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High-performance liquid chromatographic method for the determination of insulin synthesis in biological systems

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Abstract

This paper reports a two-step high-performance liquid chromatographic procedure which permits the study of the incorporation of [^{3}H]leucine into insulin in biological systems. The first step of the procedure was size exclusion chromatography, performed on a GPC-100 column, which was eluted with 0.1 *M* KH_2PO_4–methanol (9:1, v/v). By this step the bulk of both protein and radioactivity was separated from tritiated insulin. The second step, which employs reversed-phase chromatography on an octadecylsilyl column, permits the separation of insulin from other contaminants by means of a linear gradient of acetonitrile. This simple and reproducible method was employed to test insulin synthesis in cultured human retinoblastoma Y79 cells.

1. Introduction

Many authors have demonstrated the presence of insulin and its receptor in brain and in retina of vertebrates [1–10]. It has also been reported that neural tissues are capable of generating their own insulin. This conclusion is based on the observation that insulin-specific mRNA has been localized in periventricular hypothalamic cells of rat brain [11], as well as in cultured rat retinal glial cells [12]. In addition, Raizada [13] reported that neurons from primary cultures of rat brain incorporate [^{3}H]valine into immunoprecipitable insulin. In order to ascertain the synthesis of insulin in human retinoblastoma Y79 cells and the rate of its production we initially employed a procedure based on the incorporation of [^{3}H]leucine into immunoprecipitable insulin, which was performed, according to the indications of Raizada [13], by using guinea pig anti-porcine insulin as primary antibody and rabbit anti guinea-pig immunoglobulin G as secondary antibody. It was observed that in the control samples, in the absence of the primary antibody, the amount of radioactivity found in the precipitate was equal to 0.01–0.03% of the total [^{3}H]leucine added to the sample. Although this was a low percentage, it nevertheless represented too high a value of contaminant radioactivity with respect to the small amounts of tritiated insulin synthesized in the incubation samples. Because at present no other procedure is known which is capable of measuring the amount of insulin produced in a biological sys-

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tem, we have developed an high-performance liquid chromatographic (HPLC) procedure which permits separation and measurement of tritiated insulin synthesized in vitro by cultured cells incubated with [^{3}H]leucine.

A technique capable of separating eight different insulins from different species by means of reversed-phase HPLC, has been previously reported by Rivier and McClintock [14]. However, these authors did not specify whether reversed-phase HPLC was effective in separating insulin from other proteins. In preliminary experiments it was clearly shown that the use of reversed-phase HPLC alone can not be considered to be a suitable procedure for the separation of tritiated insulin from cell extracts, due to the high amount of proteins present in these samples. In the two-step procedure reported in this paper the bulk of the protein was quickly discarded by means of size exclusion chromatography; insulin was subsequently purified and assayed by using a C_{18} reversed-phase HPLC column.

2. Experimental

2.1. Chemicals

All reagents (HPLC grade) were purchased from Farmitalia Carlo Erba (Milan, Italy). Human insulin was obtained from Lilly Laboratory (Fegersheim, France). [^{3}H]Leucine (60 Ci/mmol) was from New England Research Products (Boston, MA, USA).

2.2. Instrumentation

A Gilson gradient analytical liquid chromatograph, equipped with a Rheodyne 7125 syringe-load injector (100-μl loop), a programmable UV detector (Model 112) set at 280 nm, and a programmable pump (Model 305) were used for the experiments. Chromatographic separations were performed on a Synchropak GPC-100 size exclusion column (250 × 4.6 mm I.D., 5 μm particle size, 100 Å pore diameter) and a Synchropak C_{18} RP-P reversed-phase column (250 × 4.6 mm I.D., 6.5 μm particle size, 300 Å pore diameter). Both columns were obtained from Synchrom (Lafayette, IN, USA)

2.3. Sample preparation

Y79 human retinoblastoma cells were used in the experiments. Synthesis of insulin was evaluated as incorporation of [^{3}H]leucine into insulin, measured at various specific activities of [^{3}H]leucine. Incorporation was studied in different samples incubated with identical amount of [^{3}H]leucine, but with various leucine concentrations (from 0.1 μM to 50 μM) (Table 1). For that purpose, samples containing $15 \cdot 10^6$ cells were incubated at 37°C in 10 ml of leucine-free Dulbecco's modified Eagle's medium (DMEM), supplemented with various leucine concentrations. Equilibration of the intracellular concentration of leucine with that added to the media was performed as follows: after a 1-h incubation without leucine, the media were replaced by 10 ml of identical fresh media and the incubation was protracted for one more hour. Then, the media were replaced by 10 ml of identical fresh media containing an additional 10% of fetal calf serum and 40 μCi of [^{3}H]leucine; the samples were incubated for a further 5-h period. Subsequently the media were replaced by 2 ml DMEM containing 2 mM leucine and the incu-

Table 1
[^{3}H]Leucine incorporation and insulin synthesis in Y79 retinoblastoma cells at various specific activities of [^{3}H]leucine

Leucine (μM)	[^{3}H]Leucine incorporation (dpm/10^7 cells)	Insulin synthesized (pg/10^7 cells)
0.1	1835 ± 151.1	48.7 ± 5.2
0.5	1590 ± 140.6	211.2 ± 19.9
2.0	1475 ± 128.5	784.0 ± 73.8
10.0	843 ± 68.6	2244.3 ± 213.1
20.0	420 ± 33.1	2229.1 ± 204.0
50.0	ND	

$15 \cdot 10^6$ cells were incubated for 5 h at 37°C in 10 ml of leucine-free DMEM in the presence of 40 μCi of [^{3}H]leucine at various specific activities. Synthesis of [^{3}H]insulin was evaluated as reported in Experimental. Data are the mean (± S.D.) of four separate experiments. N.D. = not detected.

bation was protracted for 5 additional min. Finally, cells were washed in saline, homogenized in H_2O (1:9, w/v), sonicated at 10 μ per 0.5 min and centrifuged at 20 000 *g* for 1 h. Appropriate amounts of authentic insulin standard were added to the supernatants and these were used for HPLC separation.

2.4. Chromatography

Size-exclusion chromatography was carried out on a GPC-100 column which was eluted with 0.1 *M* KH_2PO_4 (pH 7.0)–methanol (9:1, v/v) at a flow-rate of 0.25 ml/min. Under these conditions insulin is eluted between 11–13 min (0.5 ml). Reversed-phase chromatography was performed on a C_{18} column which was eluted first with 15% acetonitrile–0.1% trifluoroacetic acid (TFA) for 60 min at a flow-rate of 1.0 ml/min, then with 23% acetonitrile–0.1% TFA for 20 min at a flow-rate of 1.0 ml/min, and finally with a linear gradient of acetonitrile from 23 to 35%–0.1% TFA for 250 min at a flow-rate of 0.2 ml/min. Under these conditions the retention time of insulin was between 99–103 min (i.e. in the gradient section of the run). At the end of the run the C_{18} column was carefully washed with a gradient of acetonitrile from 35 to 80%–0.1% TFA for 300 min (1.0 ml/min) in order to remove any radioactive traces.

2.5. Assay of immunoreactive insulin

To test for immunoreactive insulin (IRI) we employed an insulin radioimmunoassay (RIA) (Sorin Biomedica, Saluggia, Italy), based on the competition between labelled insulin and insulin contained in the samples for a fixed number of antibody binding site. The minimum amount of IRI able to lower the binding ability of 5% was 9.61 pg.

3. Results and discussion

In the method described here, size exclusion chromatography on a GPC-100 column was performed; when the column was eluted with 0.1 *M* KH_2PO_4, pH 7.0, the retention time was found to be linearly related to the logarithm of the molecular masses for the range 2–60 kDa. However, elution of insulin was delayed, so that it appeared in the eluate after glycyltyrosine. In order to eliminate hydrophobic interactions between the solutes and the support and to improve the recovery of insulin we employed a mobile phase of potassium phosphate–methanol (9:1, v/v). The elution pattern of several standard proteins on the GPC-100 size exclusion column is shown in Fig. 1. It can be seen that insulin elutes from the column between 11 and 13 min. Similar results were obtained when methanol was substituted with acetonitrile. In order to separate [^{3}H]insulin, appropriate amounts of authentic human insulin were added to cell extracts. A 100-μl aliquot of this sample, containing ca. 160 μg of protein and 10 μg of standard human insulin, was applied to the GPC-100 size exclusion column. As shown in Fig. 2, the bulk of the radioactivity eluted from the column before insulin, which eluted together with only 0.5% of the total amount of radioactivity. Fractions between 11 and 13 min, collected from three separate runs, were combined and directly applied to a C_{18} column previously equilibrated with 15% acetonitrile. The column was eluted according to the procedure described in section 2.4. As shown in Fig. 3, a distinct radioactive peak was eluted with the linear gradient between 99–103 min. The corresponding fractions were combined and the radioactivity was evaluated in 10 ml of Ecoscint (Omnia Res., Italy). No radioactivity was detected when [^{3}H]leucine was added at the end of the incubation period or when the incubation was performed in the presence of 20 μM cycloheximide. Identification of the radioactive peak as insulin was made on the basis of the following considerations: (1) the radioactive peak eluted in perfect correspondence with authentic human insulin added to the sample (Fig. 3); (2) in some experiments insulin was not added to the sample and the fractions eluted from the C_{18} column were lyophilized and analysed by RIA. The results, shown in Fig. 4, lead to the conclusion that the radioactive peak

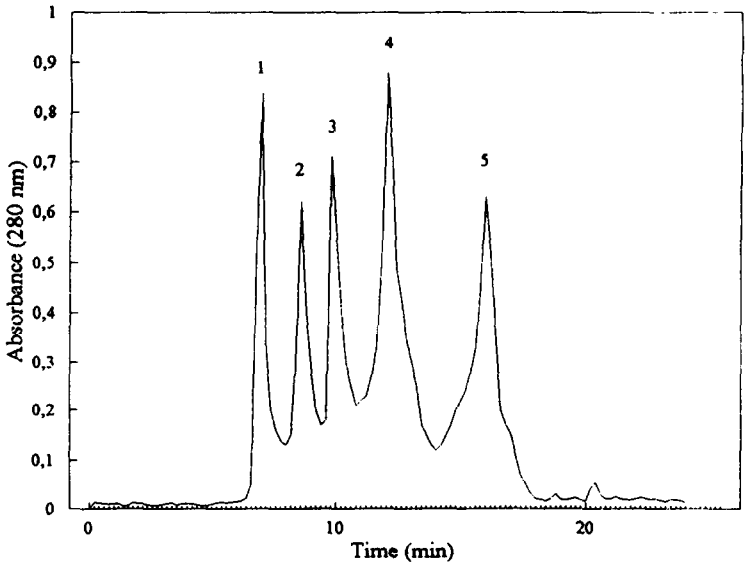

Fig. 1. Elution pattern of some standard proteins by size exclusion chromatography. Peaks: 1 = thyroglobulin (M_r 669 000); 2 = serum bovine albumin (M_r 66 000); 3 = carbonic anhydrase (M_r 29 000); 4 = insulin (M_r 5700); 5 = glycyltyrosine (M_r 238.2). Column: Synchropak GPC-100 (250 × 4.6 mm I.D.) eluted with 0.1 *M* KH_2PO_4 (pH 7.0)–methanol (9:1, v/v) at a flow-rate of 0.25 ml/min.

eluted from the C_{18} column completely coincided with the immunoreactive insulin (IRI) contained in Y79 cells; (3) in some experiments, the fractions obtained from the C_{18} column were lyophilized, reconstituted in 0.1 ml of phosphate-buffered saline and submitted to the procedure

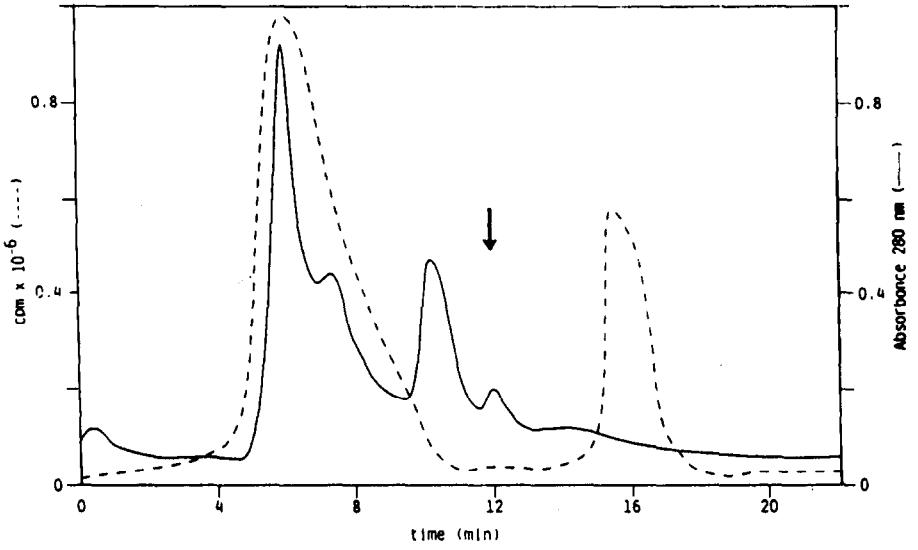

Fig. 2. Size-exclusion HPLC. A 100-μl aliquot of Y79 cell extract, prepared as reported in Experimental, with the addition of 10 μg standard insulin, was applied to a Synchropak GPC-100 size exclusion column (250 × 4.6 mm I.D.) eluted as reported in Fig. 1. Fractions of 0.1 ml were collected. Arrow indicates the insulin peak.

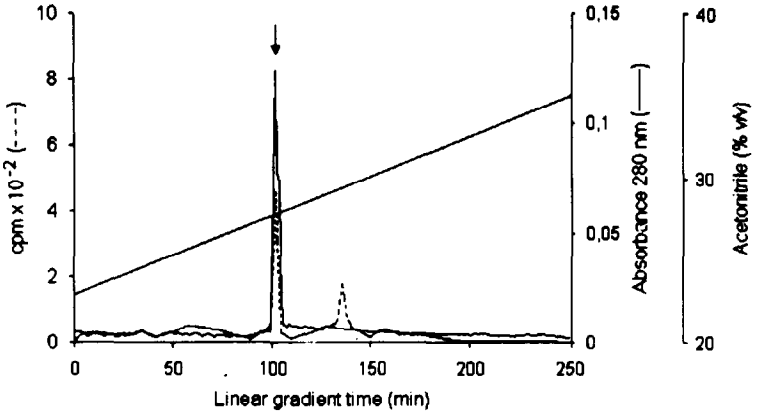

Fig. 3. Reversed-phase HPLC. Comparison with standard insulin. Fractions (11–13 min) collected from three separate runs on the GPC size exclusion column, were pooled and applied to a Synchropak C_{18} column (250 × 4.6 mm I.D.) which was eluted as reported in Experimental. Fractions of 0.2 ml were collected, at a flow-rate of 0.2 ml/min. Arrow indicates the insulin peak. Data are expressed per 10^7 cells.

described by Raizada [13] to detect immunoprecipitable insulin. The radioactivity was found in the precipitate while it was totally absent in the supernatant.

In order to ascertain the recovery of insulin with this procedure, insulin present in the eluate from the C_{18} column was measured at 280 nm. It was concluded that ca. 67.5% of the amount of human insulin (10 μg) added to the samples prior to separation was recovered.

The data reported in Figs. 1–3 refer to samples incubated with 0.1 μM leucine. Under these conditions the amount of radioactivity found in the peak was 1835 ± 151.1 dpm/10^7 cells (Table 1). Furthermore, the amount of radioactivity decreased as the specific activity diminished and could not be measured at 50 μM leucine. It is also shown that insulin synthesis increased by increasing the leucin concentration and reached a maximum at a concentration equal or superior to 10 μM. It was possible, knowing the efficiency of the radioactive count and the recovery of insulin obtained by this procedure, to calculate the total amount of insulin synthesized at various leucine concentrations, using the values for [^{3}H]leucine incorporation reported in Table 1.

In addition to tritiated insulin, tritiated proinsulin and C-peptide were also present in the cell extract. Separate elutions of authentic standards permitted us to establish that proinsulin and C-peptide were eluted from the GPC-100 column between 10.1–11.8 min and 12.8–14.3 min, respectively. Thus it seems likely that some amounts of tritiated proinsulin were present together with tritiated insulin in the GPC fractions eluting between 11–13 min. However, complete separation between insulin and proinsulin was achieved on the C_{18} column where the authentic standard of proinsulin eluted between 133–137 min of the gradient, completely separated from the insulin (99–103 min) and coinciding with the minor peak of radioactivity present in the elution pattern (Figs. 3 and 4). The observation that the material contained in the minor peak only weakly reacted with antibodies to insulin (see Fig. 4) also supported the conclusion that some amounts of proinsulin were probably present in this peak. We excluded the possibility that the C-peptide was a contaminant of both the major and the minor radioactive peaks found on C_{18} column, because the authentic standard of C-peptide was eluted between 188–196 min of the gradient, later than both insulin and proinsulin. Furthermore, since no other radioactive peaks were not found during the gradient elution of the C_{18} column, we concluded that contamination by C-peptide of GPC size exclusion fractions eluted between 11–13 min was negligible.

The method reported in this paper was also

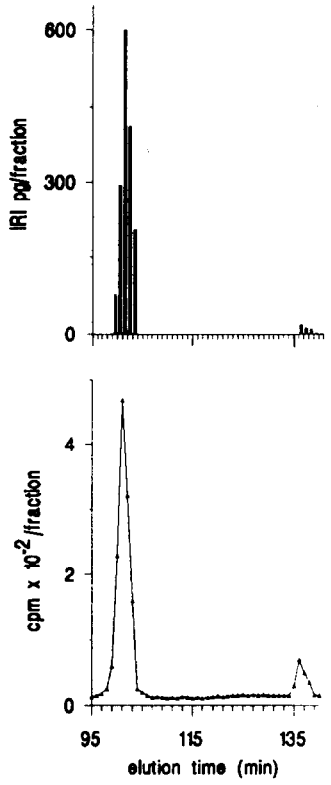

Fig. 4. Reversed-phase HPLC. Comparison with immunoreactive insulin contained in Y79 cells. The same procedure as reported in Figs. 2 and 3 was followed, except that authentic human insulin was not added to the sample; 0.2-ml fractions were collected; 0.1-ml aliquots were employed for radioactivity measurement, while the remaining 0.1 ml was lyophilized and analysed by specific RIA to evaluate the coincidence between IRI and [^{3}H]-insulin elution. Data are expressed per 10^7 cells.

employed to measure the synthesis of insulin in explants of chick embryo retinas.

In conclusion, this method seems to present a simple and reproducible procedure capable of measuring efficaciously [^{3}H]leucine incorporation into insulin in different cell systems.

Acknowledgements

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