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Note

Fluorimetric determination of rat growth hormone in pituitary cell culture by high-performance liquid chromatography with post-column derivatization

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Human growth hormone releasing factor (GRF) from human pancreatic tumour or human hypothalamic–hypophysial tissues has been reported to be a potent and specific stimulus for the release of growth hormone [1]. GRF has been found to be a diagnostic and therapeutic tool for dwarfism [2, 3]. It is composed of 44 amino acids and its chemical structure has been confirmed by synthesis [4].

The biological activity of GRF was measured in vitro [1] by determining the rat growth hormone (rGH) secreted in the medium of rat pituitary cell culture following the administration of GRF. Radioimmunoassay (RIA) of rGH has been developed to determine the rGH concentration in such a medium. However, immunological methods have several disadvantages: (1) poor reproducibility; (2) the need for antibodies and radio-isotopes; and (3) the duration of analysis. Therefore, alternative micro-analytical methods to eliminate those problems are required.

Recently, reversed-phase high-performance liquid chromatography (HPLC) has been developed for peptide or protein analysis [5]. The advantages of the post-column derivatization with *o*-phthalaldehyde (OPA) for the sensitive detection of amino acids or peptides have been reported [6]. However, reliable HPLC techniques for proteins have yet not been developed.

This paper reports the use of HPLC with post-column derivatization for sensitive and reliable analysis of rGH in rat pituitary cell culture.

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EXPERIMENTAL

Reagents and chemicals

OPA, 2-mercaptoethanol (2-ME) and Brij 35 were purchased from Wako (Osaka, Japan). Ethanol for fluorometry and acetonitrile for HPLC were obtained from Kanto (Tokyo, Japan). Trifluoroacetic acid (TFA) was purchased from the Protein Research Foundation (Osaka, Japan). Deionized and distilled water was used. Other reagents were of analytical-reagent grade.

The reaction solution for the post-column derivatization was prepared as follows: 0.2 g of OPA were dissolved in 2 ml of ethanol, and the solution was combined with 200 ml of 0.5 *M* potassium borate buffer (pH 10.0); 220 μl of 2-ME and 2 ml of 15% Brij 35 aqueous solution were added to make a reaction solution.

rGH standard solution for analysis was prepared using the rGH standard for RIA described below, which was diluted to the appropriate concentrations (1–4 μg of rGH per ml).

Apparatus

A Shimadzu LC-4A HPLC system equipped with a gradient elution module and a Shimadzu SPD-2A variable-wavelength detector was used at 220 nm (Kyoto, Japan). A reversed-phase Bio-Rad RP-304 column (C_4, 300 Å, 5 μm, 250×4.6 mm I.D.; Bio-Rad, Richmond, CA, U.S.A.) was used.

The effluent from the UV detector was mixed with the reaction solution using a reciprocating pump (Eldex, Menlo Park, CA, U.S.A.) with a damper, and the mixture was passed through a stainless-steel reaction coil (15 cm×0.5 mm I.D.) at room temperature. Fluorescence was measured with a Shimadzu spectrofluorometer (RF-530) (Kyoto, Japan) at excitation and emission wavelengths of 340 and 440 nm, respectively.

Sample preparation

Enzymatically dispersed rat pituitary cell cultures were prepared by the method of Vale et al. [7]. The cells were pre-incubated for three days with Dullbecco modified Ealge's medium, containing 0.1% bovine serum albumin. GRF was then added and, after incubation for 3 h, the medium was removed and assayed for rGH.

Radioimmunoassay

An rGH RIA Kit (rGH for standard, rGH for iodination and anti rGH serum) was supplied by the National Institute of Arthritis, Diabetes and Digestive and Kidney Diseases. rGH iodination (lot rGH I-5) was used for radiolabelling with ^{125}I and rGH RP-2 as a standard. Separations of antibody-bound and free hormones were accomplished with Staphylococcal protein A antibody adsorbent (IgG Sorb; purchased from the Enzyme Center, Malden, MA, U.S.A.). The intra- and inter-assay coefficients of variation (C.V.) were 13 and 25%, respectively.

TABLE I

OPTIMAL CONDITIONS FOR POST-COLUMN DERIVATIZATION OF rGH

Factor	Condition
pH of reaction mixture	10.0 (0.5 *M* potassium borate)
Concentration of OPA	100 mg per 100 ml
Concentration of ME	0.107 ml per 100 ml
Concentration of Brij 35	0.3 g per 100 ml
Inner diameter of reaction coil	0.5 mm
Length of reaction coil	15 cm
Flow-rate of reaction mixture	0.12 ml/min
Reaction temperature	Ambient (25°C)

High-performance liquid chromatography

The mobile phase was water–acetonitrile containing 0.1% TFA, and the flow-rate was 1.0 ml/min. A Bio-Rad RP-304 column was used for analysis. A 50-μl volume of sample or standard solution was injected into the liquid chromatograph and analysed by gradient elution. The post-column derivatization conditions are described in Table I. rGH was determined by comparing the peak heights of sample and standard solutions.

RESULTS AND DISCUSSION

A wide-pore (300 Å) C_4 reversed-phase column and a gradient elution system were used, with TFA–acetonitrile–water as eluent. In the isocratic elution mode, the separation of rGH from the components in the medium of rat pituitary cell culture was unsatisfactory. However, rGH was more strongly retained than the components in the medium, and a sharp peak for rGH was obtained by gradient elution, as shown in Fig. 1B, although it was not possible to separate the different isoforms of microheterogeneity of rGH, if any, owing to the steep ascent of acetonitrile in the mobile phase.

As shown in Fig. 1A, a steep baseline drift in UV absorbance at 220 nm is observed during gradient elution. However, the detector responses in the post-column reaction are not affected by the gradient elution, because only primary amines can be detected (Fig. 1B). Fluorimetric detection is three to five times more sensitive than UV detection, therefore post-column fluorimetric derivatization was chosen for the detection of rGH.

The chromatograms of a control sample, a spiked medium at a concentration close to the detection limit and a standard sample solution are shown in Fig. 2A–C. The control exhibited a baseline that was virtually free from interfering peaks. The detection limit for rGH at a signal-to-noise ratio of 3:1 was 3 ng (0.1 pmol) per 50 μl of injection volume (60 ppb). The calibration curve for rGH in the medium was linear in the range 20–500 ng (0.4–10 ppm). The C.V.s of five repeated analyses of two samples were 1.8 and 2.7%, respectively. The observed reproducibility was superior to that of RIA, for which the C.V. was 10–30%.

The correlation between the HPLC and the RIA methods was examined by

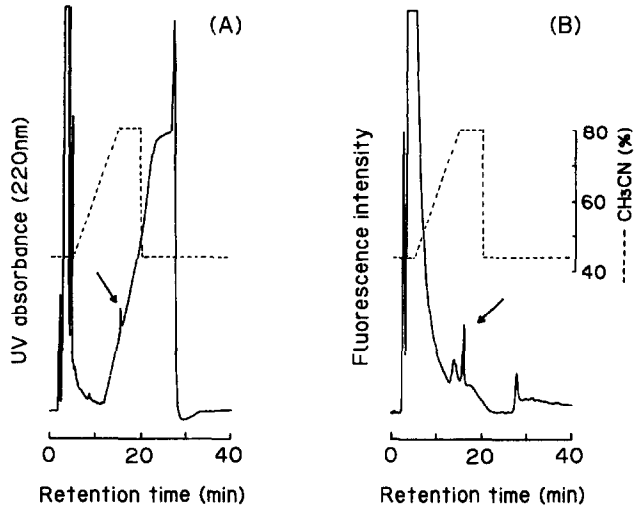

Fig. 1. Comparison of the gradient elution chromatograms of rGH detected by UV absorption and fluorimetrically. (A) UV detection (220 nm); (B) fluorimetric detection. Eluent a = 20% acetonitrile in 0.12% TFA; eluent b = 80% acetonitrile in 0.1% TFA. Gradient programme 0–5 min 40% b, 5–15 min linear gradient of b from 40 to 100%, 15–20 min 100% b. Flow-rate, 1 ml/min. Column, Bio-Rad RP-304. Column temperature, ambient. rGH content, 200 ng per injection.

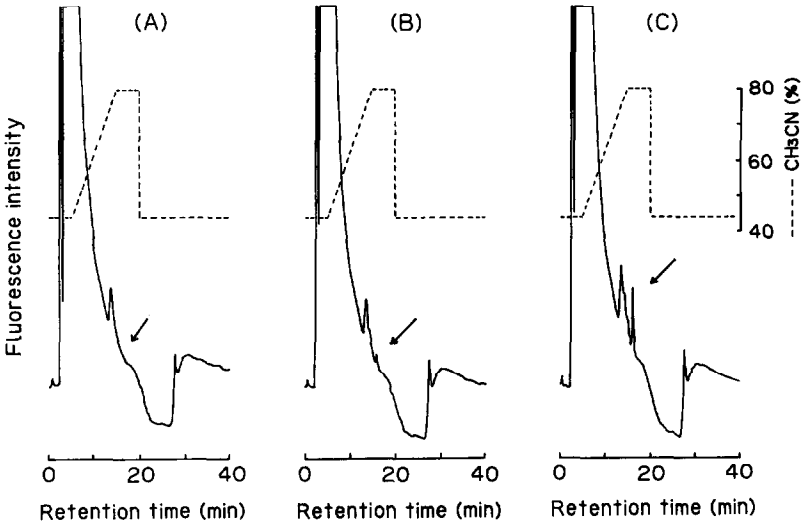

Fig. 2. Chromatograms of rGH in rat pituitary cell culture. (A) Control; (B) rGH (10 ng); (C) sample lot 9, rGH (98 ng). Chromatographic conditions as in Fig. 1B.

assays of samples from the same medium ($n = 24$). As shown in Fig. 3, a good linear correlation ($r = 0.922$) between the two assays was obtained, which indicates good separation and selective detection of rGH.

This method offers an accurate analysis of rGH with sufficient reproducibility and selectivity. It does not require any pretreatment of samples, and the analysis can be done rapidly (within 40 min). More than 500 injections of samples were carried out without any effect on column efficiency, provided column-washing

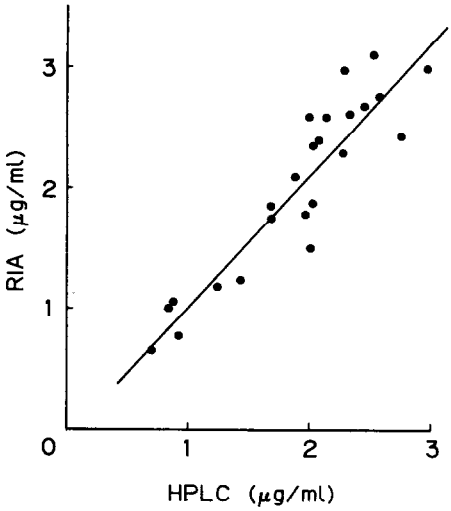

Fig. 3. Correlations between rGH content determined by the HPLC and the RIA method. Samples of the same solutions ($n=24$) were assayed by both methods. The linear regression equation was $y=1.11x-0.09$ (correlation coefficient $r=0.922$).

was done automatically with gradient elution after each analysis. The described HPLC method can be fully automated for rGH analyses in routine work.

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