Journal of Chromatography, 377 (1986) 339—344
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 3021

Note

Complementary use of gel permeation and reversed-phase liquid chromatography for the analysis of A14-[^{125}I] insulin and its degradation products in isolated human monocytes

P. MARCHETTI* and L. BENZI

*Cattedra di Malattie del Ricambio, 2nd Medical Clinic, University of Pisa, Pisa, and *CNR Institute of Clinical Physiology, Via Savi, 8, 56100 Pisa (Italy)*

V. TRISCHITTA and A. BRUNETTI

Cattedra di Endocrinologia, Institute of Medical Pathology I, University of Catania, Catania (Italy)

P. CECCHETTI and A.M. CICCARONE

Cattedra di Malattie del Ricambio, 2nd Medical Clinic, University of Pisa, Pisa, and CNR Institute of Clinical Physiology, 56100 Pisa (Italy)

V. PEZZINO and R. VIGNERI

Cattedra di Endocrinologia, Institute of Medical Pathology I, University of Catania, Catania (Italy)

and

R. NAVALESI

Cattedra di Malattie del Ricambio, 2nd Medical Clinic, University of Pisa, Pisa, and CNR Institute of Clinical Physiology, 56100 Pisa (Italy)

(First received August 9th, 1985; revised manuscript received November 12th, 1985)

After binding to membrane receptors, biologically active labelled insulin is internalized in target cells [1, 2] and subsequently metabolized [3, 4]. To evaluate the fate of insulin inside human cells, previous studies have generally employed low-sensitive methods, i.e. TCA precipitability and gel permeation chromatography [5, 6].

In this work, insulin degradation by the circulating monocyte, the human cell most often used in in vitro studies, was investigated using gel permeation

0378-4347/86/$03.50

high-performance liquid chromatography (HPLC) and reversed-phase HPLC. This procedure allowed a better resolution of the cell-processed radioactive material resulting from the internalization of labelled insulin.

EXPERIMENTAL

Preparation of pure A14-[^{125}I] insulin

Highly purified porcine insulin (NOVO Industry, Bagsvaerd, Denmark) was iodinated by the lactoperoxidase method as described previously [7], and A14-[^{125}I]insulin was subsequently separated from the iodination mixture using reversed-phase HPLC [8]. The binding activity of this labelled insulin derivative has been reported in other works from this laboratory [9, 10]. The specific activity of the tracer was 360 μCi/μg.

Cell preparation

Mononuclear leukocytes from two normal volunteers were prepared as described in ref. 11 and then incubated with A14-[^{125}I]insulin (50 ng/ml) at 37°C. After 2, 15 and 60 min, cells were centrifuged in a Beckman microfuge (2 min at 8000 *g*) (Beckman Instruments, Fullerton, CA, U.S.A.), the supernatants were discarded and the pellets resuspended in the binding buffer. Surface cell-associated radioactivity (i.e. radioactivity that was not internalized) was then removed by incubation for 6 min in barbital buffer (pH 3) at 4°C, as described in ref. 12. The internalized radioactivity was finally extracted by further incubation for 60 min in 1 ml of 0.1% Triton X-100 at 4°C.

High-performance liquid chromatography

HPLC-grade chemicals were obtained from Merck (Darmstadt, F.R.G.). Deionized, distilled water, purified with a Milli-Q System (Millipore, Bedford, MA, U.S.A.), was used for reagent preparation. Before use all eluents were degassed under vacuum. The isocratic HPLC system was obtained from Waters Assoc. (Milford, MA, U.S.A.) and consisted of a Model 510 pump, a Model U6K injector with a 2-ml loop and a Model 441 UV absorbance detector. Samples were eluted at 1 ml/min and 1-ml fractions were collected in an LKB 7000 Ultrorac collector (LKB, Bromma, Sweden). Radioactivity was counted by an Auto Gamma 500 C counting system (Packard, Downers Grove, IL, U.S.A.). Fibrinogen (mol. mass 311 000), bovine serum albumin (mol. mass 68 000), chymotrypsinogen (mol. mass 24 500), pure A14-[^{125}I]insulin and carrier-free ^{125}I were used as reference compounds.

A preliminary characterization of the radioactive material present in the fractions was performed by assessing its immunoprecipitability with a double-antibody method as described elsewhere [5].

Gel permeation high-performance liquid chromatography

An LKB UltroPac TSK-G3000 SW column (300 × 7.5 mm I.D.) was used. Samples (1 ml) of Triton X-100 solubilized cells containing the intracellular radioactivity were chromatographed. The eluent was 0.05 *M* dipotassium hydrogen phosphate containing 0.1 *M* sodium chloride and adjusted to pH 2.5 with phosphoric acid. Fractions containing radioactivity associated with low-

molecular-mass material (mol. mass <20 000) were pooled. Aliquots (1.5 ml) were prepared and 300 μl of 7 *M* urea were added to each of them. These samples were subsequently analysed by reversed-phase HPLC.

Reversed-phase high-performance liquid chromatography

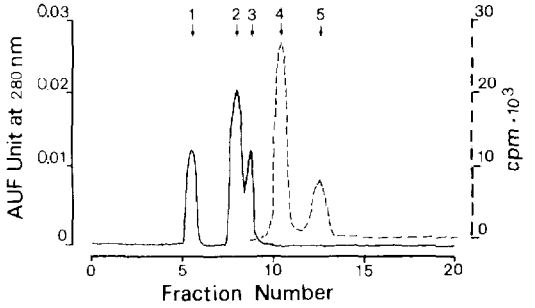
A Waters C_{18} μBondapak (average particle size 10 μm) column (300 × 3.9 mm I.D.) was used. The mobile phase used for elution was 0.01 *M* sodium phosphate buffer—isopropanol—acetonitrile (67:11:22), containing 0.15 *M* ammonium acetate (11.56 g/l of eluent) and adjusted to pH 3 with hydrochloric acid.

RESULTS

Gel permeation high-performance liquid chromatography

The elution pattern of a mixture of reference compounds shown in Fig. 1 illustrates the separation achieved among substances of different molecular mass. Fraction No. 9 (Fig. 2) was chosen as a cut-off point between samples containing material with a molecular mass lower and higher than 20 000.

Fig. 2 illustrates the chromatogram obtained by the solubilization of monocytes incubated with A14-[^{125}I]insulin for 2 min (A), 15 min (B) and 60 min (C). At all times studied, nearly 20% of the recovered radioactivity eluted in fractions 5—9, with the main peak corresponding to a molecular mass of ap-



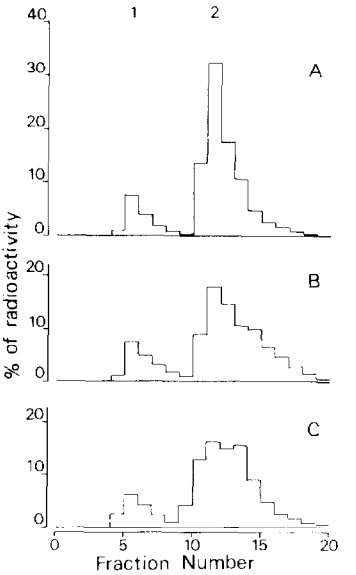


Fig. 1. Gel permeation HPLC profile of a standard mixture containing fibrinogen, mol. mass 341 000 (peak 1), bovine serum albumin, mol. mass 68 000 (peak 2), chymotrypsinogen, mol. mass 24 500 (peak 3), A14-[^{125}I]insulin (peak 4) and ^{125}I$^-$ (peak 5). Chromatographic conditions are described under Experimental.

Fig. 2. Gel permeation HPLC profiles of radioactivity extracted from mononuclear cells after incubation for 2 min (A), 15 min (B) and 60 min (C). About 20% of total radioactivity was eluted in the fractions corresponding to higher molecular mass (1). About 80% of the radioactivity was recovered in the fractions containing low-molecular-mass material (2).

proximately 300 000. The remaining radioactivity (about 80%) was recovered in fractions 10—20. The overall recovery of radioactivity was 97 ± 1.1%. As described under Experimental, fractions 10—20 were pooled and aliquots were prepared for reversed-phase HPLC analysis. High-molecular-mass radioactive material was not immunoprecipitable with the anti-insulin antibody, while radioactivity in fractions 10—20 was 30—80% immunoprecipitable.

Reversed-phase high-performance liquid chromatography

The elution times of $^{125}I^-$ and pure A14-[^{125}I]insulin in reversed-phase HPLC were 3 and 27 min, respectively (Fig. 3A). The chromatographic profile of samples previously separated by gel permeation HPLC (pooled fractions 10—20) showed two main peaks of radioactivity (Fig. 3B—D): peak 1, eluting approximately at the $^{125}I^-$ position, and peak 2, the retention time of which was identical with that of the reference A14-[^{125}I]insulin. Three additional small peaks, a, b and c, accounting for about 1.5, 4 and 1%, respectively, of the recovered radioactivity, were also observed in intermediate positions (Fig. 3). When the incubation time of A14-[^{125}I]insulin with monocytes was increased, a decrease in the insulin peak (peak 2) and a corresponding increase in peak 1 were observed; the intermediate peaks became lower with time. The overall recovery of radioactivity was 96 ± 1.5%.

To characterize further the peaks separated by reversed-phase HPLC, the immunoprecipitability of each fraction was evaluated. Peak 1 in Fig. 1 was not immunoprecipitable, whereas the sample corresponding to peak 2 always

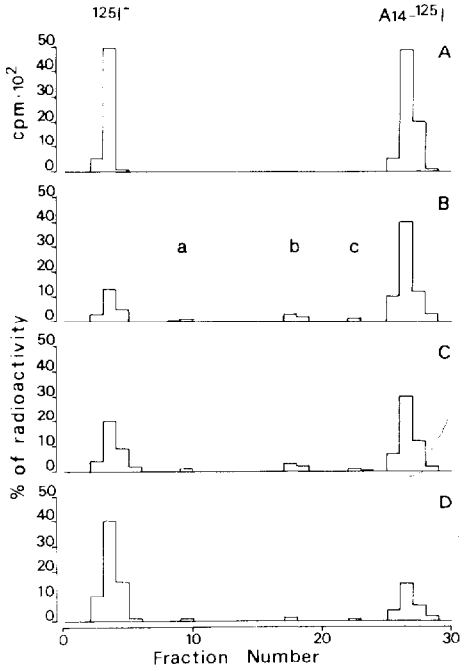


Fig. 3. Reversed-phase HPLC profiles of reference [^{125}I]- and A14-[^{125}I]insulin (A) and of low molecular mass associated radioactivity (mol. mass < 20 000) from cells incubated with the tracer for 2 min (B), 15 min (C) and 60 min (D). Intermediate peaks are indicated as a, b and c.

showed an immunoprecipitability higher than 90%, similar to that observed with pure A14-[^{125}I]insulin. As far as the intermediate radioactivity is concerned, fractions corresponding to peaks a and c were not tested because of their low radioactive content; peak b exhibited an immunoprecipitability of approx. 50%.

DISCUSSION

Most studies of insulin degradation performed using gel permeation were unable to separate the intact hormone from some of the insulin-derived fragments [11, 12]. Using these methods, many products of insulin degradation may be indistinguishable from the intact molecule.

A new method is proposed for the analysis of insulin and its degradation products involving the combined use of gel permeation and reversed-phase HPLC. It allows a faster and more efficient separation of the various radioactive insulin derivatives produced in the degradation process.

The usefulness of HPLC in the detection of degraded A14-[^{125}I]insulin has already been reported in fibroblasts [13]. In addition to the possibility of analysing the low-molecular-mass iodinated products, the present procedure also allows the study of radioactivity associated with higher-molecular-mass material by gel permeation HPLC. According to a previous report [14], this high-molecular-mass material could be the labelled insulin—receptor complex.

The chromatographic pattern of the solubilized human monocytes suggests the presence of intact A14-[^{125}I]insulin inside the cell (Fig. 3, peak 2), together with the degraded products eluting near the $^{125}I^-$ position (peak 1). Further evidence that peak 2 is intact A14-[^{125}I]insulin was obtained from immunoprecipitation. The presence of intermediate products of insulin degradation, partially retaining the immunological characteristics of intact insulin, was also shown.

The intracellular insulin degradation process was time-dependent, as indicated by the progressive conversion of intact A14-[^{125}I]insulin to degradation products (peak 1) with increasing incubation times. Both the internalization and degradation are rapid processes (2 min after incubation peak 1 is already present), and the resulting derivatives maintain in part the immunochemical properties of the intact molecule.

In conclusion, the developed method allows an accurate stepwise evaluation of the intracellular processing of insulin and a rapid characterization of the fate of insulin and its degradation products in intact human target cells.

ACKNOWLEDGEMENTS

This work was supported by the National Research Council, Special Project Diabetes, Grant No. 82.02254.56, and by the Ministero Pubblica Istruzione, Ricerca Scientifica.

REFERENCES

1 S. Terris and D.E. Steiner, J. Biol. Chem., 250 (1975) 8389—8398.

2 J. Schlenssinger, Y. Shechter, M.C. Willingham and I. Pastan, Proc. Natl. Acad. Sci. U.S.A., 75 (1978) 2659—2668.
3 R.I. Misbin, E.C. Almira and S.J. Buynitzky, J. Biol. Chem., 258 (1983) 2157—2162.
4 W.C. Duchworth and D.E. Kitabchi, Endocrinol. Rev., 2 (1981) 210—233.
5 W.C. Duckworth, F.B. Stentz, M. Heinemann and A.E. Kitabchi, Proc. Natl. Acad. Sci. U.S.A., 76 (1979) 635—639.
6 G.W. Neal and A.E. Kitabchi, Biochim. Biophys. Acta, 719 (1982) 259—266.
7 R. Navalesi, A. Pilo and E. Ferrannini, J. Clin. Invest., 61 (1978) 197—206.
8 P. Marchetti, L. Benzi, P. Cecchetti and R. Navalesi, J. Nucl. Med. All. Sci., 28 (1984) 31—34.
9 L. Benzi, P. Marchetti, P. Cecchetti, F. Caricato, A. Masoni and R. Navalesi, J. Nucl. Med. All. Sci., 28 (1984) 81—84.
10 L. Benzi, P. Marchetti, P. Cecchetti, V. Pezzino, D. Gullo, A. Masoni and R. Navalesi, J. Nucl. Med. All. Sci., 28 (1984) 277—281.
11 V. Trischitta, D. Gullo, V. Pezzino and R. Vigneri, J. Endocrinol. Metab., 57 (1983) 713—718.
12 J.M. Olefsky and M. Kao, J. Biol. Chem., 257 (1982) 8667—8673.
13 F.B. Stentz, H.L. Harris and A.E. Kitabchi, Diabetes, 32 (1983) 474—477.
14 S. Clark and L.C. Harrison, J. Biol. Chem., 257 (1982) 12239—12246.