHADEX G-50) AND (B) ODS-SILICA CARTRIDGE EXTRACTION

Sample No.	A (pg/ml)	B (pg/ml)
1	165	100
2	103	23
3	122	75
4	180	93
5	95	54
Mean	133 ± 37.7	69 ± 31.3

The use of cartridges for isolation of neuropeptides has recently been described [5, 7]. Rapid elution time has been cited as an advantage. We found ODS-silica extraction does not offer any advantage over Sephadex G-50 column chromatography. Sepharose anti-β-LPH particles did not completely remove β-LPH from spiked plasma samples, even after agitation for 24 h. Loss of radioactivity was observed at various stages of ODS-silica extraction. When acidified, β-LPH-stripped, plasma samples (n = 4) were applied to the cartridges, 22.0 ± 4.5% (S.D.) of the radioactivity was not retained by the cartridge. Sephadex G-50 chromatography of this initial cartridge eluent gave a similar chromatogram to that shown in Fig. 1A, indicating β-endorphin was not completely retained by ODS-silica. A further loss of 14.1 ± 1.3% (S.D.) of the radioactivity occurred after the acetic acid wash and 3.0 ± 0.8% (S.D.) of the radioactivity remained on the cartridge after the final wash with methanol. The mean β-endorphin level of the ODS-silica extracted plasma samples was 69 ± 31.1 pg/ml (S.D.) (Table I). This level is significantly lower than that detected by column chromatography of identical placental plasma samples (133 pg/ml).

In conclusion, a reproducible procedure for the determination of β-endorphin from small quantities of plasma (1 ml) has been developed. This

method is being applied to measurements of plasma and cerebrospinal fluid β-endorphin levels in premature infants. Chromatography of plasma samples without prior extraction or β-LPH stripping minimizes the potential loss of endorphins.

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